Methods

Prediction of $\delta^{13}C$ and $\delta^{15}N$ in plant tissues with near-infrared reflectance spectroscopy

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Summary

- Isotope measurements associated with critical plant resources, such as carbon and nitrogen, have helped deepen the ecological understanding of plant resource acquisition and plant interactions. In this study, we tested the appropriateness of near-infrared reflectance spectroscopy for the estimation of stable isotope ratios for nitrogen and carbon of plant tissues.
- $\delta^{13}C$ and $\delta^{15}N$, as well as total carbon (Ct) and nitrogen (Nt), in leaf tissues of a heterogeneous set of 72 samples of seven bog species from southern Patagonia were determined. Near-infrared reflectance spectroscopy calibrations were developed using partial least-squares regressions and tested by a cross-validation procedure. For each variable, three calibrations were calculated: one with nontransformed data and two with transformations (first and second derivative).
- Ct and Nt, as well as $\delta^{13}C$ and $\delta^{15}N$, were well predicted by our calibration models. The correlation coefficients of predicted vs actual values of the best calibration models were as follows: 0.95 (Ct), 0.99 (Nt), 0.89 ($\delta^{13}C$) and 0.99 ($\delta^{15}N$). The cross-validation procedure confirmed the high estimation quality of the calibrations.
- The results obtained underpin the great potential of the near-infrared reflectance spectroscopy technique in ecological studies as an alternative to more expensive and time-consuming standard methods.

Abbreviations: Ct, total carbon; NIRS, near-infrared reflectance spectroscopy; Nt, total nitrogen; PLSR, partial least-squares regression; RSC, standard deviation of the reference values to SECV; SEC, standard error of calibration; SECV, standard error of cross-validation.

Introduction

In ecology, stable isotope measurements have been increasingly used as an elegant method to obtain relevant information on relationships between plants and their environment (Dawson *et al.*, 2002). Stable isotopes are seen as a powerful tool for advancing our knowledge on stock cycling, both as a tracer and as a record of processes in which isotopic fractionation occurs (Bowling *et al.*, 2008). Nitrogen and carbon isotopic compositions have provided key insights into biogeochemical interactions between plants, soils and the atmosphere across a wide range of spatial and temporal scales (Robinson, 2001). For example, the $\delta^{13}C$ isotopic signature has been used successfully to disentangle physiological, ecological and biogeochemical processes within the soil–plant–atmosphere interface (Fotelli *et al.*, 2003; Bowling *et al.*, 2008; Gessler *et al.*, 2009). $\delta^{15}N$ studies have significantly improved our knowledge on nitrogen cycling pathways and nitrogen acquisition by plants (Kolb & Evans, 2002; Bragazza *et al.*, 2005; Templer *et al.*, 2007; Vallano & Sparks, 2008). However, there are still many ecological questions that may be answered, at least partially, by information that is reflected by the isotopic composition of plant material (Dawson *et al.*, 2002; Taylor, 2008).

The determination of stable isotopes in plant material requires expensive instrumentation (isotope ratio mass spectrometer), and thus, in many ecological studies, information on the isotopic composition is not provided because of a lack of access to the appropriate instrumentation. Despite this, a time- and cost-effective measurement technique of the isotopic composition of plant material may be of high scientific interest, for example, for the rapid screening of a large amount of samples to detect interesting patterns.

Over the past three decades, near-infrared reflectance spectroscopy (NIRS), as a fast and low-cost analysis technique, has been used increasingly for the analysis of pharmaceutical and agricultural products, especially in the food industry (Norris *et al.*, 1976; Clark *et al.*, 1987). By the application of NIRS, C–H, N–H and O–H bonds, as the main constituents of organic material, are induced to vibrate by near-infrared radiation (NIR). The chemical composition of the organic material determines the constitution and number of bonds (Shenk *et al.*, 2008). Therefore, the resulting spectrum contains information on the chemical composition and can be used for qualitative and quantitative analyses. The prediction of unknown samples is carried out with a model that needs to be developed with multivariate regression procedures (Workman, 2008).

The application of this technique in ecological studies is less common. However, NIRS technology still holds great potential for the analysis of various organic matter compounds which are of ecological relevance (Foley *et al.*, 1998; Coûteaux *et al.*, 2003; Aragones *et al.*, 2006; Roumet *et al.*, 2006; Chodak, 2008; Terhoeven-Urselmans *et al.*, 2008; Zornoza *et al.*, 2008).

NIRS has been proven to predict many compounds of organic material with excellent accuracy. Good results have been obtained with total nitrogen (Nt) (Bolster *et al.*, 1996; Petisco *et al.*, 2005) and total carbon (Ct) (Gillon *et al.*, 1999a,b; McTiernan *et al.*, 2003) content. Even for more complicated organic substance classes, such as fibre, tannins and phenols, NIRS calibrations have resulted in highly accurate estimations of their contents (Landau *et al.*, 2004; Petisco *et al.*, 2006; Stolter *et al.*, 2006). The application of NIRS technology for the determination of the mineral content of organic material is problematic and must be carried out carefully (Foley *et al.*, 1998). However, measurements of phosphorus, potassium, magnesium and calcium have been performed with this method and have resulted in accurate calibration models (Clark *et al.*, 1987; Gillon *et al.*, 1999a; Ruano-Ramos *et al.*, 1999; Petisco *et al.*, 2005, 2008). A limit seems to be obtained for the estimation of very poorly concentrated trace elements, such as iron, manganese, copper and zinc (Clark *et al.*, 1987; Ruano-Ramos *et al.*, 1999; Petisco *et al.*, 2005, 2008).

The atomic mass of carbon and nitrogen built into biomass should have an effect on the wave motion of bonds between the atoms or functional groups induced by NIR. A NIR spectrum may therefore contain information on the isotopic composition of a sample. This assumption was corroborated by Okano *et al.* (1983), who found indicative absorption intensities of $^{12}CO_2$ and $^{13}CO_2$ in two different infrared regions. In this study, we aimed to test whether NIRS could be used to predict $\delta^{13}C$ and $\delta^{15}N$ of plant tissue with sufficient accuracy, in order to contribute to a better utilization of the ecological information that is stored in the isotopic composition of plant biomass. In addition, the validity of NIRS for the measurement of Ct and Nt was investigated.

Materials and Methods

Sampling and chemical analysis

Plant material of seven plant species was sampled from eight different bog complexes in Chilean Patagonia. To guarantee a heterogeneous sample set of relatively high interspecific variation, these plant species belong to different functional groups. Within species, variation was also as high as possible by covering sampling sites along a steep climatic and biogeochemical gradient (Kleinebecker *et al.*, 2008). Table 1 summarizes information on the investigated taxa, including the number of samples of different bog types representing the environmental gradient.

Sampling took place in February and March 2007. Plants were randomly sampled in floristically and structurally homogeneous stands within an area of *c.* 10 m^2. The carpet-building species (cushion plants and *Sphagnum*) were collected in five to seven single samples of *c.* 10 cm^2 and were handled as one mixed sample. For *Sphagnum*, the capitula, which is the upper part with a length of *c.* 2 cm, was used for analysis. For cushion

Table 1 Species sampled and number of samples of different bog types

Species	Family	LF	SB	MB	CB	Total
Astelia pumila	Liliaceae	CP		1	9	10
Donatia fascicularis	Stylidiaceae	CP		3	6	9
Empetrum rubrum	Empetraceae	DS	4	4	2	10
Marsippospermum grandiflorum	Juncaceae	GH	6	3	7	16
Oreobolus obtusangulus	Cyperaceae	CP		5	5	10
Schoenus antarcticus	Cyperaceae	GH			7	7
Sphagnum magellanicum	Sphagnaceae	M	6	4		10
Total			16	20	36	72

SB, continental *Sphagnum* bog; MB, ecotonal *Sphagnum*-cushion mixed bog; CB, hyperoceanic cushion bog. Classification of lifeforms (LF): CP, cushion plant, cushion-forming hemicryptophyte; DS, dwarf shrub; GH, graminoid hemicryptophyte; M, moss (after Kleinebecker *et al.*, 2007).

plants, senescent leaves were eliminated and only rosettes with green leaves were analysed. Graminoids and *Empetrum* were collected in batches of 30–50 shoots at each plot and merged to a mixed sample. For the graminoid species *Marsippospermum*, only nonsenescent leaves were harvested and the green parts were used for chemical analysis. In the case of the dwarf shrub *Empetrum*, only green leaves were used.

Plant material was stored cool and dry in a dark place and then shipped to the laboratory. After drying at 105°C, samples were ground with a mill and screened through a sieve with 0.5 mm mesh wire. Ct and Nt were determined using an element autoanalyser (CARLO ERBA NA 1500, Milan, Italy). All analyses were run in duplicate, with relative differences always lower than 10% between sample pairs. $\delta^{13}C$ and $\delta^{15}N$ were determined according to Marguillier *et al.* (1997) on a Finnigan MAT Delta Plus (Finnigan, Waltham, MA, USA) with Conflo III interface. The accuracy of the analysis allows a maximum error of 0.15‰.

NIRS analysis

All milled samples were scanned with a Spectra Star 2400 (Unity Scientific, Columbia, MD, USA). Each sample was packed manually in a sample capsule with a quartz glass cover. Measurements were made at 1 nm intervals over the range 1250–2350 nm. Samples were scanned once, and one individual scan consisted of 24 single measurements, which were mediated to the resulting spectrum. The spectral data were recorded as log $1/R$ (where R is reflectance) and the first and second derivatives of log $1/R$. Because of the relatively small number of samples, we chose a cross-validation procedure to validate the quality of our calibration models (Martens & Dardenne, 1998; Terhoeven-Urselmans *et al.*, 2006). For this purpose, the set of spectra was divided into 12 segments. The spectra belonging to each cross-validation segment were selected randomly. Thus, a model was calculated using 65 spectra (11 segments) and the model was subsequently tested by the prediction of the remaining seven spectra (one segment). Analogously, predictions were made for each of the 12 segments with a calibration developed from the remaining 11 segments. To additionally prove the reliability of NIRS for the prediction of the analysed parameters, an external validation procedure was carried out. Of the total samples (72), validation sets of 13 samples were selected by arranging the respective parameters according to size and taking each fifth sample. The remaining 59 samples were utilized for calibration. This selection procedure ensured the representativeness of both the calibration and the validation set along the range of observed values.

Calibrations were calculated by partial least-squares regression (PLSR) procedures using CalibrationWorkshop (Senso-Logic Software GmbH, Norderstedt, Germany). The standard error of cross-validation (SECV) was used to determine the number of factors used for calibration. Usually, this was the number of factors at which the first minimum of SECV occurred. Calculating PLSR calibrations for each parameter, several samples were identified as outliers. The *H*-statistic is a measure of how strong is the influence of a particular spectrum on the regression model. The *T*-value determines the residual error. The statistical reconstruction error, which is obtained by attempting to recalculate the original spectrum from the selected factors, is reflected by the *S*-value. A spectrum identified as an *S*-outlier is thus notably different from the rest of the population. As suggested by the software, the limits were set to 3 (*H*- and *S*-outliers) and 2.5 (*T*-outliers). The sort and quantity of the spectra identified as outliers during the calibration process are given in Table 3. In order not to overestimate the applicability of NIRS, the total dataset, including the outliers, was used for calibration and the calculation of the respective statistical measures. The optimum calibration equations were selected on the basis of a large coefficient of multiple determination (R^2) and a low standard error of calibration (SEC) and a low SECV. SECV is a measure of the difference between the actual and predicted property values calculated over all cross-validation calibrations. SEC is exclusively based on spectra used for calibration and indicates the theoretical accuracy when using the calibration to predict unknown spectra. The general ability of NIRS to predict Ct, Nt, $\delta^{13}C$ and $\delta^{15}N$ of plant tissue was evaluated from the ratio of the standard deviation of the reference values to SECV (RSC). For good predictions, $RSC > 2$; acceptable models show $1.4 \leq RSC \leq 2$ (Chang *et al.*, 2001).

Results and Discussion

For Ct and Nt contents, as well as for $\delta^{13}C$ determined by reference methods, the samples were within relatively narrow ranges of 43.26–53.72%, 0.45–1.48% and −29.50 to −23.72‰, respectively (Table 2). $\delta^{15}N$ ratios showed a variation of more than 18‰, which is extraordinarily high for co-occurring species (Handley & Scrimgeour, 1997). The general ability of NIRS to predict Ct, Nt, $\delta^{13}C$ and $\delta^{15}N$ was documented by the RSC values. $RSC > 2$ was obtained for Ct, Nt and $\delta^{15}N$. For $\delta^{13}C$, $RSC = 1.5$, which enhances the expectation for satisfactory results (Chang *et al.*, 2001).

Although the sample size is regarded as being a borderline case, calibrations calculated for carbon and nitrogen were highly accurate (Fig. 1, Table 3) and comparable with the calibration statistics reported in the literature so far (Gillon *et al.*, 1999a; Petisco *et al.*, 2005). The best coefficients of multiple determination were found for PLSR calibrations using derivative functions, which is also in accordance with studies predicting organic compounds in plant biomass by NIRS (Petisco *et al.*, 2006). In the case of carbon, the second derivative resulted in the best calibration statistics, whereas the first derivative treatment revealed the best calibration statistics for nitrogen.

In our study, NIRS was demonstrated to predict $\delta^{13}C$ and $\delta^{15}N$ in plant biomass with surprisingly high accuracy, which corroborates the potential and relevance of this technique for ecological studies (Fig. 1). The most favourable statistics were

Table 2 Statistical summary of the sample set

		n	Range	Mean	SD
Ct (%)	*Astelia pumila*	10	45.72/46.97	46.60	0.61
	Donatia fascicularis	9	47.76/50.66	49.20	0.90
	Empetrum rubrum	10	49.39/53.24	51.12	1.12
	Marsippospermum grandiflorum	16	44.04/47.80	46.87	0.91
	Oreobolus obtusangulus	10	44.07/47.58	46.24	1.01
	Schoenus antarcticus	7	43.26/53.72	46.41	3.35
	Sphagnum magellanicum	10	45.49/47.12	46.31	0.55
	Total	72	43.26/53.72	47.55	2.10
Nt (%)	*Astelia pumila*	10	0.93/1.44	1.09	0.14
	Donatia fascicularis	9	0.60/0.81	0.73	0.08
	Empetrum rubrum	10	0.66/0.91	0.72	0.08
	Marsippospermum grandiflorum	16	0.76/1.30	1.02	0.17
	Oreobolus obtusangulus	10	0.67/0.93	0.84	0.08
	Schoenus antarcticus	7	0.81/1.48	0.96	0.23
	Sphagnum magellanicum	10	0.45/0.62	0.52	0.06
	Total	72	0.45/1.48	0.85	0.23
δ^{13}C (‰)	*Astelia pumila*	10	−26.99/−25.12	−25.98	0.61
	Donatia fascicularis	9	−26.91/−25.60	−26.37	0.45
	Empetrum rubrum	10	−29.50/−27.04	28.16	0.75
	Marsippospermum grandiflorum	16	−26.46/−23.72	−25.29	0.70
	Oreobolus obtusangulus	10	−27.40/−25.74	−26.86	0.55
	Schoenus antarcticus	7	−26.16/−24.83	−25.66	0.49
	Sphagnum magellanicum	10	−28.23/−25.90	−27.00	0.71
	Total	72	−29.50/−23.72	−26.43	1.11
δ^{15}N (‰)	*Astelia pumila*	10	−6.82/−3.07	−5.62	1.16
	Donatia fascicularis	9	−8.00/−4.71	−6.38	1.08
	Empetrum rubrum	10	−12.41/−2.04	−9.68	3.00
	Marsippospermum grandiflorum	16	−0.30/4.63	2.26	1.54
	Oreobolus obtusangulus	10	1.45/4.63	2.90	1.22
	Schoenus antarcticus	7	0.63/5.94	1.97	1.93
	Sphagnum magellanicum	10	−7.94/−2.50	−4.66	1.54
	Total	72	−12.41/5.94	−2.55	5.00

Ct, total carbon; Nt, total nitrogen; SD, standard deviation.

obtained with second derivative mathematical treatment, achieving an R^2 of 0.891 for δ^{13}C and 0.993 for δ^{15}N (Table 3). SECV values of both calibration models derived from cross-validation were 0.722 for δ^{13}C and 1.377 for δ^{15}N, which correspond to 12.5 and 7.5% of the total range within the dataset. A pseudo-correlation of Ct and Nt to their respective isotope ratios could be ruled out ($R^2 = 0.42$ and 0.38). Calibrations derived from a reduced dataset with subsequent external validation demonstrated the reliability of the models developed on the basis of the total dataset (Table 4). For all parameters and mathematical treatments, SECV was close to the standard error of prediction of the validation dataset, but generally slightly higher than SECV calculated from the total dataset (Tables 3, 4).

The calibration models also revealed accurate predictions for single species (Fig. 1). On a species' basis, we found good correlations between actual δ^{15}N and predicted δ^{15}N values (R^2 always exceeded 0.7). For δ^{13}C, we found both strong correlations ($0.5 < R^2 < 0.7$) for species that had a wide range of δ^{13}C values and weak correlations for species with a low variation in δ^{13}C values ($R^2 < 0.4$).

To our knowledge, there are only two studies in which the NIRS technique has been applied to analyse the isotopic composition of plant material. Ferrio *et al.* (2001) used NIRS to predict δ^{13}C in mature kernels of wheat. Using PLSR, they found R^2 values of 0.86 and 0.82 for the calibration and validation datasets, respectively. Clark *et al.* (1995) presented calibration models for alfalfa and some perennial grasses that were able to predict δ^{13}C with acceptable accuracy. However, both Ferrio *et al.* (2001) and Clark *et al.* (1995) emphasized the potential relevance of rapid and cost-effective δ^{13}C measurements by means of NIRS for the food industry or forage production. However, they did not recognize the relevance of their findings in other disciplines, such as ecological research. Coûteaux *et al.* (2003) found a noteworthy ability of NIRS for the prediction of ^{15}N- and ^{13}C-labelled soil organic matter fractions. Because of experimental enrichment, Coûteaux *et al.* (2003) strengthened the isotopic signal within the soil organic matter, and the datasets used for calibration showed a strongly elevated total range of ^{15}N and ^{13}C contents. Our results demonstrate the appropriateness of NIRS technology for the prediction of δ^{13}C and δ^{15}N within the natural range of isotopic composition in plant biomass, which is caused by differences in isotopic fractionation.

The easy, rapid and cost-effective assessment of the isotopic composition by NIRS may help to better capitalize on the

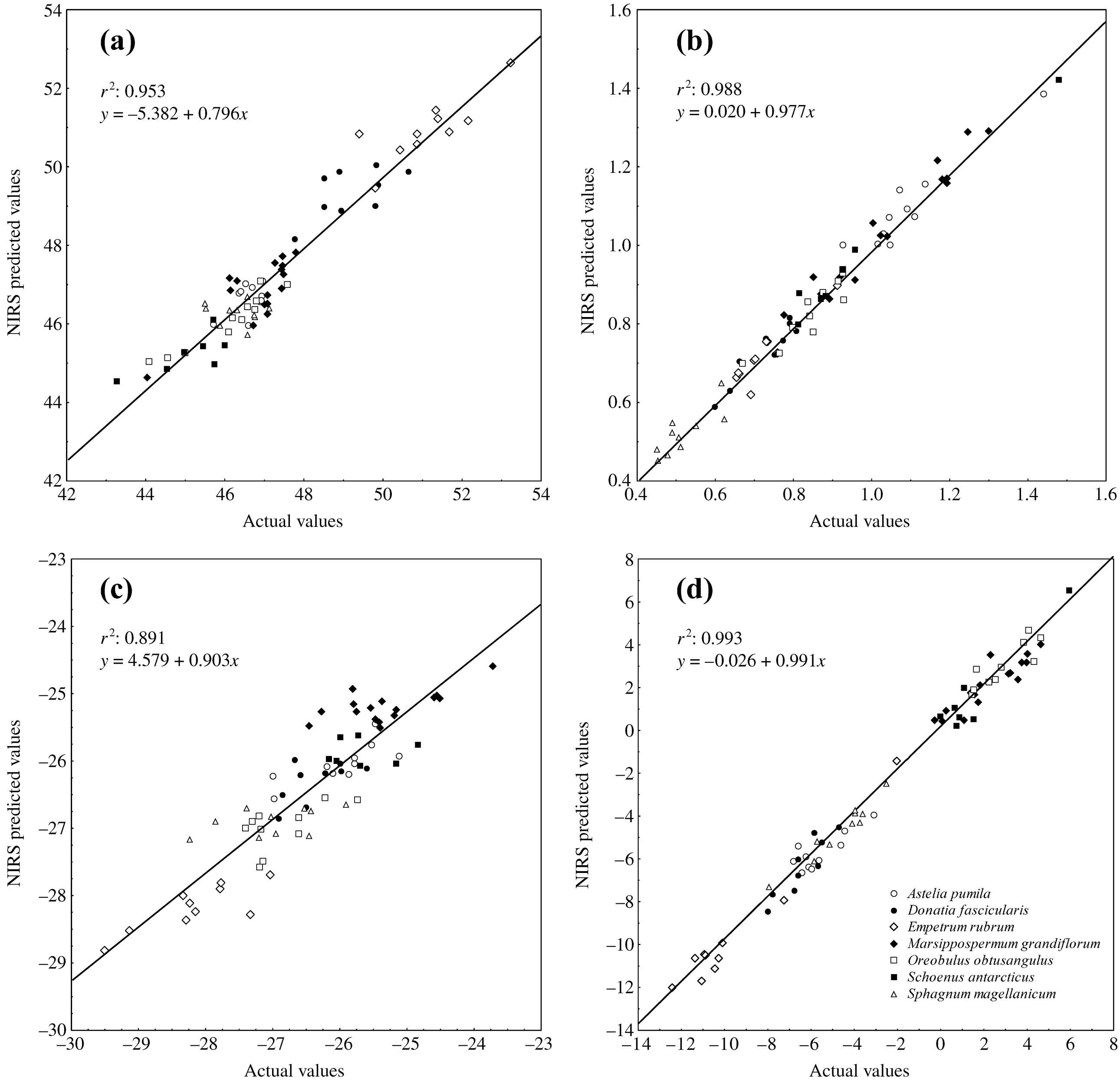

Fig. 1 Relationship between near-infrared reflectance spectroscopy (NIRS) predicted values and measurements by reference methods. For prediction, the partial least-squares regression (PLSR) calibration model with the best cross-validation statistics is presented. (a) Total carbon (%), second derivative (2D); (b) total nitrogen (%), first derivative (1D); (c) $\delta^{13}C$ (‰), 2D; (d) $\delta^{15}N$ (‰), 2D.

ecological information that is contained in the isotopic signal. The measurement of carbon and nitrogen isotopes can be combined with the measurement of other relevant biogeochemical parameters, which have already been proven to predict highly accurately with NIRS (Ruano-Ramos *et al.*, 1999; Petisco *et al.*, 2006). This might be a very useful way to achieve a rapid (the measurement of a NIR spectrum takes less than 2 min) and cheap overview of large datasets and may help to detect patterns to pose scientific questions on underlying key processes. Furthermore, the NIRS technique offers the possibility of sample screening and/or detection of analytical errors.

Our findings clearly demonstrate the great potential of NIRS technology in ecological studies. However, this study is just an initial step. Models must be proven continuously with respect to their robustness and reliability. Larger sample sets should result in even more robust and accurate predictions (Shenk *et al.*, 2008). The accuracy of prediction may also be improved by developing a set of local calibrations comprising closer ranges of $\delta^{13}C$ and $\delta^{15}N$ values and separate calibrations for individual species. Furthermore, the applicability of NIRS for the prediction of other ecologically relevant isotope ratios, such as $\delta^{18}O$ or $\delta^{34}S$, should be tested. NIRS technology was

Table 3 Near-infrared reflectance (NIR) calibration and cross-validation statistics of partial least-squares regression (PLSR) models

		Log 1/*R*	1D	2D
Ct (%)				
	No. factors	7	7	10
	R^2	0.938	0.948	0.953
	SEC	0.685	0.671	0.589
	SECV	0.774	0.768	0.730
	RSC	2.71	2.73	2.88
	Outliers	1*T*, 1*H*, 2*S*	2*T*	1*T*, 1*S*
Nt [%]				
	No. factors	13	11	6
	R^2	0.981	0.988	0.958
	SEC	0.048	0.038	0.066
	SECV	0.088	0.073	0.115
	RSC	2.61	3.15	2.00
	Outliers	1*H*, 1*S*	–	2*T*, 1*H*
$\delta^{13}C$ [‰]				
	No. factors	10	7	5
	R^2	0.848	0.864	0.891
	SEC	0.634	0.588	0.522
	SECV	0.738	0.728	0.722
	RSC	1.51	1.53	1.54
	Outliers	1*T*, 2*S*	1*T*, 3*S*	–
$\delta^{15}N$ [‰]				
	No. factors	11	9	7
	R^2	0.973	0.979	0.993
	SEC	1.260	1.098	0.620
	SECV	1.629	1.460	1.377
	RSC	3.07	3.43	3.63
	Outliers	3*S*	1*T*	–

Transformations for regression analyses: log 1/*R* (*R* = reflectance), first derivative (1D), second derivative (2D).
R^2, coefficient of multiple determination; RSC, ratio of the standard deviation of the reference values to SECV; SEC, standard error of calibration; SECV, standard error of cross-validation.

originally used for quality control in the pharmaceutical and food industries, and is the predominant measurement technique for many compounds of products to date. As shown in this study, established NIRS devices can be used to predict the isotopic composition of live leaf material. However, the practicability of commercial NIRS devices is currently restricted to sufficient sample material, which precludes the use of this promising technology for many ecological analyses, such as the analysis of single tree rings. Thus, in addition to the future improvement of calibrations to better predict the isotopic composition of organic matter, there is also much potential to optimize the technical features of NIRS to specific ecological applications.

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Table 4 External validation statistics of partial least-squares regression (PLSR) calibration models derived from a reduced dataset (n = 59 for calibration, n = 13 for validation)

		Log 1/*R*	1D	2D
Ct (%)				
	Calibration set			
	No. factors	8	7	7
	R^2	0.923	0.938	0.959
	SEC	0.853	0.763	0.598
	SECV	0.969	0.910	0.809
	Outliers	3*T*, 2*S*	2*T*	1*T*
	Validation set			
	r^2	0.925	0.933	0.941
	SEP	0.930	0.865	0.864
Nt (%)				
	Calibration set			
	No. factors	9	11	9
	R^2	0.962	0.990	0.992
	SEC	0.069	0.038	0.033
	SECV	0.094	0.079	0.140
	Outliers	1*T*, 4*S*	2*T*	1*H*, 2*S*
	Validation set			
	r^2	0.863	0.930	0.887
	SEP	0.102	0.077	0.094
$\delta^{13}C$ (‰)				
	Calibration set			
	No. factors	10	7	5
	R^2	0.858	0.862	0.909
	SEC	0.647	0.619	0.497
	SECV	0.799	0.750	0.735
	Outliers	1*T*, 1*H*, 3*S*	1*T*, 3*S*	–
	Validation set			
	r^2	0.790	0.853	0.899
	SEP	0.644	0.523	0.438
$\delta^{15}N$ (‰)				
	Calibration set			
	No. factors	10	9	6
	R^2	0.966	0.975	0.989
	SEC	1.452	1.241	0.564
	SECV	1.894	1.841	1.681
	Outliers	1*T*, 1*S*	3*T*	1*T*
	Validation set			
	r^2	0.951	0.914	0.957
	SEP	1.584	1.990	1.415

Transformations for regression analyses: log 1/*R* (*R* = reflectance), first derivative (1D), second derivative (2D).
R^2, coefficient of multiple determination; SEC, standard error of calibration; SECV, standard error of cross-validation; SEP, standard error of prediction.

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