NOTES

# Comparison of wet chemistry and near infrared reflectance measurements of carbon-fraction chemistry and nitrogen concentration of forest foliage

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Received December 5, 1990
Accepted May 15, 1991

MCLELLAN, T.M., MARTIN, M. E., ABER, J. D., MELILLO, J. M., NADELHOFFER, K. J., and DEWEY, B. 1991. Comparison of wet chemistry and near infrared reflectance measurements of carbon-fraction chemistry and nitrogen concentration of forest foliage. Can. J. For. Res. **21**: 1689–1693.

An interlaboratory comparison was designed to test the precision and relative accuracy of a method using near infrared spectroscopy for the determination of nitrogen, lignin, and cellulose concentrations in green and senescent foliage. A total of five laboratories were involved. Two methods of nitrogen analysis (Kjeldahl and CHN analysis) and two methods of carbon-fraction (lignin and cellulose) analysis were used. Equations for converting near infrared reflectance spectra into estimates of nitrogen and carbon-fraction concentrations are presented, along with estimates of relative bias between laboratories and methods. Analytical errors (variation among repeated measurements of the same sample) associated with each of the different methods are also presented. Results show that the near infrared reflectance method gives values that lie within the range of values generated by wet chemistry methods and so introduces no additional bias. Analytical errors are one-third to one-fifth those of wet chemistry methods for nitrogen concentration and one-fifth to one-eighth those of wet chemistry methods for lignin and cellulose concentration.

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Une comparaison inter-laboratoire a été effectuée afin de vérifier la précision et l'exactitude relative d'une méthode de réflectance spectrométrique dans le proche infrarouge pour la détermination des concentrations d'azote, de lignine et de cellulose dans le feuillage vert et sénescent. Cinq laboratoires ont participé à l'étude. Deux méthodes chimiques de détermination de l'azote (Kjeldahl et un analyseur CHN) et deux méthodes chimiques d'analyse des fractions carbonées (lignine et cellulose) ont été utilisées. Les équations pour convertir les spectres de réflectance dans le proche infrarouge d'azote et des concentrations des fractions carbonées sont présentées avec les estimés des biais relatifs entre les laboratoires et entre les méthodes. Les erreurs analytiques (la variation entre les mesures répétées sur un même échantillon) associées avec chacune des différentes méthodes sont aussi présentées. Les résultats montrent que la méthode de réflectance dans le proche infrarouge donne des valeurs qui reposent entre l'étendue des valeurs obtenues par les méthodes chimiques et qu'elle n'introduit pas de biais additionnel. Les erreurs analytiques sont du tiers au cinquième de celles des méthodes chimiques pour la concentration d'azote et du cinquième au huitième de celles des méthodes chimiques pour la concentration de la lignine et de la cellulose.

[Traduit par la rédaction]

## Introduction

In two recent papers (Wessman et al. 1988*a*; McLellan et al. 1991) we suggested that high spectral resolution near infrared reflectance (NIR) spectroscopy might offer an attractive alternative to traditional wet chemistry methods for the determination of nitrogen and carbon-fraction (lignin and cellulose) concentration in green, senescent, and decaying plant materials. The advantages of this method include rapid analysis (4–5 min per sample), low cost (ca. $15 per sample for total nitrogen, lignin, and cellulose), and greatly increased potential for standardization and interlaboratory comparability. NIR analysis also requires no toxic chemicals and reduces the chances of technician error.

NIR has been used for more than 20 years to measure protein, fiber, oil, moisture, and other constituents in agricultural products (Norris and Hart 1965; Norris et al. 1976; Shenk et al. 1981; Winch and Major 1981) and has become a standard method of analysis for several commercial applications. However, its application to nitrogen, lignin, and cellulose concentration of foliage of native woody plants has not been widely tested. Before this method can be adopted generally, it must be validated against the existing standard methods.

The purpose of this paper is to present data on the accuracy and precision of NIR and wet chemistry methods resulting from an interlaboratory comparison of analyses of nitrogen, lignin, and cellulose concentration of fresh and senescent foliage of woody plants. We also present new calibration equations for determining nitrogen, lignin, and

Printed in Canada / Imprimé au Canada

TABLE 1. Site, species, and collection date of samples used in the interlaboratory comparisons

| Site and species | Collection date |
|---|---|
| University of Wisconsin Arboretum, Madison, WI | |
| *Acer saccharum, Betula papyrifera, Quercus rubra, Picea glauca, Pinus strobus, Pinus resinosa* | 1980 |
| *Acer saccharum, Prunus serotina, Tilia americana, Ostrya virginiana* | 1988 |
| Blackhawk Island, WI | |
| *Acer rubrum, Quercus alba, Quercus rubra, Quercus velutina, Pinus resinosa, Pinus strobus* | 1984, 1985 |
| White Mountains, NH | |
| *Acer rubrum, Betula papyrifera, Abies balsamea, Picea rubens* | 1987 |
| Harvard Forest, MA | |
| *Acer rubrum, Quercus velutina, Prunus serotina, Castanea dentata, Pinus resinosa* | 1988 |
| Austria | |
| *Picea abies* | 1988 |

cellulose from NIR spectra. These new equations are based on a larger set of spectra obtained with a different instrument than the results presented by Wessman et al. (1988*a*).

## Methods

*Sample set development*

*Calibration data set*

A total of 255 samples were used to develop the new calibration equations for nitrogen, lignin, and cellulose concentration of fresh and senescent foliage of woody plants. These included fresh and senescent foliage samples from 18 species (6 needle-leaved conifers and 12 broad-leaved deciduous) collected from field sites in Wisconsin, New Hampshire, Vermont, Massachusetts, and Austria. All samples were analyzed for total nitrogen content using methods described later. A total of 154 of these samples were analyzed for lignin and 148 for cellulose using the methods of McClaugherty et al. (1985). Equation development was carried out using the methods reported by Wessman et al. (1988*a*) and McLellan et al. (1991). The interlaboratory comparisons presented in this paper are, in part, an attempt to validate these equations.

*Interlaboratory data set*

An additional set of 75 samples, none of which were used in the development of the calibration equations, was selected for the interlaboratory comparison. This set was chosen to cover the range of species (Table 1), constituent values (nitrogen, lignin, and cellulose, Table 2), condition (green versus senescent), and physiognomy (needle-leaved coniferous versus broad-leaved deciduous) present in the calibration set. All samples were oven-dried at 70°C for 48 h, ground to pass a 1-mm mesh, and stored in airtight containers out of direct light.

Samples were analyzed by wet chemistry procedures in a total of five different laboratories (Table 3). All samples were analyzed by NIR (see description of method later). All samples were also analyzed in at least one, and usually two, wet chemistry laboratories. Because of the expense of wet chemistry analyses of carbon-fraction content, it was not possible to analyze all samples at all laboratories.

*Replicate samples for determination of analytical error*

Analytical error was determined for the wet chemistry procedures by sending 13 blind duplicate samples to the Soil and Plant Analysis Laboratory in Madison, Wisconsin, for total nitrogen analyses by the Kjeldahl method; 16 blind duplicate samples to the Ecosystem Center laboratory, Woods Hole, Massachusetts, for carbon-fraction chemistry; and 15 blind duplicate samples to the Natural Resources Research Institute laboratory, University of Minnesota–Duluth, for both carbon-fraction and CHN analyses. For the NIR method, a total 33 duplicate samples were analyzed.

TABLE 2. Range of constituent values (%) in interlaboratory sample set

| Nitrogen | | Lignin | | Cellulose | |
|---|---|---|---|---|---|
| Min. | Max. | Min. | Max. | Min. | Max. |
| 0.45 | 2.78 | 7.8 | 29.1 | 31.8 | 56.2 |

An additional test was made of the potential for long-term drift in NIR analyses. A set of 15 green foliage samples from red spruce (*Picea rubens* Sarg.) was analyzed three times at yearly intervals (in 1987, 1988, and 1989). This procedure also tests the stability of stored samples relative to NIR spectra.

*Wet chemistry analyses*

Nitrogen was measured either by the micro-Kjeldahl technique, with digestate analyzed by automated solution chemistry (McClaugherty et al. 1985), or by CHN analyses (Cappo et al. 1987, see Table 3). Lignin and cellulose concentrations were determined by a wood-products chemistry procedure (Effland 1977) either as modified by McClaugherty et al. (1985) or by the substitution of a sonic water bath for reflux in the determination of polar and nonpolar extractable content (J. Pastor and B. Dewey, personal communication, see Table 3).

*NIR spectroscopy*

Before analysis, all samples were redried overnight at 70°C, allowed to cool for 1 h, then thoroughly mixed. Spectra were generated by an NIR Systems model 6250 near infrared spectrophotometer, which employs an oscillating concave holographic grating. This grating covers a spectral range of 1100–2500 nm at 2-nm intervals with a bandwidth of 10 nm. Reflectance ($R$) data are converted to absorbance ($A$) by the following equation:

$$A = \log(1/R)$$

Samples (1–3 g required for analysis) were scanned four times, averaging 4.5 min per sample. Each sample was scanned once, rotated 90°, scanned a second time, thoroughly mixed outside the sample cell, scanned a third time, rotated 90°, and scanned a fourth time. Spectra generated by each of the four scans actually represent the mean of 50 separate internal scans. Thus the final spectrum used is the result of 200 actual scans per sample. Each spectrum is the reflectance of the sample material expressed relative to the reflectance of an internal ceramic standard. The average of the four scans was transformed to a second difference approximation of the second derivative (Casciero and DiGiovanni 1985). Derivative spectra are frequently used in equation development to reduce errors due to base-line shifts between samples and to accentuate fine-scale spectral features (Wessman et al. 1988*a*; Hruschka 1987).

In developing the calibration equations, a stepwise forward multiple linear regression routine contained in the NIR Systems spectral analysis software was used (Casciero and DiGiovanni 1985). Two approaches can be taken to the selection of wavelengths.

In the first approach, wavelengths are selected strictly on the basis of statistical correlation. This provides the best fit to the current data set, but unless the selected wavelengths have known biophysical meaning (i.e., represents overtones of bond types known to be important in the constituent of interest), the resulting equation may not be accurate in predicting other data sets.

In the second approach, wavelengths of known biophysical importance are forced into the regression equation. This yields an equation with a strong theoretical basis, but may perhaps be less accurate for any one set of samples. It may also fail to correct for interference at particular wavelengths produced by other constituents. A combination of user- and machine-selected wavelengths

TABLE 3. Location and method of wet chemistry analyses for different subsets of the interlaboratory sample set

| | Nitrogen | | Carbon fraction | |
|---|---|---|---|---|
| Sample set | Laboratory | Method | Laboratory | Method |
| 1 | USDA Forest Service, Durham, NH | Kjeldahl | Ecosystem Center, Marine Biological Laboratory, Woods Hole, MA | Reflux |
| 2 | Natural Resources Research Institute, University of Minnesota - Duluth | CHN | Natural Resources Research Institute, University of Minnesota - Duluth | Sonic |
| 3 | Plant and Soil Analysis Laboratory, Madison, WI | Kjeldahl | University of Wisconsin, Department of Forestry | Reflux |
| 4 | Plant and Soil Analysis Laboratory, Madison, WI | Kjeldahl | Ecosystem Center, Marine Biological Laboratory, Woods Hole, MA | Reflux |
| 5 | Ecosystem Center, Marine Biological Laboratory, Woods Hole, MA | CHN | Ecosystem Center, Marine Biological Laboratory, Woods Hole, MA | Reflux |
| 6 | USDA Forest Service, Durham, NH | Kjeldahl | | |

NOTE: Carbon-fraction method denotes whether the evaporation-condensation reflux method or the sonic bath method was used for the determination of extractive content.

TABLE 4. Results of the calibration used to define wavelengths for converting near infrared spectra to nitrogen, lignin, and cellulose concentrations

| | | Statistics | | |
|---|---|---|---|---|
| Wavelength (nm) | Associated structure | $n$ | $r$ | SEC |
| | **Nitrogen** | | | |
| 2170 | Protein | 255 | 0.98 | 0.12 |
| 1686 | Aromatic | | | |
| 1978 | $CONH_2$ | | | |
| | **Lignin** | | | |
| 1438 | $CH_2$ | 154 | 0.88 | 2.53 |
| 2386 | Oil | | | |
| 2218 | CHO | | | |
| 1828 | Cellulose | | | |
| | **Cellulose** | | | |
| 2140 | CH | 148 | 0.90 | 2.63 |
| 1766 | $CH_2$ | | | |
| 1960 | Starch | | | |
| 1982 | $CONH_2$ | | | |

NOTE: Biophysical associations with wavelengths are according to Osborne and Fearn (1986). SEC, standard error of calibration; $r$, correlation coefficient of multiple linear regression.

was used for all three constituents (see McLellan et al. 1991 for a complete description of methods used in wavelength selection).

*Statistical analyses*

Analytical differences between laboratories were tested for significance using Student's $t$-test for paired samples (Snedecor and Cochran 1967).

## Results and discussion

*Calibration equations*

The calibration equations developed (Table 4) contain wavelengths of known biophysical importance for the constituent of interest and yield standard errors of calibration (SEC) in the same range as reported in two previous papers (Wessman et al. 1988*a*; McLellan et al. 1991). The specific wavelengths differ from those of previous equations, although many are in similar regions of the spectrum.

TABLE 5. Estimate of relative bias (mean of pair-wise differences between measurements) for nitrogen, lignin, and cellulose by NIR spectroscopy and wet chemistry methods from several laboratories

| Data set | $n$ | Nitrogen | Lignin | Cellulose |
|---|---|---|---|---|
| 1 | 14 | 0.057 | 0.74 | 0.78 |
| | | (0.026) | (0.79) | (0.48) |
| 2 | 74 | 0.272** | 5.45** | 2.73** |
| | | (0.018) | (0.33) | (0.38) |
| 3 | 15 | −0.249** | 7.83** | −0.15 |
| | | (0.022) | (0.89) | (0.69) |
| 4 | 15 | −0.009 | −0.27 | −0.16 |
| | | (0.041) | (0.88) | (0.81) |
| 5 | 73 | 0.006 | 2.78** | 2.99** |
| | | (0.025) | (0.44) | (0.36) |
| 6 | 36 | 0.005 | | |
| | | (0.013) | | |

NOTE: All values are expressed relative to NIR measurements (standard error of means is in parentheses). Laboratories at which analyses were performed are listed in Table 3.

**Significant at the 0.01 level (all others not significant at the 0.05 level).

Differences in wavelength selection between the present study and that of Wessman et al. (1988*a*) may be in part the result of using a different machine, which covers a larger part of the NIR spectrum (for example, the 1438 lignin wavelength was not available in the earlier analysis). Differences between these equations and those of McLellan et al. (1991) may result from the differences in types of materials analyzed (fresh versus decomposing).

*Interlaboratory comparisons*

Wet chemistry data from four of the six nitrogen sets did not differ significantly ($p > 0.05$) from NIR values (Table 5). Sample sets 2 and 3 did show significant ($p < 0.01$) differences between wet chemistry and NIR values, with one set significantly higher and the other significantly lower. Combined, there was more than a 0.5% nitrogen by weight bias between laboratories that provided data sets 2 and 3.

Results for lignin (Table 5) show the NIR analyses not to be significantly different from wet chemistry estimates for data sets 1 and 4. However, significant differences did

TABLE 6. Quality-control statistics for NIR and wet chemistry procedures (results of $n$ pair-wise comparisons of blind duplicate analyses)

| | $n$ | Sample mean (%) | Mean error (%) | Relative mean error (% of mean) |
|---|---|---|---|---|
| | | **Nitrogen** | | |
| NIR | 33 | 1.53 | 0.021 | 1.4 |
| Wet chemistry | | | | |
| Plant and Soil Analysis Laboratory | 13 | 1.98 | 0.12 | 6.1 |
| Natural Resources Research Institute | 15 | 1.68 | 0.066 | 3.9 |
| | | **Lignin** | | |
| NIR | 33 | 20.39 | 0.43 | 2.1 |
| Wet chemistry | | | | |
| Ecosystem Center | 16 | 16.12 | 2.28 | 14.1 |
| Natural Resources Research Institute | 15 | 24.02 | 1.66 | 6.9 |
| | | **Cellulose** | | |
| NIR | 33 | 42.67 | 0.28 | 0.6 |
| Wet chemistry | | | | |
| Ecosystem Center | 16 | 49.41 | 2.10 | 4.3 |
| Natural Resources Research Institute | 15 | 43.25 | 2.19 | 5.1 |

NOTE: Sample mean refers to mean value for all samples analyzed. Mean error is the mean of the absolute differences between pairs of analyses. Relative mean error is the mean error divided by the sample mean.

TABLE 7. Changes in estimates of nitrogen, lignin, and cellulose concentration (%) of 15 green *Picea rubens* foliar samples over a 2-year period by NIR analysis

| | 1987 | 1988 | 1989 |
|---|---|---|---|
| Nitrogen | 1.104 | 1.183 | 1.176 |
| Lignin | 19.77 | 19.47 | 20.91 |
| Cellulose | 39.35 | 39.80 | 40.39 |

occur between NIR and laboratory values for data sets 2, 3, and 5. For cellulose (Table 5), significant differences occur between NIR data and measurements from laboratories 2 and 5.

The close agreement with data sets 1 and 4 might be expected in that the samples were processed at the same laboratory from which the calibration set wet chemistry data came. As none of the samples used in the interlaboratory comparison were also in the calibration set, this represents a validation of the calibration equations. Data set 5 was also analyzed at the Woods Hole laboratory, but by a different operator.

The relatively high lignin values obtained from laboratories that produced data sets 2, 3, and 5 represent a relative bias in the analysis. The difference with laboratory 2 may result from the different efficiencies of the two methods used for removing polar and nonpolar extractives (reflux versus sonicating water bath). Differences between results from laboratories 3 and 5, and NIR values, are an example of bias that may be introduced by different operators using the same technique.

Because all of the methods described here are "proximate analyses," no one method can be said to give the correct answer. If the NIR method had been calibrated against measurements from the sonicating water bath method, for example, it would be expected to validate well against that method and to show biases relative to the reflux method. Lignin and cellulose analyses have value in that highly predictive relationships have been developed between these parameters and important ecosystem processes such as litter decomposition and nitrogen cycling (e.g., Melillo et al. 1982; Wessman et al. 1988*b*). However, for carbon-fraction data to have general meaning, it must be possible to compare data from different studies knowing that the methods used yield comparable results.

Wet chemistry procedures for lignin and cellulose determination are time-consuming and difficult to replicate. Several opportunities exist for minor differences in the procedures to alter results. The data presented here suggest that important biases do indeed exist between laboratories and that the standarization provided through NIR analyses may increase the intercomparability of results.

*Analytical error*

Results from the NIR and two of the wet chemistry laboratories suggest that the NIR technique also reduces analytical error (Table 6). Relative mean error (mean of absolute values of differences between paired analyses divided by the overall mean of all samples) with the NIR method was 1.4% for nitrogen, 2.1% for lignin, and 0.6% for cellulose (Table 6). Values for wet chemistry procedures ranged from 3.9–6.1% for nitrogen, 6.9–14.1% for lignin, and 4.3–5.1% for cellulose (Table 6).

Repeated NIR measurements of the same sample set at yearly intervals showed no significant drift with time

(Table 7). This suggests that long-term dry storage in the dark does not affect the reflectance characteristics of samples and also that drift in the NIR analysis did not occur over a 2-year period.

## Conclusion

NIR analysis produces results with analytical errors considerably lower than those associated with wet chemistry methods. The interlaboratory comparison suggests that the substitution of standardized NIR procedures could increase the intercomparability of results between studies. NIR analysis also reduces the cost of carbon-fraction chemistry significantly, increasing the numbers of samples that can be collected and measured. We conclude that NIR spectroscopy is a viable alternative to wet chemistry methods for determining nitrogen, lignin, and cellulose concentrations in plant materials.

## Acknowledgements

This research was supported by the National Science Foundation (grant BSR 87-06203) and the National Aeronautics and Space Administration (grant NAS5-30771). We thank Sue Katt and Chris Basanta for assistance with NIR analyses and Martha Downes and Andrea Rica for wet chemistry analyses.

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