

# Revising the Acetyl Bromide Assay to Optimize Lignin Determinations in Forage Plants

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## Introduction

The acetyl bromide method was originally developed to provide a rapid, yet sensitive, spectrophotometric method to determine lignin concentrations in small wood samples (Johnson et al.). It has since been modified a number of times mostly to adapt the procedure to herbaceous plants. Most recent modifications have utilized perchloric acid as a catalyst to aid in the total solubilization of plant cell walls (Iiyama and Wallis). We were interested in using this method for small forage samples and lignin fractions which are not amenable to forming insoluble residues (i.e., Lignin-carbohydrate complexes, etc.). However, difficulties which arose during routine sample analysis prompted us to re-evaluate the standard procedure to improve the reliability of lignin determinations.

## Materials and Methods

For all experiments, the acetyl bromide reagent was 25% (v/v) acetyl bromide in glacial acetic acid. All samples were dried overnight at 50 °C before weighing into glass culture tubes (16 x 150 mm) fitted with Teflon lined screw caps. Weighed samples were placed in a vacuum desiccator over P<sub>2</sub>O<sub>5</sub> with the caps off for at least 18h before analyzing. The standard procedure involved removing the samples from the desiccator, adding 2.5 mL of acetyl bromide reagent (prepared fresh), capping immediately, and heating at either 50, 60 or 70 °C. Some samples also contained 100 µL of perchloric acid. Heating times varied from 15 minutes to 4h depending upon the nature of the experiment. After heating, the samples were quantitatively transferred, with the aid of acetic acid, to 50 mL volumetric flasks that contained 10 mL of 2 M NaOH and 12 mL of acetic acid. Hydroxylamine (350 µL of 0.5 M) was added to each flask and samples were diluted to 50 mL with acetic acid. Absorption spectra (250 to 350 nm) were determined for each sample and used to

determine the absorption maxima at 280 nm. Samples included corn rind walls, alfalfa stem walls, corn cell culture walls (lignified to varying degrees), and polysaccharides (polygalacturonan, arabinoxylan, and cellulose). All experiments were run in triplicate with duplicates for each treatment.

## Results and Discussion

Initially, several parameters were tested to determine their impact upon the lignin values obtained from standard samples (corn rind and alfalfa stem cell walls). These experimental parameters included concentration of acetyl bromide, heating time, heating temperature, perchloric acid, and use of hydroxylamine. Of these parameters, acetyl bromide concentration appeared to have the least impact as long as the concentration was maintained at 15 to 30% (v/v). It was also determined that addition of hydroxylamine, as described in the original method, gave the most consistent results (i.e., removal of polybromide anion that forms during the reaction). As expected, decreasing the reaction temperature decreased the rate of reaction that could be compensated for by increasing the reaction time.

The impact of varying the reaction temperature is shown in Figure 1. Samples were heated at 50 °C for 2 h, 60 °C for 1 h and 70 °C for 0.5 h with or without the addition of perchloric acid. Theoretically the increased reaction time should compensate for the decrease in reaction temperature. This would appear to be the case for samples without the inclusion of perchloric acid (Fig. 1 dashed lines). The inclusion of perchloric acid resulted in significantly higher absorption values as the temperature increased (Fig. 1 solid lines). This could be interpreted to mean that increased temperature and the addition of perchloric acid are required to solubilize all of the wall bound lignin. Alternatively the increase

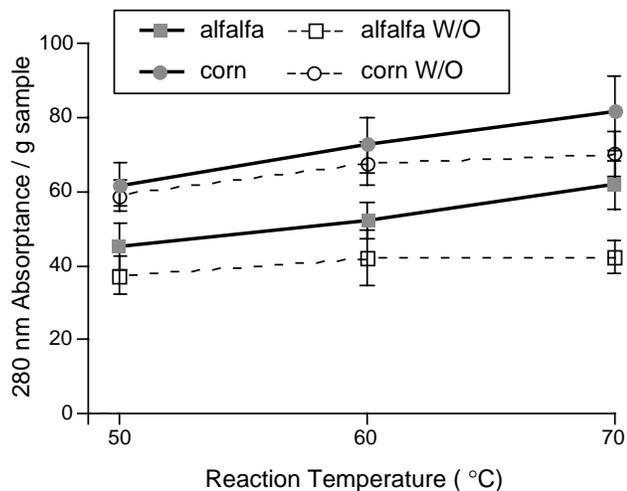


Figure 1. Changes in the total absorbance at 280 nm after treatment of cell walls with acetyl bromide reagent for 2 h at 50 °C, 1 h at 60 °C and 0.5 h at 70 °C. Solid lines represent samples with perchloric acid added and dashed lines were without perchloric acid.

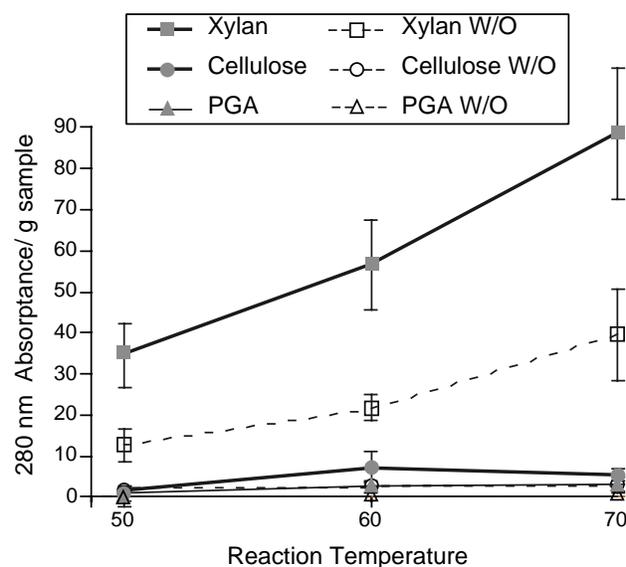


Figure 2. Reaction of wall polysaccharides with acetyl bromide at increasing temperatures. Solid lines represent samples with perchloric acid added and dashed lines were without perchloric acid. Heating times were 2 h at 50 °C, 1 h at 60 °C and 0.5 h at 70 °C.

in absorbance could be due to increased polysaccharide degradation.

Treatment of selected structural polysaccharides with acetyl bromide, following the same regime as above, revealed that xylans were particularly susceptible to degradation in the acetyl bromide reagent (Fig 2). Increased temperature accelerated this degradation and was further enhanced by the addition of perchloric acid. Although the absorption spectra for degraded xylans were different from lignin, there was a strong absorbance at 280 nm, the absorption maximum used for lignin. It is likely that a good deal of this increased absorbance at higher temperatures, and especially in the presence of perchloric acid, was due to xylan degradation. Dehydrogenation polymers of coniferyl alcohol (DHP, artificial lignins) formed in the absence of cell wall polysaccharides did not show the same temperature dependent reaction. All temperatures gave the same absorbance values (Fig. 3). There was only a slight increase when perchloric acid was added to the reaction. These findings indicate that higher absorbance values, especially with added perchloric acid, may be due to xylan degradation.

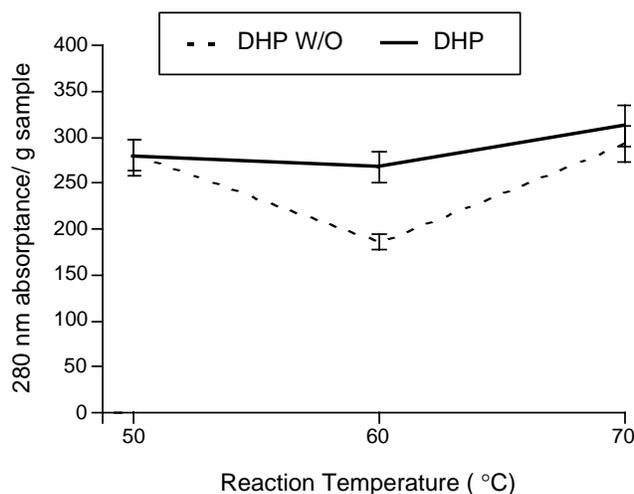


Figure 3. Comparison of DHP lignins treated with acetyl bromide reagent (heating times as in Fig. 1). Solid lines = perchloric acid added. Dashed lines = without perchloric acid.

## **Conclusions**

The acetyl bromide assay for lignin is a convenient procedure for small samples which may not be suitable for Klason or acid detergent lignin methods. However, xylan degradation should be considered as a possible interference that would overestimate total lignin concentrations. This can be minimized by using a lower temperature such as 50 °C even though the reaction time is increased to 2 to 4 hours depending on the difficulty in wall solubilization.

## **Impact**

Improved methods for determining lignin concentrations, especially on small samples, allow a better understanding of lignin's influence on wall digestibility. Detailed information concerning the interactions of wall components leads to development of genetic approaches (traditional and molecular) to improve forage cell wall digestibility.

## **References**

- Johnson, D.P., Moore, W.E., and Zank, L.C. 1961. The spectrophotometric determination of lignin in small wood samples. *Tappi* 44: 793-798.
- Iiyama, K., Wallis, A.F.A. 1990. Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. *J. Sci. Food Agric.* 51:145-161.