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NOTE

Error analysis of the determination of carbon stable isotope ratios in lignin and cellulose from plant samples

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To determine the accuracy and precision limits associated with carbon stable isotope ratio ($\delta^{13}C$) measurements of cellulose and lignin from plant samples, experiments were performed using differing extents of treatment for separating these two components. Laboratory reagent lignin and cellulose were used, together with model mixtures of both components. The resulting data were fitted to empirical functions. It was found that the $\delta^{13}C$ of cellulose could be determined with higher accuracy (<0.1‰) than that of lignin (<0.25‰). The corresponding precision values were better than 0.04‰ and 0.08‰, respectively. Longer treatment times do not always result in better accuracy of the isotope data for either component.

Keywords: plants, carbon isotope ratio, lignin, cellulose, error analysis

INTRODUCTION

Plant tissues contain two principal organic constituents, cellulose and lignin, characterized by carbon isotope ratios that differ systematically from each other (Wilson and Grinsted, 1977; Loader *et al*., 2003; Akagi *et al*., 2004). Isolation of cellulose, with subsequent $\delta^{13}C$ measurement of this component, is a standard procedure for investigating the carbon isotopic composition of plants (e.g., Brendel *et al*., 2000; Mazany *et al*., 1980). Numerous published studies have reported the precision of carbon isotopic measurements of cellulose and lignin isolates from specific plant samples (Benner *et al*., 1987; Loader *et al*., 1997, 2003; Brendel *et al*., 2000; Mazany *et al*., 1980; Hobbie and Werner, 2004; Fernandez *et al*., 2003; Schweizer *et al*., 1999; Wilson and Grinsted, 1977; Leavitt and Danzer *et al*., 1993). It is not surprising, however, that, to the best of our knowledge, no studies reported or estimated the accuracy of the isotopic data, because of the lack of appropriate isotopic reference materials.

Incomplete isolation of the respective components is one potential source of error in such isotopic characterization studies. Brendel *et al*. (2000) applied spectroscopic methods to investigate the effectiveness of the methods used to separate lignin and cellulose, and reported that a small amount of lignin always remained in the cellulose isolates. Another potential source of error is variation of the respective abundances of cellulose and lignin, which depends mainly on the nature of the tissues (Schweizer *et al*., 1999; Akagi *et al*., 2004) and extent of decomposition (Benner *et al*., 1987; Schweizer *et al*., 1999; Akagi *et al*., 2004, Fernandez *et al*., 2003). Furthermore, varying proportions of hemi-cellulose and α-cellulose, whose isotopic signatures have not been well documented, also provides a potential source of error. To estimate the errors due to those factors, isolation of the individual components prior to isotopic measurements may be insufficient, because the accuracy of the $\delta^{13}C$ values of the pure isolates is not known. In order to evaluate the errors associated with determining the carbon isotopic analysis of plant materials, and validate the data obtained, it is useful to formulate the errors as a function of composition.

The aim of our study was to use cellulose and lignin (laboratory reagents), and a model mixture of these, to identify factors controlling the accuracy and precision of the isotopic data. We recognize, however, that application of the results of this investigation directly to plant samples is likely to be complicated by the presence of hemi-cellulose in natural samples, besides compositional variations of the lignin, but suggest that our results provide a useful baseline for further studies.

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METHODS

Samples

The experimental samples (lignin only, cellulose only, and a 1:1 mixture thereof by weight) were prepared using laboratory reagents cellulose (fibrous, medium, Sigma-Aldrich^{TM}) and lignin (alkali, Sigma-Aldrich^{TM}). Both reagents were used as supplied. Mixture samples were prepared by mixing 5 mg of each reagent thoroughly on a sheet of aluminum foil. To assess the respective carbon isotopic homogeneity, $\delta^{13}C$ measurements were made of 10 replicate samples of each pure reagent, sampled from different positions of the reagent bottle. It was found that the standard deviation of the measurements was as low as that obtained from replicates of CO_2 samples that we routinely prepare from conversion of our laboratory standard graphite. We are therefore confident that both reagents were practically homogenous with respect to carbon isotopic composition. The respective $\delta^{13}C$ values were determined as –27.38 ± 0.08‰ (cellulose) and –28.33 ± 0.07‰ (lignin), relative to VPDB. The standard procedure is well documented (Akagi *et al*., 2004; Haraguchi, 1985; Nakano and Iizuka, 1994) and is summarized below. For our study, some of the isolation conditions were modified, to investigate their effect; details are provided in the following sections.

Cellulose separates

The following procedure is to isolate holo-cellulose, which consists of α-cellulose and hemi-cellulose. To 10 mg of the experimental samples, 45 ml of water, 0.3 g of sodium chloride and 0.06 ml acetic acid were added, and the mixture heated at 80°C for an hour. The procedure was repeated with a new addition of the same amounts of sodium chloride and acetic acid, then replicated a specific number of times (0, 1, 3, 5 and 7), to examine the effect of increasing the time at which the sample was treated at 80°C: 1, 2, 4, 6 or 8 hours. (For the standard procedure, the replication number is 3.) The residue was then collected on a glass filter (GF/C, Whatman Co. Ltd.), washed with Milli-Q water, dried at 80°C in pre-weighed aluminum sheets, and weighed. Carbon isotope ratio measurements were made using a dual-inlet, gas source mass spectrometer (SIRA-10, VG Ltd., UK) after appropriate conversion of the samples to CO_2 (Akagi *et al*., 2004).

Lignin separates

10 mg of the experimental samples were treated with 1 ml of 72% sulfuric acid for 4 hours at room temperature, after which the sulfuric acid was diluted to 1.7% by the addition of Milli-Q water. Samples were heated further at approx. 100°C for different periods (0, 1, 2, 4, 6 or 8 hours). (For the standard procedure, the heating time is 4 hours.) The residue was then collected on a glass filter (GF/C, Whatman Co. Ltd.) and washed with Milli-Q water. It was then dried at 80°C and analyzed for carbon isotopic composition in the same manner as for the extracted cellulose.

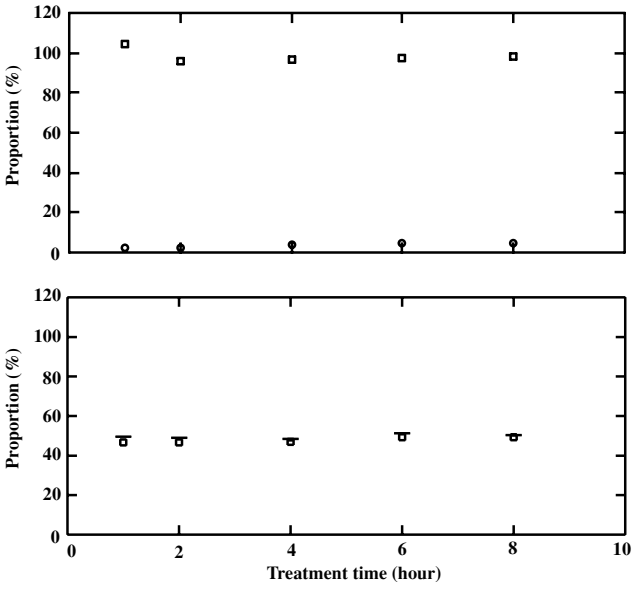

Fig. 1. Weights of substances recovered as cellulose from cellulose-only samples (squares in upper diagram), from lignin-only samples (circles in upper diagram) and from mixture samples (lower), with different times of treatment for lignin decomposition. The weights are expressed as proportions of the total sample weight. Bars show the initial (starting) proportions; squares show recovered proportions.

RESULTS AND DISCUSSION

Weight of substance recovered as cellulose

The weight of the substance recovered as cellulose, expressed as a proportion of the total sample weight, is plotted on the ordinate axis of Fig. 1. From the cellulose-only samples, cellulose recovery ranged from 95 to 100 wt%, except in the case of a one-hour treatment (104.3 wt%, Fig. 1 upper). This quantitative recovery, under a wide range of conditions, indicates that cellulose is quite stable to the treatment used for decomposition of lignin. From the lignin-only samples, the amount of material recovered as cellulose was less than 5 wt% under all conditions (Fig. 1, upper). In accord with expectations, lignin was found to decompose relatively quickly under the experimental conditions used. In the case of the mixtures, the weights of substance recovered as cellulose were almost identical to, or slightly less than, the initial weights of cellulose (Fig. 1, lower); the difference was less than 2% under all the conditions adopted. The results for the

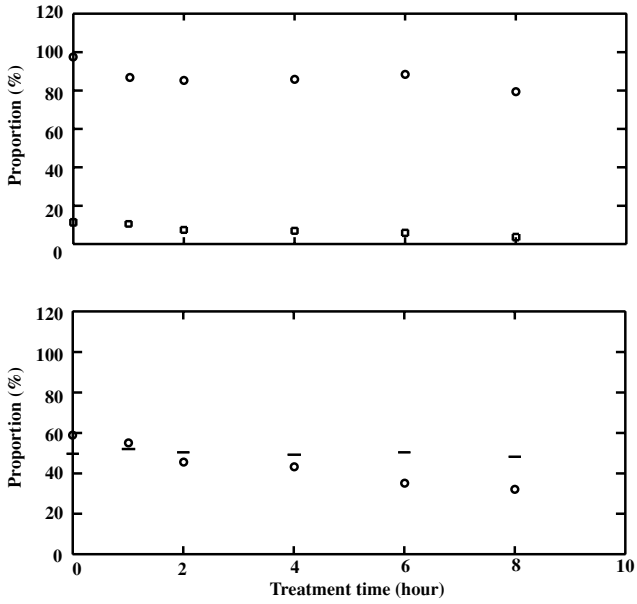

Fig. 2. Weights of substances recovered as cellulose from cellulose-only samples (squares in upper diagram), from lignin-only samples (circles in upper diagram) and from mixture samples (lower), with different times of treatment for cellulose decomposition. The weights are expressed as proportions of the total sample weight. Bars show the initial (starting) proportions; squares show recovered proportions.

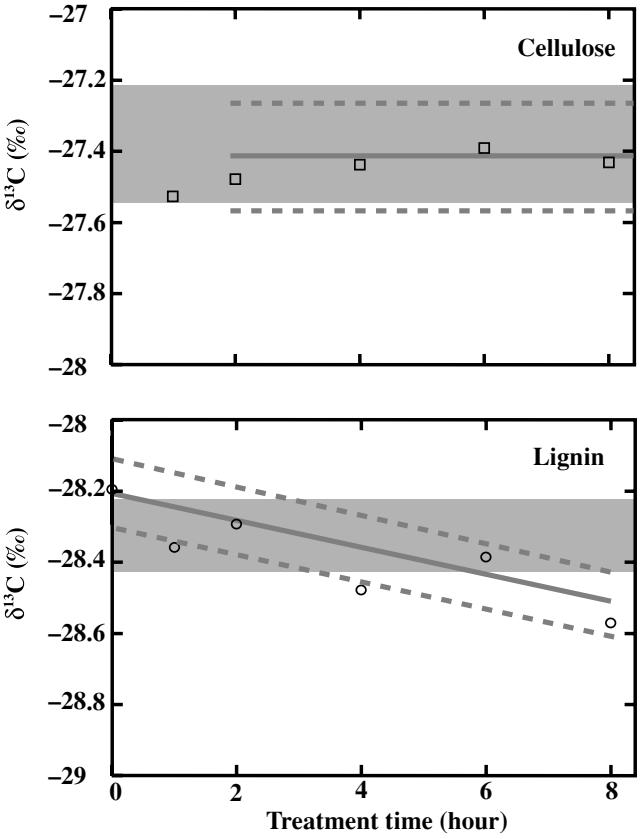

Fig. 3. The measured $\delta^{13}C$ value and estimated $\delta^{13}C$ value (solid line) with 1σ errors (broken lines) of the substances recovered as cellulose (upper) and as lignin (lower) from the mixture samples. The shadows show the range of $\delta^{13}C$ data of the corresponding reagents.

cellulose/lignin mixtures were consistent with the comparative results obtained using cellulose-only and lignin-only samples.

Weight of substance recovered as lignin

The weight of the substance recovered as lignin, expressed as a proportion of the total sample weight, is plotted on the ordinate axis of Fig. 2. From the lignin-only samples, the lignin yield was around 80–90 wt% under all the conditions, except for the zero hours treatment (Fig. 2, upper). From the cellulose-only samples, the amount of the substance recovered as lignin decreased from 10 to 2 wt% with increasing treatment time (Fig. 2, upper). Decomposition of cellulose was rapid during the first hour, after which the rate of decomposition suddenly decreased. In the case of the mixture samples, the amount of substance recovered as lignin was greater than the initial weight of lignin, if the sample was treated for less than 1 hour. However, the weight decreased gradually with increasing treatment time (Fig. 2, lower). When the length of the treatment was insufficient, the material recovered might have contained undecomposed cellulose. When the treatment was too long, lignin may be also decomposed by the sulfuric acid. The results demonstrate the difficulty of removing cellulose without losing a proportion of lignin.

The carbon isotope ratios of substances recovered from the mixture samples

The purpose of the component separation is not to determine the respective amounts of cellulose and lignin, but to correctly determine the corresponding carbon isotope ratios. Even if recovery of a component is low, this does not represent a problem if the separated component is pure and has not changed isotopic composition during the course of the separation procedure.

The measured carbon isotopic composition of the substances recovered as cellulose and lignin from the mixture samples are plotted in Fig. 3. The shadows show the measured $\delta^{13}C$ ranges of the corresponding reagents. The values of the recovered substances as cellulose (Fig. 3, upper) were distributed within the range of the standard deviation of the cellulose value, but tended to increase slightly to approach the mean value of the cellulose reagent (–27.38‰) with increasing treatment time. The results are in accordance with progressive removal of lignin as the treatment time at 80°C is extended; a lengthy treat-

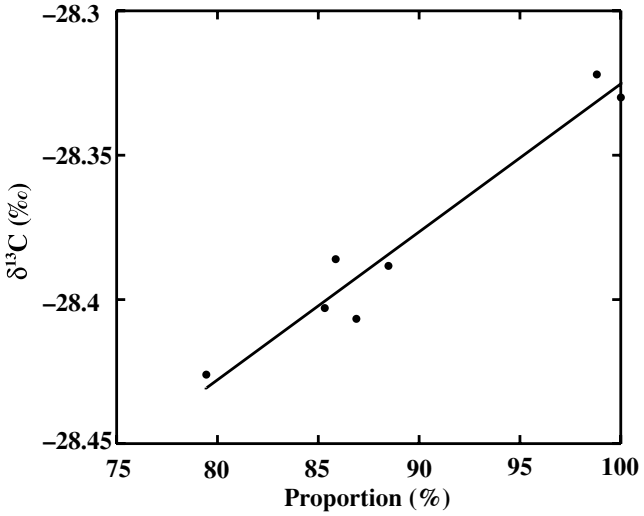

Fig. 4. The relationship between $\delta^{13}C$ value and recovery of lignin in the extraction experiment of lignin from the lignin-only samples.

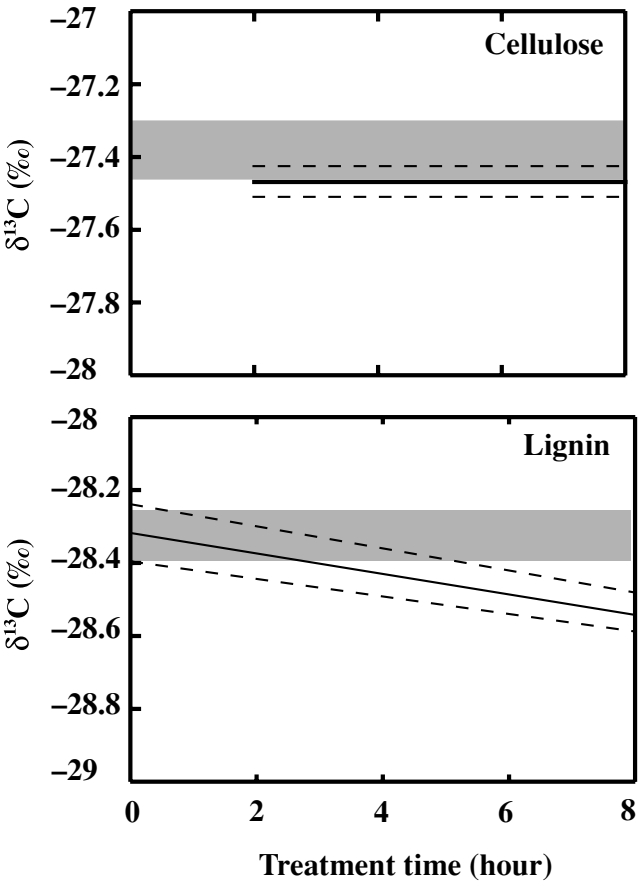

Fig. 5. Estimated $\delta^{13}C$ values of cellulose (upper) and lignin (lower) from natural plant samples, for various isolation conditions, using model equations obtained by this study (see text). Solid line indicates the mean values (corresponding to accuracy); broken lines show the 1σ variation (corresponding to precision). Shadows show the ranges of "true" values $\pm 1\sigma$ for ideal separation.

ment time is needed to obtain an accurate isotope ratio value.

The $\delta^{13}C$ values of the substances collected as lignin showed a variation of 0.4‰ (Fig. 3, lower). For the untreated sample (zero hours of treatment), the $\delta^{13}C$ value was significantly higher than the measured range of the lignin reagent (–28.33 ± 0.07‰). This can be explained by mixing with cellulose of a higher isotope ratio. The $\delta^{13}C$ values tended to decrease with increasing treatment time; samples treated for 4 and 8 hours showed significantly lower values than the range of measured values of the lignin reagent. The isotope ratios of the substances collected as lignin from the lignin-only sample showed a change which depended on the extent of recovery of lignin (Fig. 4). Probably, lignin contains isotopically different components, or is composed of isotopically different groups, and the components or groups with a higher $\delta^{13}C$ tend to be removed preferentially during the course of the lignin separation. The lower values in the experiment using the mixture samples (Fig. 3, lower) imply that a lignin component with a higher isotope ratio was decomposed progressively as treatment time was extended.

The errors involved in the determination of the carbon isotope composition of cellulose and lignin in plant samples

One of the aims of this study is to evaluate how closely the measured $\delta^{13}C$ values of cellulose and lignin isolates are to the respective true values, by fitting the empirical data to equations which could then be applied to natural plant samples to understand the accuracy and precision involved in those isotope ratio determinations.

Cellulose The isotope ratio change in the experiments using the mixture samples (Fig. 3, upper) indicates a slightly decreasing contribution of lignin with extended heating time. In the case of cellulose extraction, however, no significant dependence of recovery of both cellulose and lignin on the length of treatment time was found, except for the result of 1-hour treatment (Fig. 1, upper). We assumed that 4 ± 1% of lignin was constantly maintained in the mixture experiments, from the results of the cellulose isolation experiment using lignin-only samples. Then Eq. (1) expresses the $\delta^{13}C$ of the model mixture:

$$\delta^{13}C_{\text{estimated cellulose}} = \frac{(0.96 \pm 0.01) \times \delta^{13}C_{\text{cellulose}} + (0.04 \pm 0.01) \times \delta^{13}C_{\text{lignin}}}{(0.96 \pm 0.01) + (0.04 \pm 0.01)}. \quad (1)$$

The expected $\delta^{13}C$ value and the precision for the substances recovered as cellulose components from the model mixture are shown in a solid line and two broken lines in Fig. 3 (upper part). The expected data for less than 2 hours treatment is not given in Fig. 3 (upper part), because the observed data for 1 hour, where recovery exceptionally exceeded 100%, was not used in the estimation. The expected $\delta^{13}C$ values of the substances recovered as cellulose were lower, compared to the true values, by 0.03 ± 0.07‰ (1σ). The observed $\delta^{13}C$ values for all lengths of treatment time fall within the range of the expected $\delta^{13}C$ ± 1σ, showing that the estimation is appropriate.

In the case of natural plant samples, we have to consider two other parameters as sources of error, namely: variation in the lignin and cellulose contents, and also variation in isotopic difference between cellulose and lignin:

$$\delta^{13}C_{\text{estimated cellulose}} = \frac{f_{\text{cellulose}} \times (0.96 \pm 0.01) \times \delta^{13}C_{\text{cellulose}} + f_{\text{lignin}} \times (0.04 \pm 0.01) \times \delta^{13}C_{\text{lignin}}}{f_{\text{cellulose}} \times (0.96 \pm 0.01) + f_{\text{lignin}} \times (0.04 \pm 0.01)}. \quad (2)$$

In Eq. (2), cellulose and lignin relative proportions (f) by mass in natural plant samples were assumed to be 0.5 ± 0.1 and the $\delta^{13}C$ value of lignin was assumed to be smaller than that of cellulose, by 2.5 ± 0.5‰ (1σ) (Ooki *et al*., unpublished data).

Using Eq. (2), the accuracy was estimated to be –0.08‰ and the precision was about 0.15‰ (Fig. 5, upper). The error due to the isotopic difference between cellulose and lignin is much more influential than that due to the constituent variation. The $\delta^{13}C$ of substances recovered as cellulose would be significantly lower than the true value.

Lignin The weight of the substances recovered as lignin from the mixture samples as well as from the lignin-only sample decreased gradually as treatment time was extended (Fig. 2, upper and lower). The decomposition of cellulose showed a clear dependence on the treatment time (Fig. 2, upper), and a significant amount of cellulose was considered to remain in the substances recovered as lignin. We assumed that the difference between the weight of recovered substances from the mixture samples (Fig. 2, lower) and that of recovered substances from the cellulose-only samples (Fig. 2, upper) would give the weight of lignin in the recovered substances. Then, the change in the weight of lignin in separates was fitted using an exponential function against treatment time. More complicatedly, the $\delta^{13}C$ of recovered substances as lignin changed, depending on recovery (Fig. 4). The $\delta^{13}C$ change of lignin was fitted using a linear function of recovery.

Considering all the effects mentioned above, the expected value of $\delta^{13}C$ was formulated as a function of treatment time (Eq. (3)) and mean value and 1σ of the substance recovered as lignin from the mixture samples were estimated (Fig. 3 lower). The precision (1σ) of the estimation was obtained based on the errors involved in the fittings (Eqs. (3) and (4)).

$$\delta^{13}C_{\text{estimated lignin}} = \frac{\left(1.0213\, e^{-0.066 \times \text{treatment hour}} \pm 0.05\right) \times \delta^{13}C_{\text{lignin}} + \left(0.1095\, e^{-0.1432 \times \text{treatment hour}} \pm 0.003\right) \times \delta^{13}C_{\text{cellulose}}}{\left(1.0213\, e^{-0.066 \times \text{treatment hour}} \pm 0.05\right) + \left(0.1095\, e^{-0.1432 \times \text{treatment hour}} \pm 0.003\right)}, \quad (3)$$

$$\delta^{13}C_{\text{lignin}} = 0.5125 \times \left(1.0213\, e^{-0.066 \times \text{treatment hour}} \pm 0.05\right) - 28.838 \pm 0.07. \quad (4)$$

The expected values are different by –0.24 or +0.13‰ from the true value, with a standard deviation of 0.07‰. The observed $\delta^{13}C$ of the substances recovered as lignin usually fell within the range of the expected $\delta^{13}C$ ± 1σ, showing that the estimation is fairly satisfactory.

In the case of natural plant samples, Eq. (5) was introduced to incorporate the two other variations:

$$\delta^{13}C_{\text{estimated lignin}} = \frac{f_{\text{lignin}} \times \left(1.0213\, e^{-0.066 \times \text{treatment hour}} \pm 0.05\right) \times \delta^{13}C_{\text{lignin}} + f_{\text{cellulose}} \times \left(0.1095\, e^{-0.1432 \times \text{treatment hour}} \pm 0.003\right) \times \delta^{13}C_{\text{cellulose}}}{f_{\text{lignin}} \times \left(1.0213\, e^{-0.066 \times \text{treatment hour}} \pm 0.05\right) + f_{\text{cellulose}} \times \left(0.1095\, e^{-0.1432 \times \text{treatment hour}} \pm 0.003\right)}. \quad (5)$$

In Eq. (5), relative mass proportions (f) of cellulose and lignin in natural samples were assumed to be 0.5 ± 0.1 and it was also assumed that the $\delta^{13}C$ value of lignin was smaller than that of cellulose by 2.5 ± 0.5‰ (1σ) (Ooki *et al*., unpublished data).

Based on our model study, the accuracy and precision of the isotopic measurement of lignin from a natural plant are shown in Fig. 5, lower. The accuracy was estimated to range from –0.2 to 0.02‰, and precision from 0.05 to 0.08‰. The reason for the accuracy deterioration at longer treatment time is the isotopic change involved in the decomposition of lignin. The slightly better precision at longer treatment time is due to a relatively smaller contribution of undecomposed cellulose in the treated sample. The error caused by the constituent variation is negligibly small and the significant error results mainly from the isotopic difference between lignin and cellulose. The $\delta^{13}C$ of the substances recovered as lignin would not correspond to the true value (within the range of precision) when the treatment time is longer than 2 hours. It should be noted that the higher accuracy obtained by a treatment time of two hours is an "artifact" resulting from balancing lignin isotopic change (error in negative direction) against an error incurred from cellulose contamination (error in positive direction).

Incidentally, the accuracy and precision both deteriorate when the component of interest is present only as a minor constituent. Application of the present equations estimates the attendant errors. When cellulose abundance is one tenth that of lignin, the accuracy and precision of cellulose isotope measurement would be –0.3‰ and 0.2‰, respectively. When lignin abundance is one tenth of that of cellulose, the accuracy and precision for lignin would be 0.4‰ and 0.2‰, respectively.

Conclusions

Our experiments have been performed using laboratory reagent cellulose and lignin, but the application of the findings to natural plant samples may be affected by the following considerations:

- Structural and/or compositional variations of lignin and hemi-cellulose, which are likely to be associated with isotopic variations. Unlike alpha-cellulose, hemicellulose and lignin are macromolecules with various spatial structures, which may affect their resistance to the chemical treatments described in this paper.
- The extent to which hemi-cellulose is present with the cellulose.
- The way of mixing or combining of those substances to compose natural tissues.

Therefore we summarize our conclusions as rather qualitative remarks:

1. Cellulose is preserved by the treatment used for cellulose extraction and isolation, whereas lignin is readily removed by the procedure. The accuracy of the $\delta^{13}C$ determination of cellulose is governed by the extent of contamination by undecomposed lignin. The $\delta^{13}C$ values of cellulose extracted from plant samples will always be smaller than the corresponding true value, by about 0.1‰.

2. The treatment for isolation of lignin is accompanied by a partial loss of lignin, which also results in a change of $\delta^{13}C$ value to the lignin itself. Complete removal of cellulose is difficult without losing also a proportion of lignin. The accuracy of $\delta^{13}C$ data is limited by both the residual quantity of undecomposed cellulose and by the change in isotopic composition that is accompanied by lignin loss. Model calculations show that a $\delta^{13}C$ value obtained from a plant sample would be significantly smaller than the corresponding "true" value, when a sample is treated for longer time.

3. Based on model estimates, the accuracy and precision of the isotopic data become worse for samples that contain the component of interest in reduced amounts.

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