A Semi-micro Method for the Determination of Lignin and its Use in Predicting the Digestibility of Forage Crops

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The lignin content of dried grasses can be measured, after prior removal of interfering phenolic materials, by dissolving the residue in 25% acetyl bromide in acetic acid and determining the absorption at 280 nm. The absorption values can also be used to predict the nutritive value of dried grasses, hays and silages.

1. Introduction

Lignin is a complex aromatic polymer which occurs in plant cell walls in close association with cellulose and the hemicellulosic polysaccharides. Johnson, Moore and Zank\(^1\) have published a method for the spectrophotometric determination of lignin in small wood samples whereby the wood, or wood product, is dissolved in a mixture of acetyl bromide in acetic acid and the absorption of the resultant solution is measured at 280 nm. It is this method that has been adapted for dried grass samples.

A knowledge of the nutritive value of a feed is of fundamental importance in animal nutrition experiments. The \textit{in vitro} digestibility technique as devised by Tilley, Deriaz and Terry\(^2\) is probably the most accurate method for indirectly estimating nutritive value but it has disadvantages especially in the length of the operation and the need for a source of rumen liquor. A more rapid method, even if less accurate as a predictor, offers certain advantages for laboratories that do not have the \textit{in vitro} method running continually. Progressive lignification is generally considered to be the major cause of the decline in digestibility as a herbage matures and, of all the grass components that can readily be measured by chemical means, lignin gives the best correlation coefficient with \textit{in vitro} digestibility values.\(^3\text{--}^5\)

2. Experimental

2.1. Preparation of the samples

Five grasses were used, the diploid and tetraploid strains of S23 and S24 ryegrass and diploid S37 cocksfoot. The grasses were cut at either four or five stages of growth and 12 of these cuts were separated into leaf and leaf sheath–stem fractions. The leaf sheath was included with the stem as they have been found to be closely related with respect to both lignin content and digestibility.\(^6\) All samples were dried within 1 h of cutting in a forced draught oven at 100 to 110 °C for 45 min, ground to pass a 0.7 mm screen and...
stored in sealed glass jars at 4 °C. The method of Ellis, Matrone and Maynard, as modified by Waite, Johnston and Armstrong, was used to determine reference lignin contents. Determinations of the \textit{in vitro} digestibility were made by the method of Tilley and Terry as modified by Alexander as McGowan.

2.2. Analytical procedure

Dried grass (approximately 2 g of known ash content) was heated with distilled water (150 ml) at 60 to 65 °C for 30 min with occasional shaking, then filtered hot through a Whatman No. 52 filter paper. The residue was washed thoroughly with water, ethanol, acetone and diethyl ether until no further colour appeared in the filtrate and the preparation was dried at 47 °C overnight. The volumes of the reagents used for washing varied depending on the state of maturity of the grass but did not exceed 200 ml of each. The yield and dry matter content of this crude cell wall preparation were determined and samples (40 to 50 mg) were heated at 70 ± 0.1 °C for 30 min in glass-stoppered test tubes with 5 ml of 25% acetyl bromide in glacial acetic acid. After cooling to 20 °C, the material was transferred to a 250-ml volumetric flask containing 4.5 ml of 2 N-sodium hydroxide in 25 ml acetic acid. Acetic acid was used to wash the residue from the tube and to make the volume up to approximately 200 ml 8 ml of 0.5 M-hydroxylammonium chloride solution was added, the flask was made to the mark with acetic acid, shaken and allowed to stand for at least 1 h to allow the protein sediment to settle before reading the optical density of the solution at 280 nm in 10-mm silica cells. A reagent blank was run with every set of estimations. Fresh reagents were prepared if the reagent blank had an optical density greater than 0.050 units. The absorption values (A) were calculated from the equation:

\[ A = \frac{\text{O.D.}_S - \text{O.D.}_B}{c} \text{ cm}^{-1}\text{g}^{-1}, \]

where \( \text{O.D.}_S \) = optical density of the sample, \( \text{O.D.}_B \) = optical density of the blank, \( c \) = concentration of dry organic matter in the final solution (gl⁻¹).

3. Results

3.1. Effect of preliminary extractions

The work of Johnson, Moore and Zank demonstrated that the method was suitable for measuring the lignin content of extracted woods, but preliminary investigations were necessary to ascertain whether materials similar to those normally found in the grass cell wall would exhibit any absorption in this reagent at 280 nm. The results are given in Table 1. The absorption values obtained from the two cellulose samples and the pectin sample are not significant as will be discussed later.

The scheme, described by Waite and Gorrod, for the comprehensive analysis of grasses which employs successive treatment with azeotropic ethanol–benzene, water at 60 °C and acid pepsin was examined as the final residue is known to be free from low molecular weight phenolic materials and most of the protein. Grass samples were treated in this way and the residue after each extraction was examined by the acetyl bromide procedure. A typical set of results is given in Table 2 and it is clear that the
Digestibility of forage crops and lignin content

Table 1. The u.v. absorption values (A) at 280 nm of some materials similar to those found in grass cell walls

<table>
<thead>
<tr>
<th>Material</th>
<th>A (cm(^{-1})lg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose powder (Balston’s)</td>
<td>0.222</td>
</tr>
<tr>
<td>Filter paper (Whatman No. 1)</td>
<td>0.127</td>
</tr>
<tr>
<td>Grass hemicellulose (arabino-4-O-methyl-glucuronoxylan)</td>
<td>0.048</td>
</tr>
<tr>
<td>Pectin (apple)</td>
<td>0.278</td>
</tr>
<tr>
<td>Protein (casein)</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Table 2. The effect of extractive treatment on the u.v. absorption values (A) at 280 nm of a grass sample

<table>
<thead>
<tr>
<th>Material</th>
<th>A cm(^{-1})lg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried grass after extraction with:</td>
<td></td>
</tr>
<tr>
<td>(a) Ethanol-benzene (1:2)</td>
<td>3.864</td>
</tr>
<tr>
<td>(a) Ethanol-benzene (1:2) and (b) water</td>
<td>3.342</td>
</tr>
<tr>
<td>(a) Ethanol-benzene (1:2) (b) water and (c) acid pepsin</td>
<td>2.767</td>
</tr>
<tr>
<td>(a) Acetone and (b) water</td>
<td>2.720</td>
</tr>
<tr>
<td>(a) n-Hexane and (b) water</td>
<td>2.741</td>
</tr>
<tr>
<td>(a) Chloroform–methanol (1:1) and (b) water</td>
<td>2.677</td>
</tr>
<tr>
<td>(a) Chloroform–methanol (2:1) and (b) water</td>
<td>2.659</td>
</tr>
<tr>
<td>(a) Methanol and (b) water</td>
<td>2.853</td>
</tr>
<tr>
<td>(a) Water alone</td>
<td>2.778</td>
</tr>
</tbody>
</table>

In all experiments, the residue after the water extraction was thoroughly washed and dried through ethanol, acetone and ether.

Treatment with acid pepsin can be omitted even though it removed about 75% of the protein originally present in the samples. It was noticed that all grass or extracted grass samples left a sediment after being treated with acetyl bromide reagent. By centrifuging off this sediment from a grass with a high protein content, it was found to contain more than 98% of the protein nitrogen originally present in the grass. No other cell wall components could be detected in the suspension.

Complete extraction with boiling ethanol–benzene requires about 18 h, so to speed up the pretreatment of the grass, other solvents were tried which were known to be good extractants for lipids, waxes and phenolic materials. The absorption values obtained from the residues of several such extractions on the same grass sample as above are also given in Table 2. Two other grass samples, the first at a very young stage of growth, the other very mature, gave absorption values of 1.073 and 3.543, respectively, for the residues obtained after ethanol–benzene followed by water extractions while values of 1.038 and 3.591 were obtained from the residues after extraction with water alone, provided the residues were washed with ethanol, acetone and diethyl ether until no further colour appeared in the filtrate.
3.2. Effect of duration of heating at 70 °C

Although Johnson, Moore and Zank\(^1\) recommended an acetyl bromide reaction time of 30 min at 70 °C for wood samples, this required confirmation as grass lignins are known to be structurally distinct from wood lignins. The u.v. absorbance from woods at 280 nm rose rapidly for about 10 min, the rate of increase then declined but the absorbance was still rising even after 90 min reaction time. They chose 30 min as the optimum time of reaction because heating beyond this period produced a second absorption maximum at about 325 nm which they attributed to carbohydrate degradation products. The full spectra, between 250 and 400 nm, of a grass sample heated at 70 °C for various times is shown in Figure 1 and gives some indication of how grass and wood lignins differ on treatment with acetyl bromide. In this grass sample about 75% of the u.v. absorbance at 280 nm was produced rapidly with little or no heating, the intensity increased for about 15 min and then remained fairly steady for a further 25 min. Thereafter, another rapid increase in intensity was noted and the intensity was still increasing even after 2 h. The optimum reaction time was taken as 30 min, to be near the centre of the steady period. The initial rise in absorption at 280 nm was caused by the phenolic chromophore of lignin as it dissolved in the reagent. Grass lignin would appear to be more soluble in the reagent than wood lignins from the intensity of absorption produced without heating. The further increase in intensity after 40 min was due

![Figure 1. Effect of different acetyl bromide reaction times on u.v. spectrum of grass solution heated at 70 °C. Times given against curves are in minutes.](image-url)
to a second absorption area with a maximum centred near 310 nm. At 2 h this peak had a greater intensity than the one at 280 nm. As neither purified cellulose or hemicellulose gave this second absorption maximum, we suggest that it is probably produced by acetolysis of the lignin molecule.

3.3. Interpretation of results

To put the results on a quantitative basis should only require the absolute absorptivity of a grass lignin standard. However, attempts so far to prepare a pure grass lignin have been unsuccessful and all the preparations have been contaminated with 5 to 10% carbohydrate which could not be removed by precipitation or other fractionating techniques. The alternative method that has been used entails the analysis of a number of samples for both acetyl bromide absorption value and lignin as measured by Waite, Johnston and Armstrong and calculation of the regression equation. A series of 55 dried grass samples, made up of 31 whole samples, 12 leaf and 12 leaf sheath-stem

![Figure 2](image-url)

Figure 2. Relationship between acetyl bromide absorption values and lignin content. ●, Whole samples; ▲, leaf sheath–stem samples; ■, leaf samples.

subsamples were analysed in this way. The results shown in Figure 2, gave rise to the regression equation:

\[
\text{lignin (\% organic matter)} = 3.36A - 1.11,
\]

where \(A\) = absorption value.

The correlation coefficient \((r)\) for these samples was +0.976 and the standard error of estimate was ±0.66. The acetyl bromide absorption values given in Figure 2 and of all subsequent results are the mean of three determinations, the within sample difference
being less than ±2%, a value which did not show any significant variation at high or low lignin levels.

Holocellulose samples were prepared from 12 of the dried grasses and both acetyl bromide absorption values and lignins were determined. The regression equation for these samples was:

\[
\text{lignin} \left( \% \text{ organic matter} \right) = 2.47A - 0.36 \quad r = +0.922.
\]

The significant difference between these two regression equations will be discussed later.

The relationship between the lignin absorption values and \textit{in vitro} digestibility of the organic matter was investigated in the dried grass samples already described and also in 19 samples of dried hay and 20 samples of dried silage. The results for all these samples are plotted in Figure 3 and gave rise to the following regression equations.

![Figure 3. Relationship between acetyl bromide absorption values and \textit{in vitro} digestibility.](image)

- All samples: \text{digestibility} = 99.75 - 13.34A, \quad r = -0.923
- Dried grasses: \text{digestibility} = 102.47 - 15.54A, \quad r = -0.965
- Hays: \text{digestibility} = 100.75 - 12.54A, \quad r = -0.882
- Silages: \text{digestibility} = 104.97 - 14.31A, \quad r = -0.867.

For the same population of dried grasses, the \textit{in vitro} digestibility and lignin content, as determined by Waite, Johnston and Armstrong, were related by the equation:

\[
\text{digestibility} = 96.61 - 4.49L \quad r = -0.953,
\]

where \( L \) = lignin (\% organic matter).
For the hay and silage samples, the in vitro digestibility and lignin content, as determined by the method of Deriaz,\textsuperscript{12} were related by the equations:

- **Hays:** digestibility $= 89.09 - 3.52 L$, $r = -0.703$
- **Silages:** digestibility $= 86.98 - 3.21 L$, $r = -0.815$

where $L = \text{lignin (\% organic matter)}$.

### 4. Discussion

The preliminary investigations demonstrated that low molecular weight phenolic materials, as was expected, gave very strong absorption maxima in the region of 280 nm when brought into solution by the acetyl bromide reagent and so interfered with the estimation of lignin. However, these materials could readily be removed by solvent extraction to yield crude cell wall preparations which were sufficiently pure to be used to determine lignin. Cell wall polysaccharides gave very low absorption values (Table 1). By using the derived regression equation for holocelluloses, the filter paper would appear to contain no lignin while the cellulose powder would have a content of around 0.2\%. Indeed the cellulose powder was shown to contain a small amount of lignin by using the method of Waite, Johnston and Armstrong.\textsuperscript{8} The somewhat high value obtained from apple pectin is not really significant when it is remembered that grasses contain only about 2\% pectin.\textsuperscript{8} Proteins were expected to cause the greatest interference as they may contain high proportions of the aromatic amino acids which do absorb strongly in the region of 280 nm. Three experiments indicated that such interference did not occur. Acid casein, a protein mixture containing approximately 12.5\% by weight of the aromatic amino acids, gave virtually no absorption value. Digestion of the crude cell walls with the proteolytic enzyme pepsin removed more than 75\% of the protein from different samples but the absorption value of a typical sample after this treatment was only reduced by 0.7\%. The insoluble residue that remained after the acetyl bromide reaction accounted for more than 98\% of the protein nitrogen from the original sample and consisted almost entirely of protein indicating that all other cell wall components were soluble in the reagent. It is relevant to comment here on some of the problems associated with the determination of lignin by the method of Ellis, Matrone and Maynard.\textsuperscript{7} There are numerous reports\textsuperscript{13,14} that lignin is partly soluble in mineral acid probably as a result of degradation. Grass samples can have very high protein contents and, under acidic conditions, insoluble residues can result from condensation reactions between protein and carbohydrate degradation products. Even the use of proteolytic enzymes in preliminary hydrolysis does not remove all the protein\textsuperscript{7,15} and it is customary to adjust the values obtained for lignin to correct for this nitrogenous impurity. The use of detergents has also been suggested by Van Soest\textsuperscript{16} but they too cannot solubilise all the protein and are reported to form colloidal suspensions of lignin which are lost on filtration.\textsuperscript{17}

Although grass lignins are chemically distinct from wood lignins by possessing $p$-hydroxyphenyl residues as well as the coniferyl and sinapyl moieties found in wood lignins, the full u.v. spectra of the acetyl bromide reaction products are similar in having an absorption maximum in the region of 280 nm and the experimental conditions used.
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for determining lignin in wood can be used also for grass lignin. The absorption maximum probably arises from both the free and bound phenolic elements that make up the lignin polymer.

The two regression equations obtained when twelve of the dried grass samples were compared with the holocelluloses derived from them were significantly different and suggest that the residual lignin in these holocelluloses had a different structure from the whole lignin. The absorption was higher in the holocellulose per unit of lignin which suggests that more free phenolic groups are present per molecular unit. This is consistent with the belief that cross-linkages in the lignin network are broken down prior to complete dissolution of lignin during the preparation of a holocellulose.

All of the results given previously are based on concentration of ash free sample. If the ash value is not known, an accurate estimation of lignin can still be made although a different regression equation to the one given previously would have to be used, namely:

\[
\text{lignin (\% dry matter)} = 3.37A - 1.05,
\]

where \(A\) = absorption value of dry matter. The correlation coefficient \((r)\) for these samples was +0.977 and the standard error of estimate was ±0.61.

In schemes that have been proposed for the analysis of animal feeds, much emphasis has been placed on high predictability of nutritive value. In vivo techniques are impractical on a large scale while the in vitro determination of Tilley and Terry\(^9\) and Alexander and McGowan,\(^18\) although giving a very good prediction of nutritive value, is still lengthy to perform. Many authors have used chemical parameters, the two most successful being fibre and lignin. Van Soest\(^16\) found that the correlation coefficients between digestibility and acid-detergent fibre, crude fibre and detergent lignin were −0.81, −0.75 and −0.78, respectively. Mowat, Kwan and Winch,\(^20\) using Van Soest’s method showed that if grasses and legumes were separated the correlation coefficients between digestibility and lignin could be as high as −0.94 and −0.95.

When the in vitro digestibility and acetyl bromide lignin contents of 94 grass samples comprising 55 oven dried grasses, 19 hays and 20 grass silages were compared a correlation coefficient of −0.923 was obtained. Separation of the results into individual populations improved the correlation coefficient for the dried grasses to −0.965. This is little different from the correlation coefficient between digestibility and lignin as measured by the method of Waite, Johnston and Armstrong,\(^8\) for the same samples but was obtained in a much shorter time. The individual hay and silage populations had reduced correlation coefficients of −0.882 and −0.867, but these values were higher than those obtained by comparing digestibility and lignin as measured by the method of Deriaz,\(^12\) namely −0.703 and −0.815. The correlation coefficient for the silage values is the lowest obtained but these analyses were carried out after drying the wet silage by heat and it may be noted that Alexander and McGowan\(^20\) showed that fresh undried silages gave a much better correlation coefficient between in vivo and in vitro digestibilities than did heat dried silages. The dried hay and silage samples had been contributed by another laboratory and had necessitated subsampling bulks of forage after the in vitro digestibility and lignin determinations had been made.

The method described offers distinct advantages over other methods of estimating
lignin and digestibility in that, in addition to the simplicity of the method and the relatively short working time involved, the determinations can be made on milligram quantities. Indeed, the sample size can be reduced to 10 mg, with corresponding reduction in the reagents used, without loss of accuracy but with a higher standard error. This should be useful in measuring lignin content or predicting the digestibility of material removed from different parts of the digestive system of experimental animals. The accuracy of the method is enhanced if the regression equation employed is derived from similar types of material to the experimental samples, and to this end it is intended to determine relationships given by legumes and grass–legume mixtures.

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References