Evaluating the use of near-infrared reflectance spectroscopy as a proxy measure of carbon and nitrogen isotopes in the leaves of Black Cottonwood (*Populus trichocarpa*)

by

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Abstract

Stable isotopic measurements are one of the more powerful tools used to help advance our understating of plants and their environment. Yet this tool is underutilized because of the large amount of resources and time it takes to extract this information. In this study, I evaluated if near-infrared reflectance spectroscopy can be used as a faster and more economical way to estimate the ratios of carbon and nitrogen isotopes in tree leaves. The $\delta^{13}C$ and $\delta^{15}N$ values were determined with samples of Black Cottonwood leaves *(Populus trichocarpa)* taken from 3 different clones and grown in two different CO_2 concentration conditions. The samples were scanned with a near-infrared reflectance spectrometer (NIRS) to create calibration models. These models are created using partial least-squares regressions and tested by cross validation procedures. The resulting calibration models were unable to accurately predict the amount of $\delta^{13}C$ and $\delta^{15}N$ in the leaves as none of the models could produce a high correlation coefficient. The reflectance spectra produced by the NIRS was able to differentiate the two different CO_2 concentration treatments, and was also able to classify clones from different origins based on their reaction to the CO_2 concentration treatments.

Key words: $\delta^{13}C$, $\delta^{15}N$, isotopic composition, leaf tissue, near-infrared reflectance spectroscopy, partial least-squares regression.

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Introduction

Stable isotope methods have recently emerged as one of the more powerful tools for advancing the understanding of relationships between plants and their environment (Dawson et al., 2002).They are used extensively to examine physiological, ecological, and biogeochemical processes and provide information at a variety of temporal and spatial scales (Bowling et al., 2008). Isotopes with higher mass are usually discriminated against in a reaction because they are less reactive than their lighter counterpart due to the strength of the bonds, which will cause isotopic fractionation between substrate and the product (Farquhar et al., 1989). For example, understanding the ratio of carbon 13 in relation to total carbon in plants cells has been used to classify higher plants into the 3 photosynthetic modes: C3, C4 & CAM (Farquhar et al., 1989). The isotopic ratio of carbon 13 can also be used to assess water-use efficiency of conifers trees and other plants with different environmentally induced and genetic differences (e.g., Sun et al., 1996).

Traditionally, determining the variation in isotopic composition of a sample requires expensive and not very portable instrumentation like an isotope ratio mass spectrometer. Also samples are destroyed after the analyses are done and analysis can be time-consuming. Because of these limitations, many workers are not able to use isotopic composition as one of their measurements for rapid screening of a large amount of samples to detect interesting patterns (Kleinebecker et al., 2009).

As technology develops, near-infrared reflectance spectroscopy (NIRS) has been introduced as a fast low-cost alternative for analysis of the chemical composition of organic materials, especially in the food industry (Clark et al., 1987). A near-infrared spectrometer is very portable compared to the mass spectrometer and costs a fraction of the price. But the biggest advantages of NIRS over mass spectrometry are the speed of analysis, there is little or no sample preparation and that the sample is not burnt or used up after the analyses. Near-infrared radiation is use to induce vibration between C-H, N-H, and O-H bonds which will reflect a different amount of radiation back at different frequency resulting a spectrum (Shenk et al., 2008). To predict the composition of

unknown samples, a model is then made from these spectrums of samples with known chemical composition (Workman, 2008).

Work has been done by Kleinebecker et al. (2009) to show that there is a strong correlation between predicted versus actual values of carbon 13 and nitrogen 15 isotopes using the calibration models they constructed with leaves from seven bog species from southern Patagonia. The goal of my project is to see if there are such correlations between predicted versus actual values of carbon 13 and nitrogen in Black Cottonwood (*Populus trichocarpa*) leaves. Towards this goal, I had also attempted to isolate the specific part of the reflectance spectrum responsible for carbon 13 and nitrogen 15 isotopes.

Methods

The *Populus trichocarpa* leaf samples used in this analysis was collected from a previous experiment done by Buschhaus (2007) on the tissue $\delta^{15}N$ of *Populus trichocarpa* grown in steady-state NH_4^+ nutrition. Three different clones from each latitudinally dispersed populations of *Populus trichocarpa* from Jasper River, OR (44^oN), Quesnel River, BC (52^oN) and Bell-Irving River, BC (56^oN) were chosen for the experiment. Uniform, 5 cm cuttings were rooted and grown in hydroponic medium containing 400 µM of $(NH_4)SO_4$. Half of the samples were then grown in a growth chamber with ambient CO_2 concentration of 400 µL L^{-1} while the other samples were grown in a different chamber with all the same nutrients except with enriched CO_2 concentration of 800 µL L^{-1} inside. The tank CO2 used for these experiments was depleted in 13C relative to normal air by ~24‰, ensuring a large difference in isotopic composition of the plants. After 6 weeks of growth, leaves of each sample were collected and freeze-dried before being ground into fine powder using a ball-mill (Fritsch Laborgerätebau, Terochem Scientific). The $\delta^{13}C$[‰] and $\delta^{15}N$[‰] values of each leaf sample were determined on a Europa ANCA-SL preparation module and a Europa Hydra 20/20 isotope ratio mass spectrometer (University of California Stable Isotope Facility, Davis, CA). There were four characters on the label of each samples. The first two characters identified the clone's origin. The last two characters were the unique identifier for each replicate.

The leftover fine powder samples from the previous analysis was then stored in the laboratory at room temperature and humidity. Samples were then dried by keeping them in air-tight containers partly filled with desiccant for three days before the NIRS analysis (this was found to be essential to obtain good reproducibility). Each sample was then manually packed into the sample capsule with a quartz glass cover and scanned with a QualitySpec Pro Vis/NIR Spectrometer (Analytical Spectral Devices, Inc., Boulder, CO, USA). Measurements were made at 1 nm intervals over the range 1250-2350 nm. Each sample was scanned once per day for three separate days, and each individual scan consisted of 24 single measurements. All of the scans from one sample were then averaged into one resulting spectrum. The spectral data were recorded as reflectance

values and it was transformed to log 1/R (where R is reflectance) before it was used by the calibration modelling software. Full cross validation procedure was used to validate our model because of the relatively small sample size (Martens & Dardenne, 1998; Terhoeven-Urselmans et al., 2006). Each set of spectra was divided into 12 segments and a calibration model was calculated for each segment by only leaving one sample out. Then the model calculated was used to predict the value of the left-out sample to see how close it was to its mass spectrometer value. This process was then repeated for all 12 segments and until every sample had been left out once.

Calibrations were calculated by partial least-squares regression (PLSR) procedures using The Unscrambler (CAMO Software AS. Oslo, Norway). All the samples including the outliers were used for calibration and the related statistical analysis. The optimal number of Principal Components (PCs) used in the model was selected based on the least amount of PCs used while keeping the largest coefficient of multiple determinations for the calibration model (R^2) and the lowest residual variance found. Standard error of prediction (SEP) is a measurement of the difference between the actual and predicted property values calculated over all cross validation calibrations (Kleinebecker et al., 2009). Standard error of calibration (SEC) is exclusively based on spectra used for calibration and indicates the theoretical accuracy when using the calibration to predict unknown spectra (Kleinebecker et al., 2009). The ratio of standard deviation to root mean standard error of the prediction (RPD) was used as a measurement for the model best fit. Models with RPD larger than 2 are consider to have good predictions, and acceptable models should have a RPD value between 1.4 to 2 (Chang et al., 2001).

Analysis was also done with spectra of each sample to try to isolate the section of wavelength most sensitive to the changes of the amount of $\delta^{13}C$[‰] and $\delta^{15}N$[‰] values. This was done by dividing the spectrum of the ambient CO_2 treated clone to the spectrum of its enriched CO_2 treated counterpart. An average of all the ambient CO_2 treated spectra was also divided by the average of all the enriched CO_2 treated spectra for this analysis.

Results

The $\delta^{13}C$ values used for this calibration model have a very wide variation of 19.49‰ and $\delta^{15}N$ values have a very narrow range of 3.05‰ (Table 2). The RSD values for both the $\delta^{13}C$ and $\delta^{15}N$ calibration models were below the acceptable 1.4 limit to be used as adequate models (Table 1). The calibration models generated were unable to accurately predict $\delta^{13}C$ and $\delta^{15}N$ due to their high SEP values and low coefficients of multiple determinations for the cross validation models (r^2) (Table 1, Fig 1 a-b). Four more separate calibration models were calculated using only ambient or enriched CO_2 treated samples to predict $\delta^{13}C$ and $\delta^{15}N$ values. Even with the narrower range of the $\delta^{13}C$ values used in the calibrations, the accuracy of the ambient models only improved slightly while the enriched model did not. All of those models had RPD values below or too close to the 1.4 limit (Table 1, Fig 1 c-f).

With the exceptions of samples from the Quesnel River clone, almost all ambient CO_2 treated samples have higher reflectance values than their enriched counterparts (Fig 2 a). When I divided the ambient CO_2 treated clone's spectrum over its enriched CO_2 treated counterpart, the most dramatic change in reflectance for most samples occurred within the 2050 to 2150nm range. All of the samples from different clones have different change in reflectance patterns yet some samples from the same clones have similar changes in their reflectance patterns (Fig 2 b-g).

Table 1: NIR calibration and cross validation statistics of PLSR models

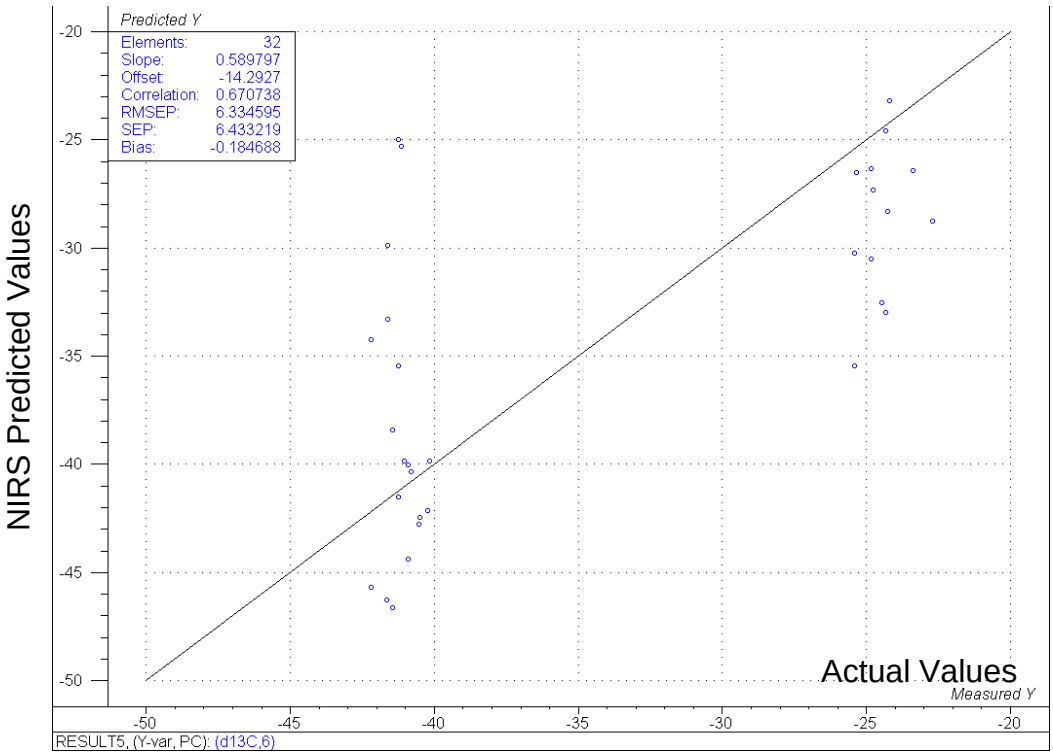
$\delta^{13}C$[‰]	Log 1/R			$\delta^{15}N$[‰]	Log 1/R		
	Complete	Ambient	Enriched		Complete	Ambient	Enriched
No. Factors	6	5	1	**No. Factors**	6	9	2
R^2	0.863	0.954	0.076	**R^2**	0.856	0.999	0.549
SEC	4.221	0.234	0.569	**SEC**	0.446	0.029	0.776
r^2	0.671	0.709	-0.690	**r^2**	0.630	0.624	0.282
SEP	6.433	0.571	0.634	**SEP**	0.727	0.608	0.928
RMSEP	6.335	0.549	0.617	**RMSEP**	0.716	0.586	0.903
RPD	0.090	1.422	0.925	**RPD**	1.296	1.323	1.027
Outliers	2	1	0	**Outliers**	14	2	3

Transformations for regression analyses: log 1/R (R = reflectance), R^2, coefficient of multiple determination; SEC, standard error of calibration; r^2, coefficient of multiple determination for the cross validation model; SEP, standard error of prediction by cross validation; RMSEP, root mean square of the SEP; RPD, ratio of the standard deviation of the reference values to RMSEP.

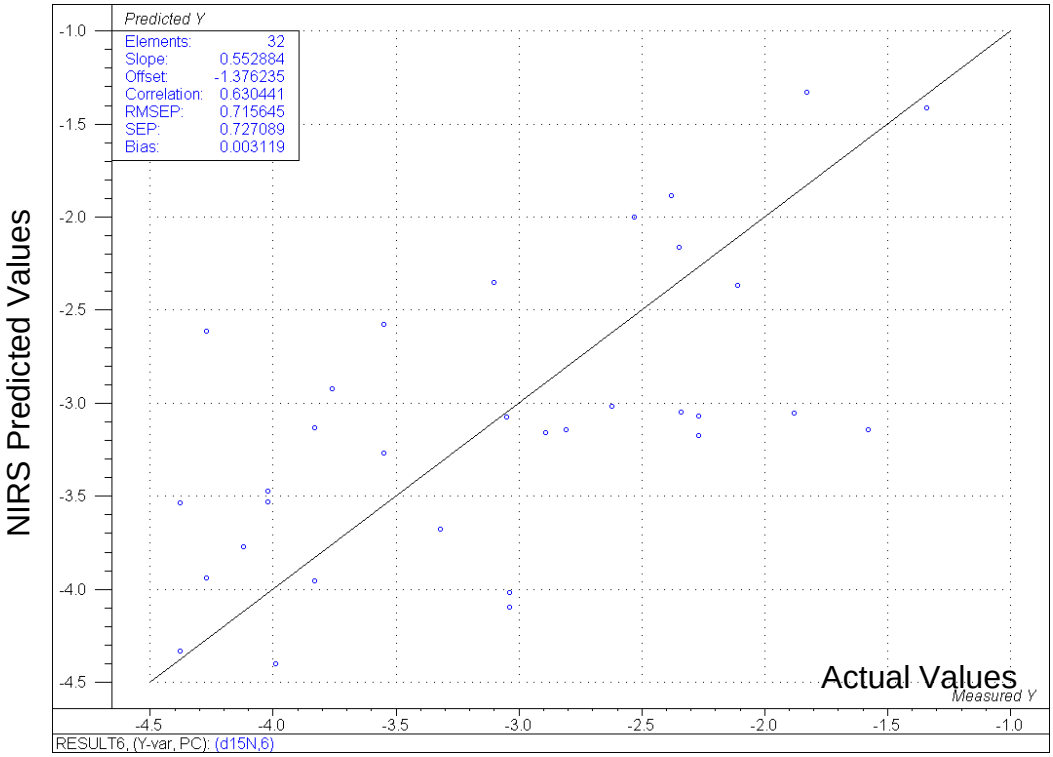
Table 2: Statistical summary of the sample set

Sample	CO2	Leaf $\delta^{15}N$ [‰]	Leaf $\delta^{13}C$ [‰]
I1BL	Ambient	-1.88	-24.48
I1BL	Enriched	-1.58	-40.25
I1CL	Ambient	-2.62	-24.18
I1CL	Enriched	-2.11	-41.03
I1LA	Ambient	-4.12	-24.76
I1LA	Enriched	-2.35	-40.54
I1LB	Ambient	-3.10	-23.39
I1LB	Enriched	-2.38	-40.17
I1LC	Ambient	-2.53	-22.72
I1LC	Enriched	-2.34	-41.65
J1BL	Ambient	-4.38	-24.33
J1BL	Enriched	-4.27	-41.61
J1LA	Ambient	-3.99	-25.35
J1LA	Enriched	-2.81	-40.82
J1LB	Ambient	-4.38	-24.33
J1LB	Enriched	-4.27	-41.61
J2AL	Ambient	---	---
J2AL	Enriched	-4.02	-42.20
J2LA	Ambient	---	---
J2LA	Enriched	-4.02	-42.20
J2LB	Ambient	---	---
J2LB	Enriched	-3.76	-41.46
J4AL	Ambient	-3.04	-24.85
J4AL	Enriched	-3.55	-41.24
J4BL	Ambient	-3.83	-25.41
J4BL	Enriched	-2.27	-40.92
J4LA	Ambient	-3.04	-24.85
J4LA	Enriched	-3.55	-41.24
J4LB	Ambient	-3.83	-25.41
J4LB	Enriched	-2.27	-40.92
Q2LB	Ambient	-3.32	-24.28
Q2LB	Enriched	-2.89	-41.14
Q3LA	Ambient	---	---
Q3LA	Enriched	-1.34	-41.45
Q3LB	Ambient	---	---
Q3LB	Enriched	-1.83	-40.52
Q4LA	Ambient	---	---
Q4LA	Enriched	-3.05	-41.26

	Leaf $\delta^{15}N$	Leaf $\delta^{13}C$
For All Samples		
Number	32	
Mean	-3.09	-34.39
Standard Deviation	0.893	8.350
Range	-4.38	-42.20
	-1.34	-22.72
Ambient Samples Only		
Number	13	
Mean	-3.390	-24.487
Standard Deviation	0.775	0.781
Range	-4.384	-25.412
	-1.876	-22.716
Enriched Samples Only		
Number	19	
Mean	-2.877	-41.171
Standard Deviation	0.928	0.571
Range	-4.270	-42.201
	-1.339	-40.168

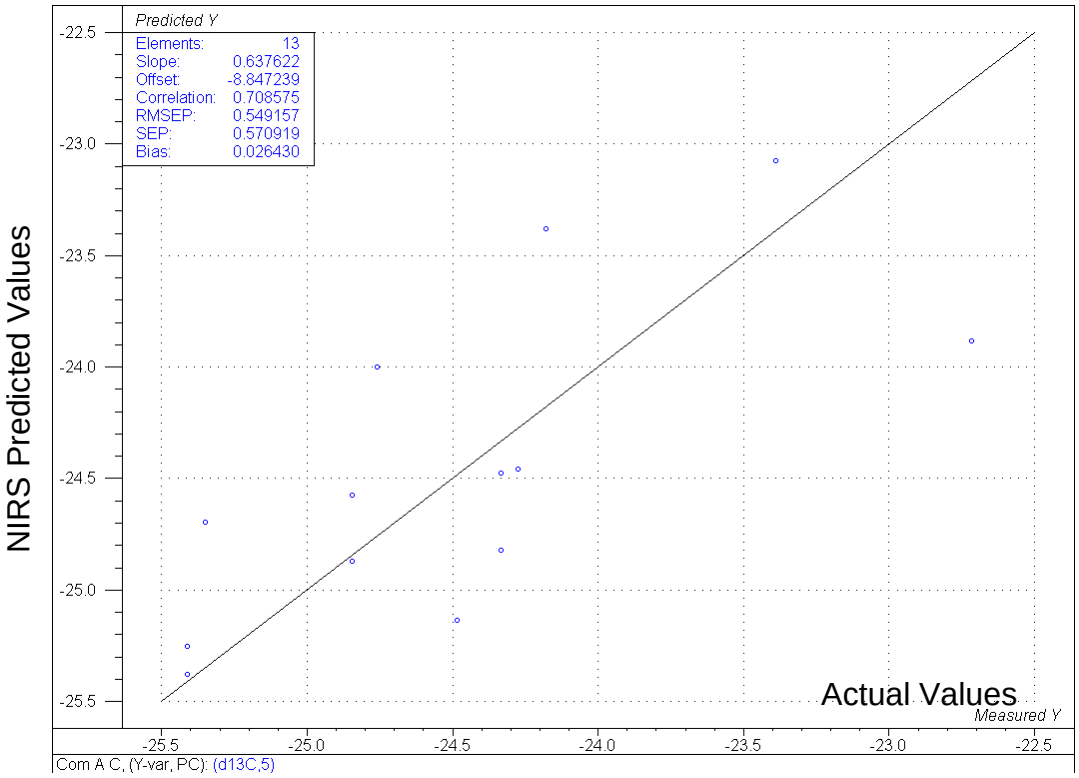


(a) Predicting $\delta^{13}C$ [‰] using all the samples.

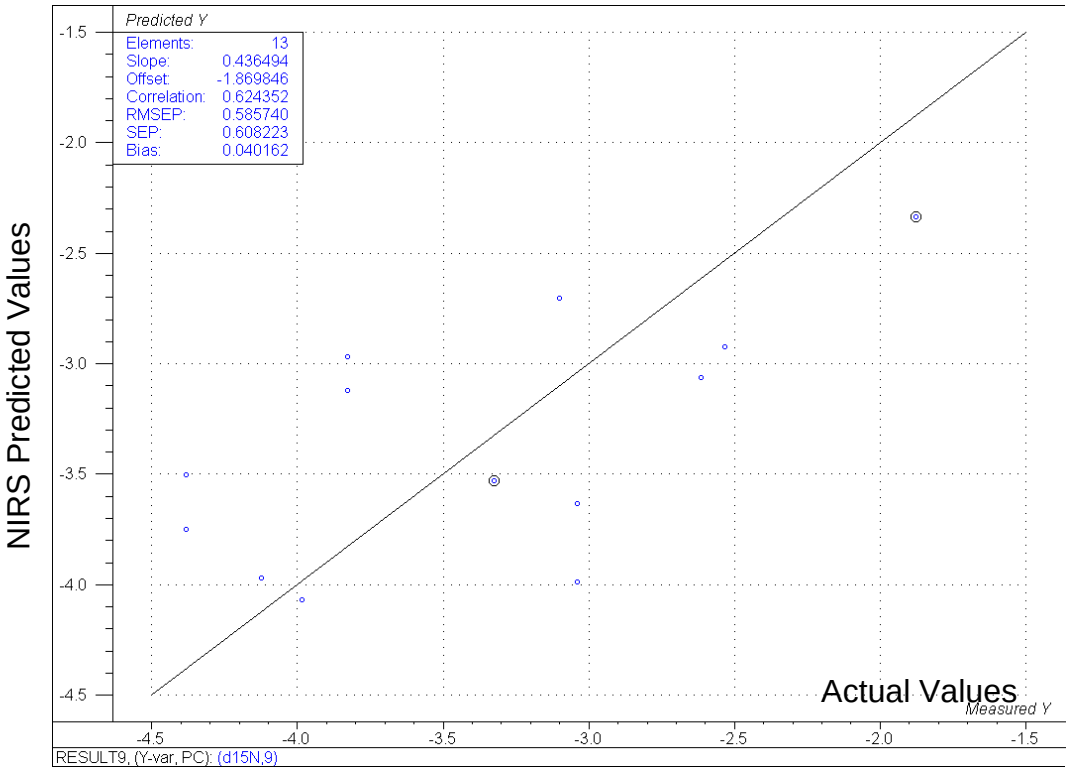


(b) Predicting $\delta^{15}N$ [‰] using all the samples.

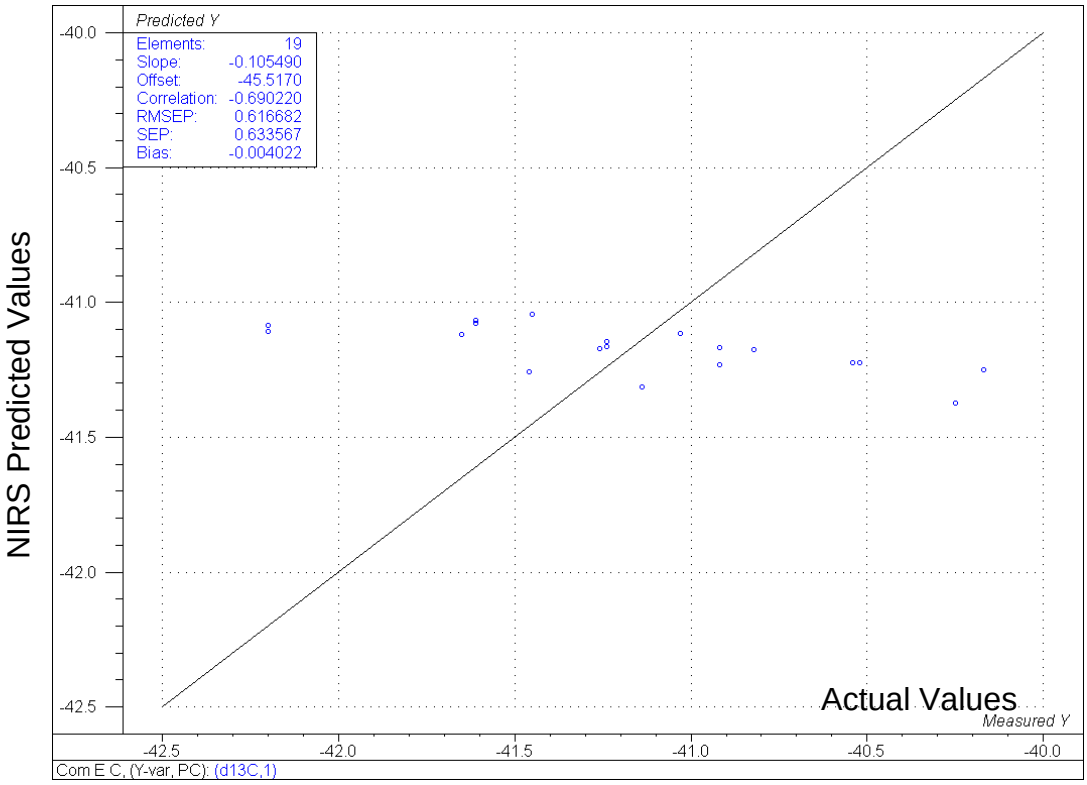
Figure 1: Relationship between NIRS predicted values and measurements by reference methods. For prediction, the partial least-square regression (PLSR) calibration model with the best cross validation statistics is presented.



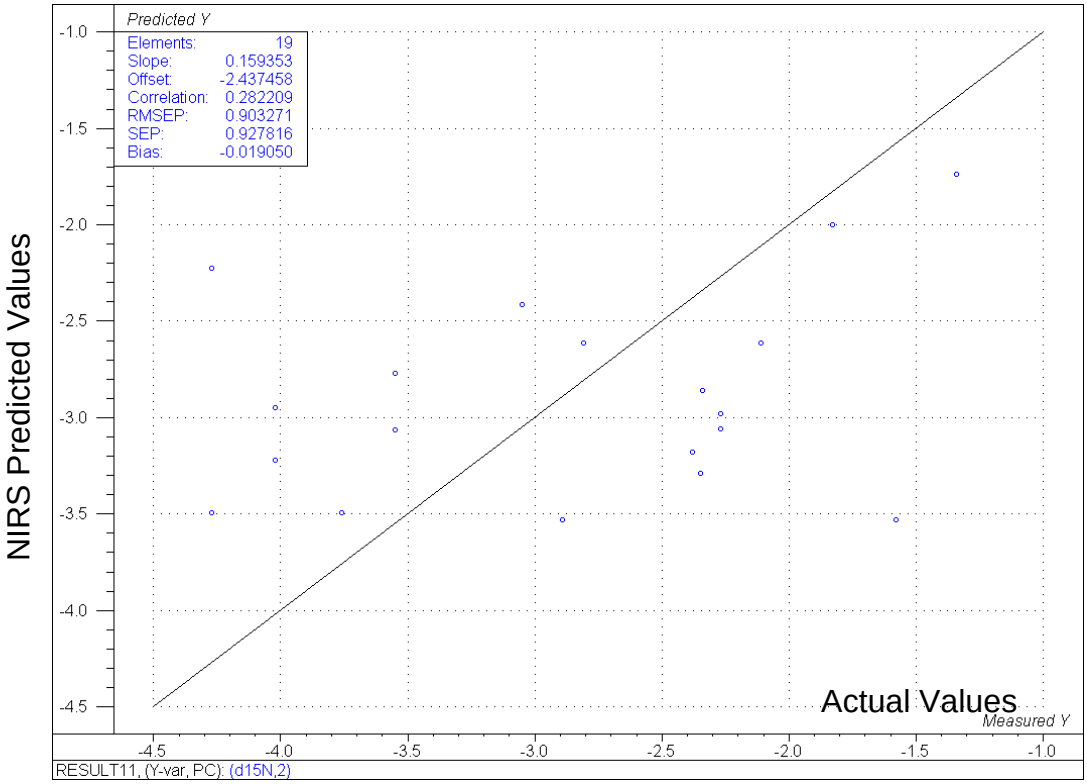
(c) Predicting $\delta^{13}C$ [‰] using ambient CO_2 treated samples only.



(d)) Predicting $\delta^{15}N$ [‰] using ambient CO_2 treated samples only.



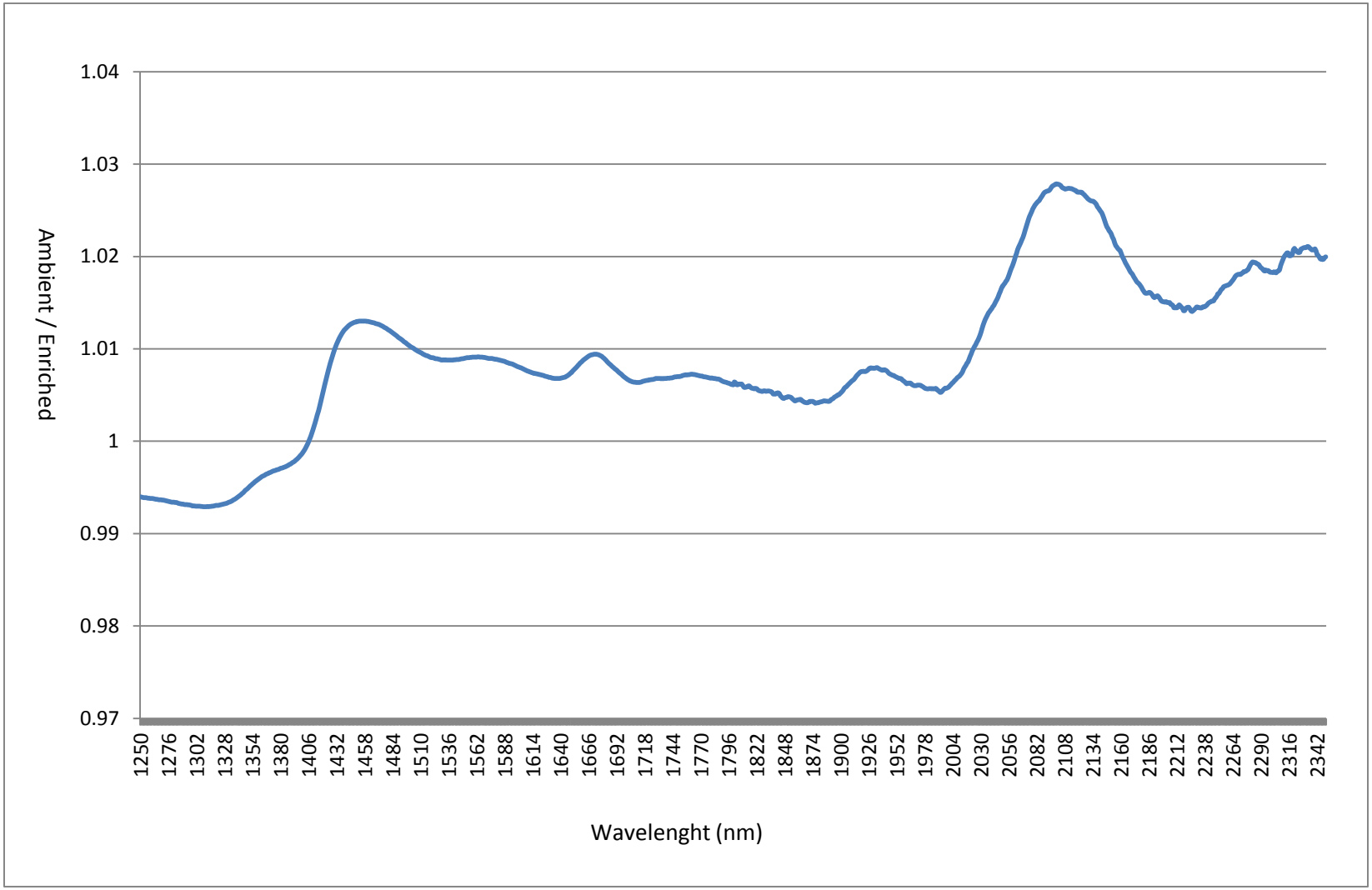
(e) Predicting $\delta^{13}C$ [‰] using enriched CO_2 treated samples only.

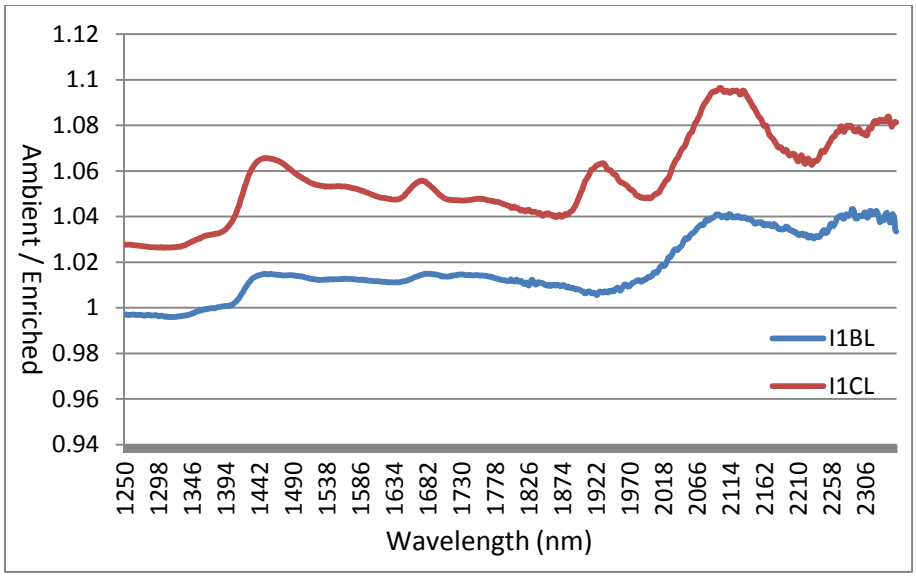


(f) Predicting $\delta^{15}N$ [‰] using enriched CO_2 treated samples only.

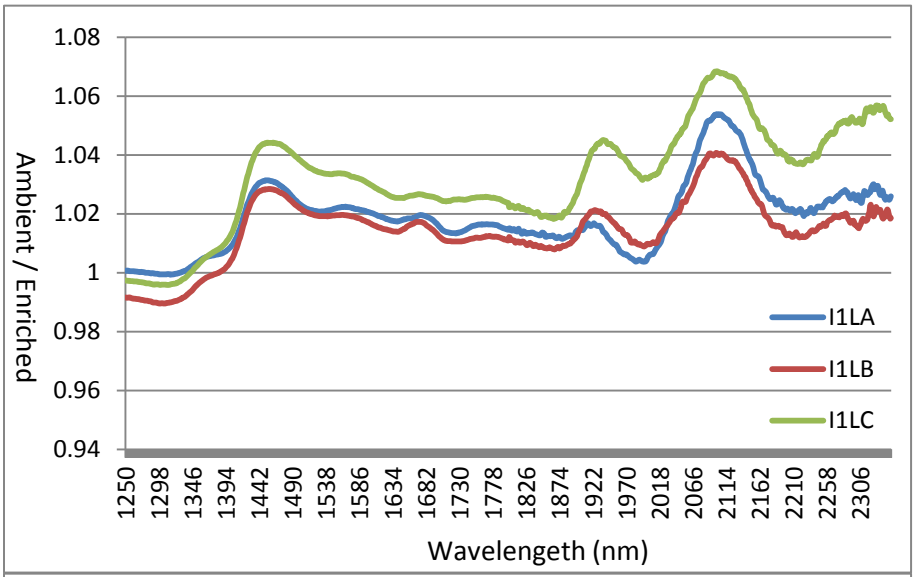
Figure 2: Ambient / Enriched CO_2 treatment changes in reflectance spectra graphs

(a) Average ambient CO_2 treated samples / average enriched CO_2 treated samples

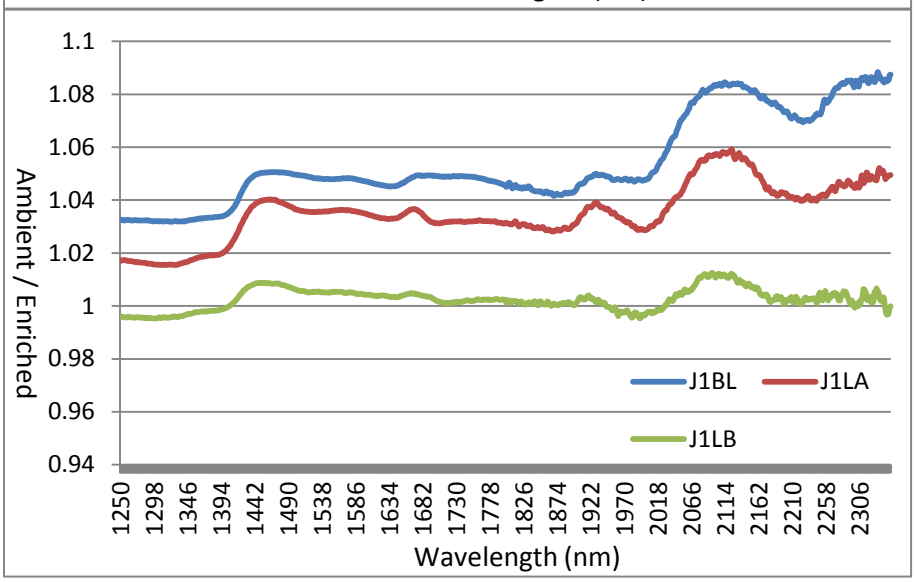




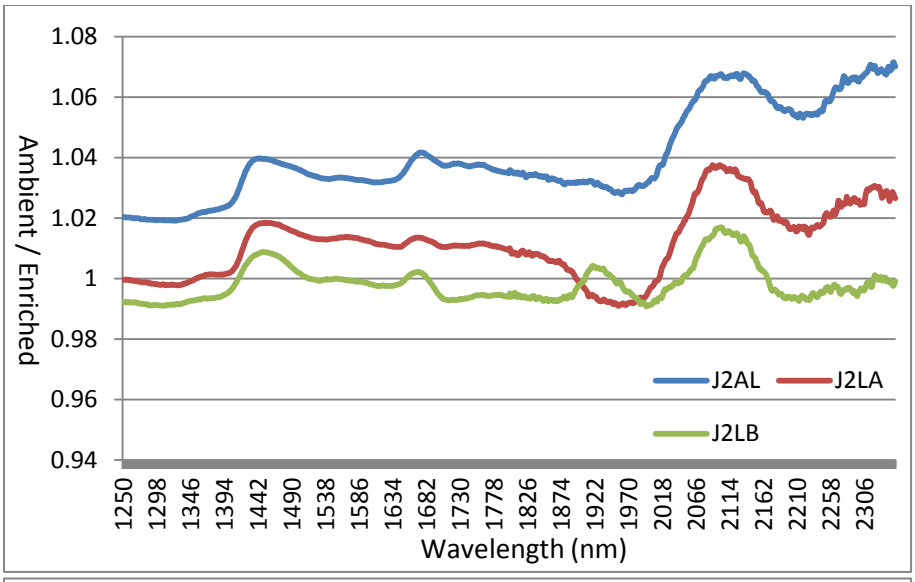
(b) Ambient / enriched CO_2 treated samples from the Bell-Irving River clone replicates (I1)



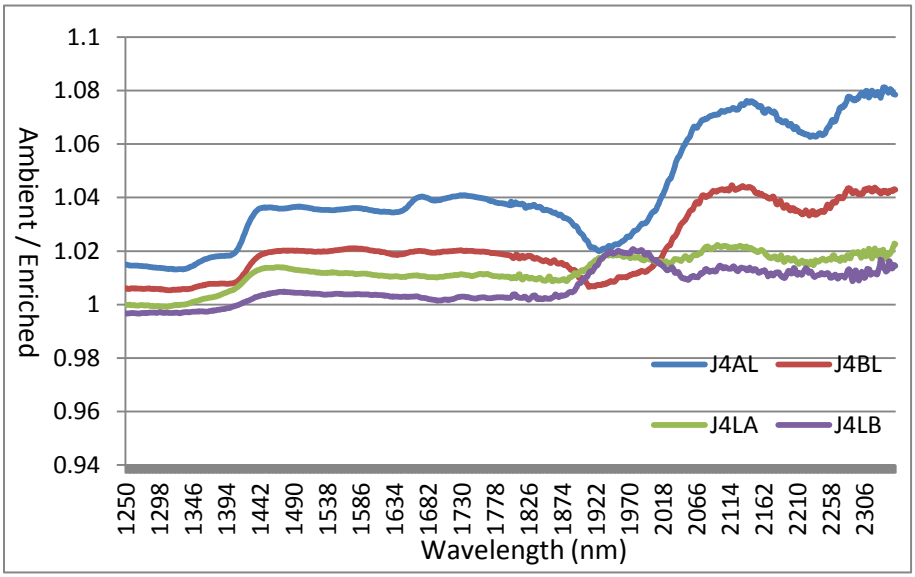
(c) Ambient / enriched CO_2 treated samples from Bell-Irving River clone replicates (I1L)



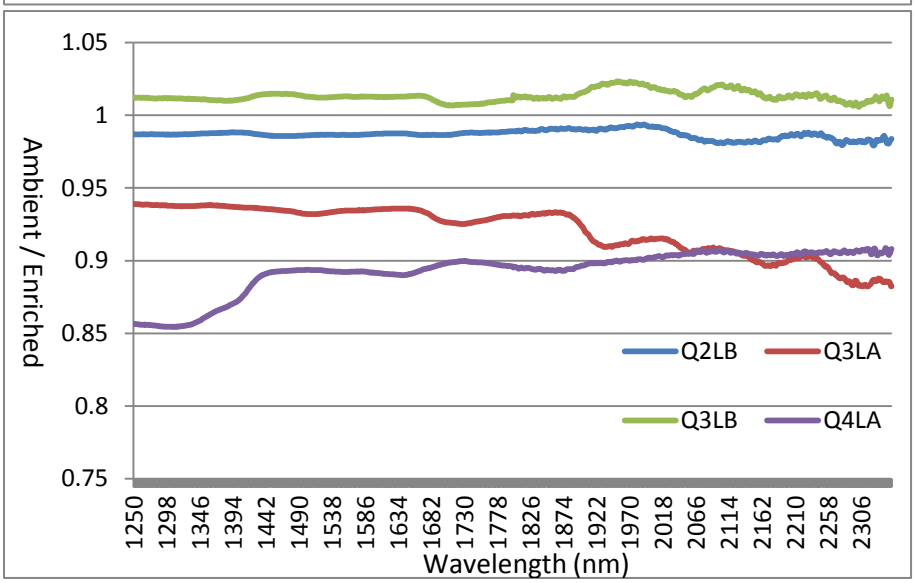
(d) Ambient / enriched CO_2 treated samples from the Jasper River clone replicates (J1)



(e) Ambient / enriched CO_2 treated samples from the Jasper River clone replicates (J2)



(f) Ambient / enriched CO_2 treated samples from the Jasper River clone replicates (J4)



(g) Ambient / enriched CO_2 treated samples from the Quesnel River clone replicates

Discussion

Although I was unable to find a calibration model that can accurately predict the $\delta^{13}C$ and $\delta^{15}N$ values in *Populus trichocarpa* leaves, the NIRS was able to differentiate samples that have been treated with enriched CO_2 concentration from its ambient counterparts. This is evident as almost all ambient CO_2 treated samples have higher reflectance value than its enriched counterpart (ambient / enriched > 1) (Fig 2 a).

I had also found that the calibration models with only ambient CO_2 treatment samples outperformed the calibration models with only enriched samples at predicting $\delta^{13}C$ and $\delta^{15}N$ values (Table 1). This suggests that the CO_2 treatment has a tremendous effect on the reflectance properties of the sample that are not dependent on the $\delta^{13}C$ and $\delta^{15}N$ values. This result was consistent with the findings reported by Clark et al. (1995) where they found the NIRS could correctly identify the $\delta^{13}C$ value of alfalfa (Medicago sativa L.) and several cool-season perennial grass samples 77 to 82% of the time but only for the $\delta^{13}C$ values that were in the lower 20% of their dataset. The CO_2 enriched treatment not only interfered with the $\delta^{13}C$ calibration model but with the $\delta^{15}N$ model as well. According to Chang et al. (2001), the influence of wavelength on the correlation between reflectance intensity and total carbon and nitrogen content were similar. They believed that the similarity in spectral response for carbon and nitrogen was due to their high intercorrelation.

Further study is needed to be done on the CO_2 effect in order for us to have a more accurate prediction of $\delta^{13}C$ and $\delta^{15}N$ values in leaves with higher carbon content. An experiment with more than two CO_2 concentration treatments and more in-depth analysis from the mass-spectrometer would help create calibration models with many different reference values. This may hopefully all us to understand what affects the reflectance intensity the most in leaves with high carbon content.

The NIRS not only picked up the effect of CO_2 concentration on the samples, it also picked up the variables that were more dependent on the location of where the samples were originated. As shown in Figure 2 b to g, each samples from different clones reacted to the CO_2 treatment differently but samples of the same clone replicates seem

to follow a distinct pattern. Chang et al. (2001) in their experiment on soil, also noted that there was a tendency for regional similarity in NIR reflectance spectra of soils. They noted that all the soil had peaks in their spectra at similar wavelengths but soil originating from different regions peaks at different intensity. Sun et al. (2012) were also able to classify lamb meat between pastoral regions from agricultural region using NIRS. They also had an 80% accuracy in classifying lamb meat samples from five different geographical origins.

Perhaps a better calibration model for predicting $\delta^{13}C$ and $\delta^{15}N$ values can be produced if both the effect of CO_2 concentration and clonal variation can be minimize. More samples are needed from each population to minimize the effect of clonal variations. Clone replicates subject to CO_2 with different isotopic compositions without changing the overall CO_2 concentration can also minimize the effect of CO_2 concentration. Through the above suggestions, the resulting calibration model should yield results similar to those that are done by Kleinebecker et al. (2009). This will then translate to more accurate predictions of $\delta^{13}C$ and $\delta^{15}N$ values with samples of various species at from different locations.

Although there are no research performed on predicting $\delta^{13}C$ and $\delta^{15}N$ values from woody plant species to this day, a study done by Petisco et al. (2005) was performed by a NIRS to predict the nitrogen, phosphorus and calcium content in leaf samples in of several woody species from various locations. In that study, they were able to successfully predict the total nitrogen content in leaves, and they were also able to conclude that wavelengths between 2,024 and 2,176 nm to be the most relevant in their nitrogen calibration. They came to that conclusion by looking at the equations used by the calibration processes. At another study done on lamb meat, Sun et al. (2012) found success at predicting $\delta^{13}C$ and $\delta^{15}N$ values. They suggested the spectra ranged between 1380 and 1530 nm to be most relevant in their calibration model. This was consistent with the data of this study as the second highest change in the reflectance spectra of the average ambient over enriched CO_2 treatment also occurs at the spectra ranged between 1380 and 1530 nm (Figure 2 a). Sun et al. (2012) theorized that the strong absorptions observed at the 1380 and 1530 nm relates to the C-H combination

vibrations and N-H first overtones. They believed that those bond vibrations were probably associated with carbohydrate and protein compounds in the defatted lamb meat. Based on the above findings, locating the wavelengths most relevant to $\delta^{13}C$ and $\delta^{15}N$ values for plants should be possible as long as there is an accurate model that can predict these values.

Neglecting the fact that NIRS was unable to perform isotopic analysis for woody plant species, NIRS is still a very powerful tool for plant physiology. The use for this technology are endless as NIRS is a proxy measurement that have many different correlations with many different elements. The limiting application of NIRS is in finding a suitable data pre-treatment and calibration strategies (Chang et al., 2001). One relatively quick and non-destructive scan of NIRS can estimate the total nitrogen, lignin, cellulose and many other different properties in a leaf of a woody plant from a sample set with several species coming from clearly different environmental conditions and leaf morphology and physiology (Petisco et al., 2006). Therefore NIRS has the potential to be used as a quick-screening and high-throughput phenotyping tool for leaf chemistry and many other plant physiology applications.

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