Spectrophotometric Investigations on Lignin in Wheat (*Triticum aestivum* L.): Influence of Cell Wall Preparation, Solvent, and Standard

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Absorbance at 280 nm was used to determine lignin concentrations in solutions of cell walls from winter wheat whole-crop silage, stems, and straw. Three different cell wall preparations (hot water−organic solvent fiber, ethanol−benzene residue, neutral detergent fiber) were prepared and dissolved in either HCl−triethylene glycol or acetyl bromide. Three phenolic standard solutions were used to calculate lignin concentrations in the wheat materials from absorbance readings: mixtures of phenolic monomers based on aldehydes (HIGUCHI), aldehydes and acids (REEVES), and washed Indulin (WIND). Gravimetric Klason lignin determination on the cell wall preparations consistently gave higher values than spectrophotometric lignin analysis. Material recovered as cell wall was slightly greater for hot water−organic solvent fiber than for neutral detergent fiber and was lowest for ethanol−benzene residue, probably due to partial dissolution of cell wall components with the latter procedure. Acetyl bromide shifted UV spectra about 10 nm to lower wavelengths, which complicates quantification of absorbance readings. Additionally, triethylene glycol is easier to handle and less toxic. Lignin concentrations related to the REEVES standard were more consistent across cell wall preparations and solvents than other standards. It appears that the REEVES standard was best suited for winter wheat whole-crop silage, stems, and straw.

Keywords: *Triticum aestivum; lignin; triethylene glycol; acetyl bromide; ultraviolet spectra*

INTRODUCTION

Lignin, an integral component of the cell wall of forage crops, is the chemical component which is most commonly associated with the reduced digestibility of fiber (Van Soest, 1982). The Klason lignin (Sarkanen and Ludwig, 1971) and acid detergent lignin (ADL; Van Soest, 1963) procedures are gravimetric and describe lignin as the residues that are found after treatment of the plant material and of the acid detergent fiber, respectively, with H_2SO_4. Results are affected by the solubilization of acid soluble lignin and(or) by contamination with proteins bound to lignin (Van Soest, 1982) or other condensation products like polyphenolics from the cell contents (Iiyama and Wallis, 1988). The loss of acid soluble or dispersible lignin in the preparatory step for ADL was up to 50% of the lignin present in tropical grasses (Lowry *et al.*, 1994). The loss is much smaller in Klason lignin procedures, in particular when the analyses are performed on pre-extracted material, e.g., neutral detergent fiber (NDF; Sewalt *et al.*, 1996) or starch-free, alcohol insoluble residues (Hatfield *et al.*, 1994).

Alternatively, lignin may be determined after oxidation with $KMnO_4$ (Van Soest and Wine, 1968) or nitrobenzene (Sarkanen and Ludwig, 1971). In these procedures, non-core lignin components can be included in the lignin estimates, because *p*-coumaric acid and ferulic acid are oxidized and thus mimic core lignin components (Lapierre *et al.*, 1989). No gravimetric method is capable of indicating differences in lignin composition or structure although combinations of gravimetric methods and functional group analysis, e.g., methoxyl content determined in Klason lignin (Sewalt *et al.*, 1996) or pyrolysis−gas chromatography/mass spectrometry of isolated lignin (Reeves and Galletti, 1993), can indicate compositional or structural differences. It is of great importance to find a practical method to measure the amount of lignin in plant cell walls and to identify the characteristics of different lignin types. The varying chemical composition of the lignin polymer (Monties, 1989; Monties and Calet, 1992) causes the variability in the type of bonds between lignin and cell wall carbohydrates. Therefore, lignin macromolecular structure and composition affect digestibility probably more than the amount of lignin itself (Porter and Singleton, 1971; Reeves, 1985, 1987; Jung, 1989; Sewalt *et al.*, 1996).

Spectrophotometric methods have possibilities for identifying variability in lignin composition, because of the specific absorbance of the individual phenolic constituents (Harborne, 1989). Commonly, acetyl bromide is used to solubilize lignin prior to spectrophotometric determination (Morrison, 1972a,b). Alternatively, HCl-activated triethylene glycol may be used (Grondal and

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Zenczak, 1950; Zenczak, 1952). Although this procedure has been proposed (Monties, 1989) as an alternative assay to the acetyl bromide method, it has so far only been used to determine lignin gravimetrically as the loss upon treatment of the acid detergent fiber residue with HCl-activated triethylene glycol (Edwards, 1973; Reeves, 1993). Cell wall fractions of the material under consideration must be prepared to avoid interference by other absorbing substances, e.g., aromatic amino acids, flavonoids, isoflavones, or pigments. Several possibilities exist to remove these substances, but each has the risk of removing some of the original lignin. The formation of colloidal suspensions with soluble lignin, which is lost during the isolation procedure, can occur when neutral detergent contains sodium sulfite (Van Soest and Robertson, 1980) or in particular when acid detergent is used (Porter and Singleton, 1971). Additionally, not all of the cell wall bound protein can be removed by detergent solutions (Fukushima *et al.*, 1991).

In spectrophotometric methods, reference lignin values need to be obtained. Morrison (1972a,b) recommended the use of values of ADL or Klason lignin determinations, and Selvendran *et al.* (1989) found ADL values were closely related to those obtained spectrophotometrically as acetyl bromide soluble lignin. Klason lignin and ADL values do not represent the original amount of lignin because acid treatments which characterize these procedures partly solubilize lignin, though to different extents, and condensation products are formed during lignin preparation (Van Soest, 1982; Iiyama and Wallis, 1988). Native (isolated) lignin (Brauns, 1939) also differs from "true" lignin due to the chemical changes during preparation. Fukushima *et al.* (1991) used an industrial alkali-lignin, Indulin, to calculate lucerne lignin concentrations from absorbance readings. Individual phenolic monomers or mixtures may represent part of the lignin polymer. Vanillin has been identified as the main aromatic compound when grass lignins were subjected to nitrobenzene oxidation (Higuchi *et al.*, 1967; Lapierre *et al.*, 1989). Higuchi *et al.* (1967) and Reeves (1985) utilized mixtures of phenolic monomers to mimic monomeric composition of the plant lignins under consideration.

The present experiment was undertaken to study spectrophotometric lignin determination in three winter wheat materials (whole-crop silage, stem, straw) as related to (1) preparation of cell wall fractions, (2) type of solvent, and (3) type of standard used for calculating reference lignin values.

MATERIALS AND METHODS

Cell Wall Preparations. Three different morphological fractions from winter wheat (variety Ares), grown on the experimental farm "Schädtbek" of the Federal Dairy Research Center near Kiel, Germany, were used. Whole-crop silage and stem material was harvested on July 13, 1989, at early dough stage, according to code 82 in the staging system of Zadoks *et al.* (1974). Agronomic and forage quality characteristics of these two materials have been previously reported (Südekum *et al.*, 1991). The wheat straw was the residue from combine harvesting of wheat grain between August 24 and 27, 1991. Stems and whole-crop silage were lyophilized prior to grinding. Stems, whole-crop silage and straw were successively ground to pass screens with 3- and 1-mm sieves. ADL was then determined according to Goering and Van Soest (1970). Three cell wall fractions were prepared from each of the three materials. First, hot water−organic solvent (diethyl ether, 95% ethanol, and acetone)-extracted materials (HWO) were prepared by the method of Morrison (1972a). Second, ethanol−benzene residues (EBR) were prepared according to Bagby *et al.* (1973) with modifications. Samples were extracted in a Soxhlet apparatus for 18 h with a 1:2 (v/v) ethanol−benzene mixture. Samples were then washed with approximately 1 L of deionized water. Third, NDF was prepared according to Goering and Van Soest (1970) except that 2 g of all materials were used and sodium sulfite was omitted, because it may dissolve aromatic compounds (Van Soest and Robertson, 1980). The samples were filtered through a Whatman No. 52 filter paper and washed with deionized water. To avoid condensation products by Maillard reactions, which may occur when samples are dried at temperatures greater than 50 °C (Van Soest, 1964), all residues from the cell wall preparations were lyophilized. Residues were analysed for nitrogen, starch, and Klason lignin. Nitrogen was determined using the standard Kjeldahl procedure with K_2SO_4 and $CuSO_4$ as catalysts. Crude protein (CP) was calculated as N $\times$ 6.25. Free and α-linked glucose (starch) content was determined by enzymatic hydrolysis of starch to glucose, employing a heat-stable α-amylase (Termamyl 120 L; Novo Industrials, Bagsværd, Denmark) as a starch-solubilizing agent (Brandt *et al.*, 1987). Klason lignin was determined as the residue remaining after treatment in H_2SO_4 (720 g/kg) at 30 °C for 1 h, followed by autoclaving at 121 °C in H_2SO_4 (30 g/kg) for 1 h (Kaar *et al.*, 1991). Klason lignin was calculated as the difference in weight of the residue before and after ashing at 450 °C for 6 h. No correction was made for N contamination of Klason lignin.

Solvents. The three cell wall preparations were digested in either HCl−triethylene glycol or acetyl bromide under the same conditions to yield triethylene glycol soluble lignin (TGSL) and acetyl bromide soluble lignin (ABSL), respectively. The procedures were as follows.

(1) Triethylene Glycol Soluble Lignin. Triethylene glycol was mixed with HCl (370 g/kg; 1.6 mL in 250 mL of triethylene glycol). Cell wall material (25 mg) was weighed into screwtab tubes and dispersed with 1.5 mL of methanol (1000 g/kg). HCl−triethylene glycol reagent (20 mL) was added, and the tubes were heated in an autoclave (120 °C) for 60 min. After being cooled in ice water the samples were filtered through glass sinter crucibles (porosity 1). The filtrate (5 mL) was diluted with methanol to give a final volume of 20 mL. The contents were thoroughly blended, and the absorbance of the solutions was measured at 280 nm (UV/vis spectrometer Lambda 2, Perkin Elmer, Überlingen, Germany).

(2) Acetyl Bromide Soluble Lignin (Fukushima et al., 1991, Modified). An amount equivalent to about 8 mg of ADL was weighed into the tubes with Teflon-layered screwtabs. The material was dispersed with 1.5 mL of acetic acid (990 g/kg) and mixed with 10 mL of acetyl bromide in acetic acid (250 g/kg). The samples were kept in an autoclave (120 °C) for 60 min. After cooling in ice water, 20 mL of acetic acid were added to each tube, the tubes were tightly closed and thoroughly shaken, and the contents were filtered through glass sinter crucibles (porosity 1). Another series of 25-mL tubes was prepared, each containing 5 mL of acetic acid and 5 mL of 0.3 M NaOH. The filtrate (5 mL) was added, and the contents were thoroughly blended. To each tube was added 1 mL of 0.5 M hydroxylamine hydrochloride, and acetic acid was added to the contents to a final volume of 25 mL. Samples were mixed again and allowed to stand for 45 min before the absorbance was read at 280 nm. The spectra of each sample were also recorded. Both solvents, activated triethylene glycol and acetyl bromide, were prepared freshly each day.

Standards. To compare the influence on apparent lignin concentrations by using standards differing in phenolic composition, and hence in absorbance at 280 nm, three different standards were prepared. All phenolics were purchased from Sigma (Munich, Germany; reference numbers in parentheses).

(1) A mixture of phenolic aldehydes, namely, vanillin (V2375), syringaldehyde (S0138), and *p*-hydroxybenzaldehyde (H5630), which are frequently found as lignin oxidation products obtained by nitrobenzene. The composition of this standard was based on studies on grasses by Higuchi *et al.* (1967) and designated HIGUCHI.

(2) A mixture of phenolic aldehydes (as above) and the phenolic acids syringic acid (S6681), vanillic acid (V2250),

Table 1. Composition of Phenolic Standards for Spectrophotometric Lignin Determination

standard	method: triethylene glycol soluble lignin (g/L of methanol)		method: acetyl bromide soluble lignin (g/L of acetic acid)
HIGUCHI	1	total phenolics	10.02
	0.44	vanillin	4.410
	0.34	syringaldehyde	3.406
	0.22	*p*-hydroxybenzaldehyde	2.204
REEVES	0.874	total phenolics	8.72
	0.059	*p*-coumaric acid	0.570
	0.045	ferulic acid	0.428
	0.030	vanillic acid	0.300
	0.046	*p*-hydroxybenzaldehyde	0.452
	0.066	syringic acid	0.640
	0.268	syringaldehyde	2.719
	0.360	vanillin	3.611
WIND	0.02		0.1

p-coumaric acid (C9008), and ferulic acid (F3500). The composition of this standard was from Reeves (1985) except for the non-core lignin components *p*-coumaric acid and ferulic acid. The ratio between these two acids in the standard mixture was taken from winter wheat straw data reported by Mason *et al.* (1988). This standard was named REEVES.

(3) An alkali-lignin obtained from wood, Indulin AT (I6384), was purified by heating 5 g of Indulin with 0.7 L of deionized water at 70 °C for 60 min. The suspension was filtered through a glass sinter crucible (porosity 1) and washed with hot water until the filtrate was colorless. The residue was freeze-dried and hereafter referred to as washed Indulin (WIND).

Standards were prepared in methanol for the TGSL method and in acetic acid for the ABSL method. The concentrations of the standards in their respective solvents are given in Table 1.

Standards in acetic acid had higher concentrations of their phenolic constituents because the dilution during preparation for the spectrophotometric analysis was greater. Standards were stored protected from light and, if dissolved in methanol, kept at 5 °C. Standard curves were recorded by measuring five different dilutions of each standard at 280 nm. Ultraviolet spectra of the three standards in methanol and acetic acid were recorded.

Spectrophotometric Analysis. Each combination of cell wall preparation, solvent and standard was analysed in triplicate and appropriate duplicate blanks were included in each series of measurements. Lignin concentrations were calculated from the absorbance at 280 nm.

Statistical Analysis. Lignin concentrations derived from spectrophotometric analysis were subjected to analysis of variance according to a 2 × 3 × 3 factorial design separately for the three wheat materials (whole-crop silage, stem, straw). Sums of squares were partitioned into main effects of solvent, method of cell wall preparation and standard and the respective two- and three-way interaction(s). Because the extent of our study was limited and we did not want to stress our data too much, drawing conditional inferences from our data appeared preferable over further separate statistical examination of main effects (Lowry, 1992).

RESULTS AND DISCUSSION

Cell Wall Preparation. The yield of the cell wall preparations HWO, NDF, and EBR and the ADL contents increased in the order whole-crop silage, stem, and straw (Table 2). The crude protein, starch, and Klason lignin concentrations in the cell wall preparations of the wheat materials are reported in Table 3. The Klason lignin values were always greater than the ADL values. The magnitude of the difference was the same as that reported by Hatfield *et al.* (1994) for the stems of a legume and two grass species. The differences in the yields of cell wall among the wheat materials can largely be explained by varying morphological composition (whole-crop silage versus stem) and maturity stages (stem versus straw) of the materials. Irrespective of the wheat sample, the highest yield of cell wall was found by HWO, followed by NDF; EBR yielded the least quantities. In the latter procedure, application of benzene and the duration of the extraction most probably resulted in partial dissolution of cell wall compounds. Moreover, EBR contained more CP than the other two cell wall preparations and at least as much starch as HWO and NDF (Table 3). In whole-crop silage, preparation of NDF resulted in the lowest concentrations of CP and, in particular, starch. The high concentrations of starch in HWO indicates that a pre-extraction of starch must be performed before HWO

Table 2. Contents (g/kg of Dry Matter) of Cell Wall Preparations and Acid Detergent Lignin (ADL) in Wheat Materials[a]

material	cell wall preparation[b]: HWO	EBR	NDF	ADL
whole-crop silage	654	450	628	30
stem	743	652	689	46
straw	898	761	835	71

[a] Values are means of duplicate determinations. [b] Cell wall preparations: HWO, hot water−organic solvent fiber; EBR, ethanol−benzene residue; NDF, neutral detergent fiber.

Table 3. Composition of Cell Wall Preparations of Wheat Materials (g/kg of Cell Wall Dry Matter)[a]

cell wall preparation[b]	item[c]	wheat material: whole-crop silage	stem	straw
HWO	crude protein	17.1	12.4	13.7
	starch	214.5	12.4	10.9
	Klason lignin	170.9	235.3	227.5
	KLDM	111.8	174.8	204.3
EBR	crude protein	30.8	23.2	22.7
	starch	232.4	12.0	11.4
	Klason lignin	163.9	212.8	241.5
	KLDM	73.8	138.7	183.8
NDF	crude protein	3.7	2.1	8.9
	starch	15.4	11.1	11.0
	Klason lignin	219.3	218.5	232.3
	KLDM	137.7	150.5	193.9

[a] Values are means of duplicate determinations. [b] Cell wall preparations: HWO, hot water−organic solvent fiber; EBR, ethanol−benzene residue; NDF, neutral detergent fiber. [c] Item: KLDM, Klason lignin expressed as g/kg of dry matter of the original material.

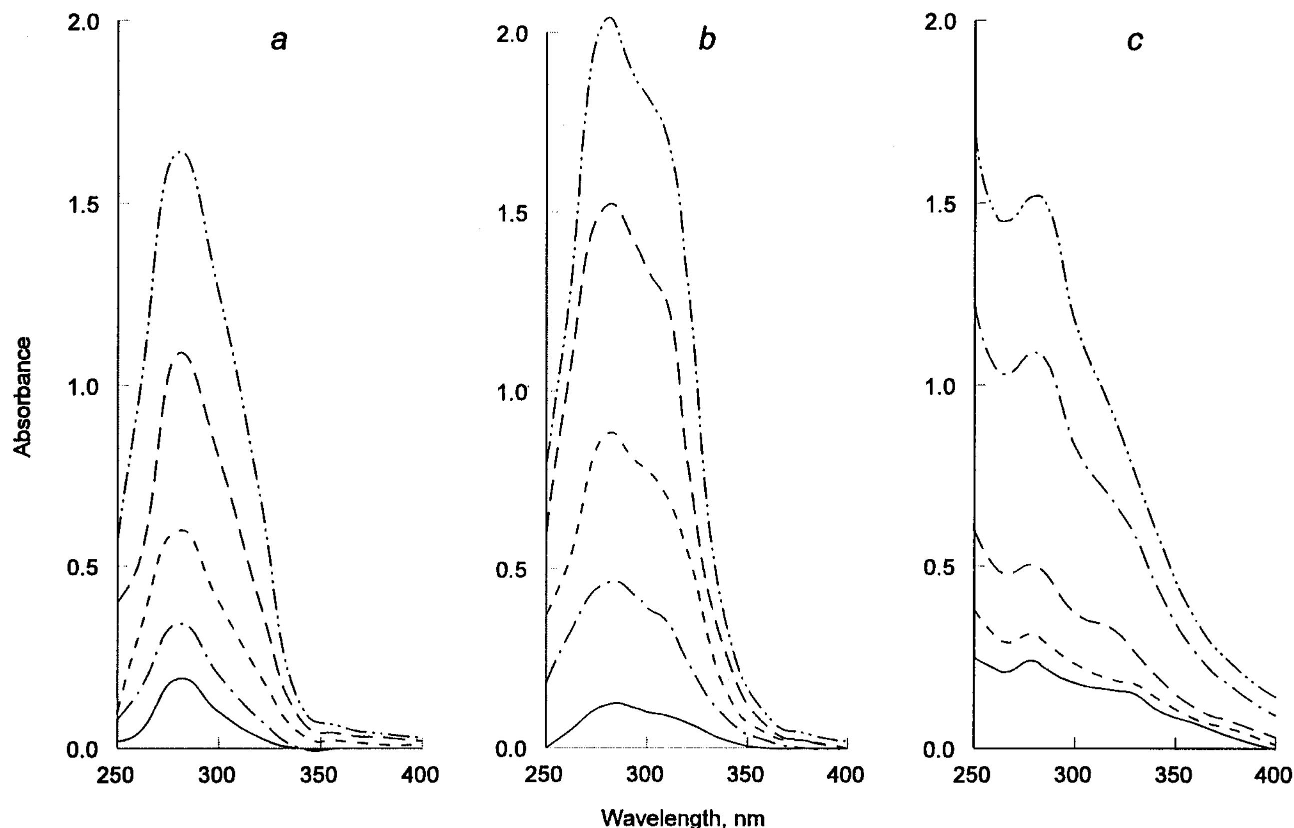

Figure 1. Ultraviolet spectra of phenolic standards in methanol (triethylene glycol soluble lignin procedure) at different concentrations (for composition of standards see Table 1). (a) HIGUCHI: 8.72, 12.21, 17.44, 26.16, and 34.88 mg/L. (b) REEVES: 7.62, 15.24, 22.87, 30.49, and 38.11 mg/L. (c) WIND: 5.08, 10.16, 15.24, 20.33, and 30.49 mg/L.

preparation from whole-crop silage can be conducted. In stem and straw, starch contents of NDF and HWO were similarly low and CP content of HWO was only slightly higher than that of NDF (Table 3). Reeves (1993) reported similar CP concentrations (14 g/kg of dry matter) in HWO of wheat straw. Though a small amount of cell wall bound proteins obviously remained in the NDF and HWO, it is unlikely that they greatly influenced absorbance at 280 nm, because N bound in aromatic amino acids forms only a small part of total amino acid N in cell walls. Morrison (1972a) found that acetyl bromide treatment shifted the peak in absorbance of aromatic amino acids from 280 to near 260 nm, which would further lower possible interference of aromatic amino acids in absorbance readings at 280 nm, at least for HWO with the ABSL procedure. Because NDF and HWO yields from stem and straw (Table 2) were more divergent than could be explained by differences in starch and CP contamination of the cell wall preparations (Table 3), it appears that HWO was slightly superior to NDF for preparing cell walls for lignin determination in winter wheat stems and straw, whereas NDF obviously was the best preparatory step in whole-crop silage.

Spectrophotometric Analysis. When triethylene glycol was used as solvent, all three standards had a clear maximum close to 280 nm (280.5−282 nm; Figure 1a−c). In acetic acid, the maxima of the spectra were shifted about 10 nm to the shorter wavelength and maxima were found between 269.2 and 272.2 nm (Figure 2a−c). Iiyama and Wallis (1989) regarded the shifting of the spectra after treatment with acetyl bromide as typical for all 4-substituted phenolics. Harborne (1989) reported that upon ionization of phenolics with alkali there is normally a large (15−50 nm) bathochromic shift of ultraviolet spectra. Absorption coefficients (absorbance at 280 nm under 1 cm thickness of a solution containing 10 g/kg of the respective standard) were derived from the absorbance readings of the standards presented in Figures 1 and 2. The absorption coefficients for the standards in methanol (TGSL-procedure) were 423 (HIGUCHI), 235 (REEVES), and 513 (WIND). The respective values for the standards in acetic acid (ABSL procedure) were 686, 569, and 270.

One purpose of this study was to examine the influence of standards differing in composition and thus in absorbance on resulting lignin concentrations. Washed Indulin (Fukushima *et al.*, 1991) was chosen as a standard. It has the disadvantages that it is obtained from wood (Fukushima *et al.*, 1991) and not from grasses and that its monomeric composition is unknown. Mixtures of phenolic monomers offer the advantage that they can be varied in their relative composition and theoretically be best adapted to forage lignin. Thus, we examined two standards that approximated lignin composition in *Gramineae* and wheat, respectively, namely HIGUCHI and REEVES, to calculate lignin concentrations in the forage materials. It must be considered, however, that even these standards represent only approximations to the native forage lignin, which is partly due to high variability in forage lignin composition. Phenolic constituents of core lignin are normally determined after oxidation of the lignin by nitrobenzene or $KMnO_4$. Vanillin, *p*-hydroxybenzaldehyde, and syringaldehyde are oxidation products of coniferyl alcohol, *p*-coumaryl alcohol, and syringyl alcohol, respectively, and relative yields of the oxidative degradation products are dependent on time and temperature of nitrobenzene oxidation (Billa *et al.*, 1996).

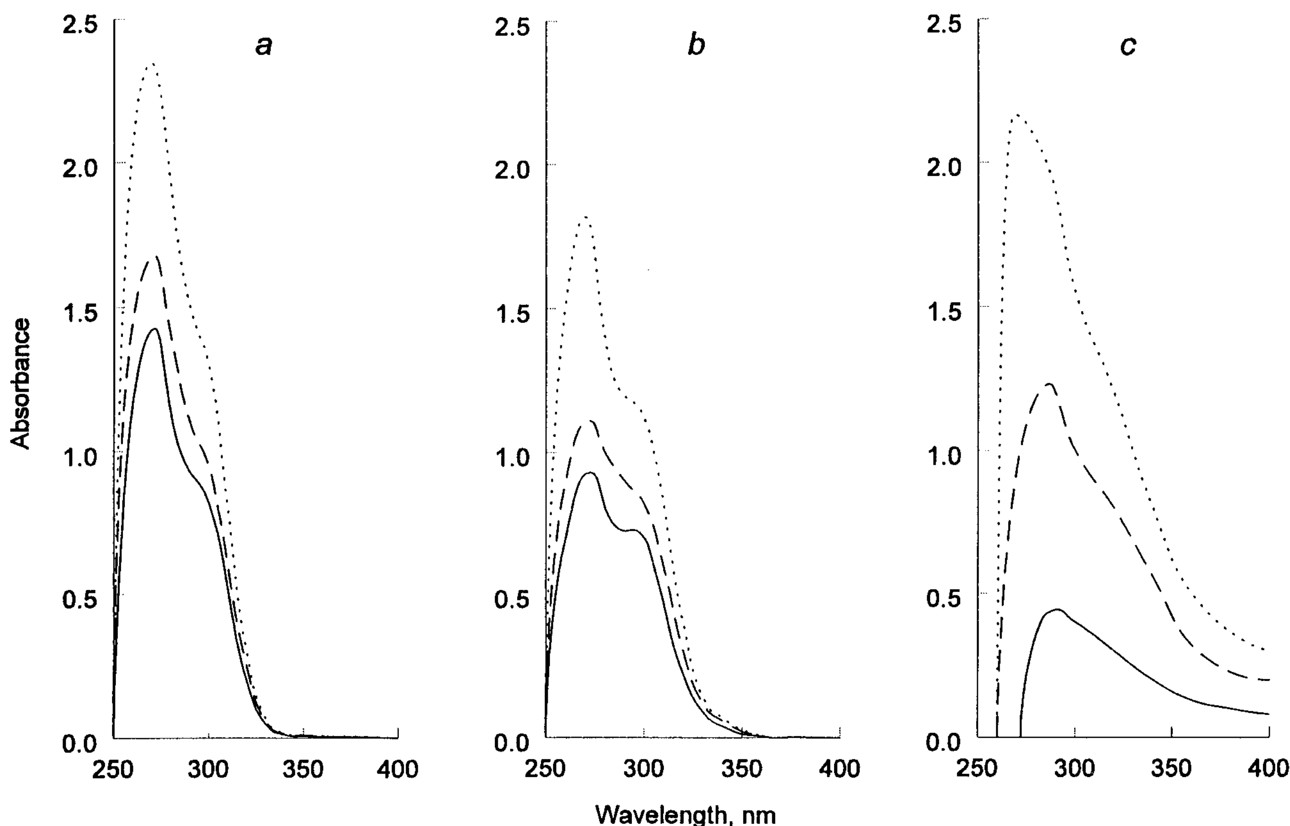

Figure 2. Ultraviolet spectra of phenolic standards in acetic acid (acetyl bromide soluble lignin procedure) at different concentrations (for composition of standards see Table 1). The spectra for the lowest and highest concentration of each standard that are reported below were not recorded by the printer connected to the spectrometer. Because the absorbances were recorded regularly they were included in estimates of standard curves that were used for calculation of absorption coefficients. (a) HIGUCHI: 9.54, 19.09, 22.90, 28.63, and 38.17 mg/L. (b) REEVES: 8.31, 16.60, 19.90, 24.90, and 33.20 mg/L. (c) WIND: 12.70, 25.40, 50.70, 76.10, and 101.80 mg/L.

Moreover, oxidation of the non-core lignin compounds *p*-coumaric and ferulic acid could also lead to the production of *p*-hydroxybenzaldehyde and vanillin that are subsequently included in estimates of core lignin composition and concentration. Higuchi *et al.* (1967) observed a reduction in amount to less than 50% of *p*-hydroxybenzaldehyde after oxidation of saponified residues compared to untreated cell wall residues. They reported that more than half of the total apparent core lignin aldehydes of different species of the *Gramineae* family could be attributed to *p*-coumaric acid and a major part of total vanillin to ferulic acid. More recent studies conducted by Hartley and Keene (1984) on maize and barley stems and wheat bran and Lapierre *et al.* (1989) on wheat straw confirmed the earlier observations.

Although concentrations of phenolic constituents in standard solutions for absorbance readings were slightly different between triethylene glycol and acetic acid, a comparison of Figures 1 and 2 indicate differences between triethylene glycol and acetic acid in peak shape and height related to the standards. The main compound in standard HIGUCHI and standard REEVES is vanillin, which, among others, is responsible for the strong absorbance at 280 nm. Standard REEVES additionally contained *p*-coumaric acid and ferulic acid, though both acids together did not contribute more than 120 g/kg of total phenolics in the standard mixtures. The presence of the non-core lignin compounds can be seen especially in the spectra of the TGSL method (Figure 1b) as a slight shoulder at the characteristic wavelength of 310–330 nm (Harborne, 1989). This shoulder was reduced in intensity when acetyl bromide was the solvent (Figure 2b), which can be explained by the effect of the solvent on phenolics. This phenomenon has already been discussed for 4-substituted phenolics (Iiyama and Wallis, 1989). The composition of the industrial alkali-lignin Indulin is not known. However, the shape of the ultraviolet spectrum is similar to that of spruce lignin obtained by the same method in this laboratory (spectrum not shown), which may indicate a phenolic monomer composition typical of wood. Although Fukushima *et al.* (1991) reported that WIND is a suitable standard for lignin determination in lucerne, our data indicate that WIND is not suitable to quantify lignin in wheat forage materials.

The ultraviolet spectra after digestion with HCl–triethylene glycol reagent of whole-crop silage (Figure 3), stem (Figure 4), and straw (Figure 5) coincided in shape with the standard spectra. Each had a maximum at 280 nm and a second maximum that was probably due to *p*-coumaric acid and ferulic acid at about 315 nm. In accordance with the spectra of the standard solution (Figure 2), treatment with acetyl bromide shifted both maxima to shorter wavelengths and led to a reduction in height of the second maximum (not shown). Morrison (1972a) reported that prolonged heating (120 versus 60 min) of samples in acetyl bromide gave an increase in the absorbance above 300 nm. Although these conditions were probably less severe than the 60 min at 120 °C used in this study, we observed no increase in absorbance above 300 nm. Iiyama and Wallis (1989) claimed that conjugated carbonyl groups rather than esterified phenolics are responsible for the peak at 315 nm. The uniform shifting of the maxima of the standard (Figure 2) and the wheat materials spectra (not shown)

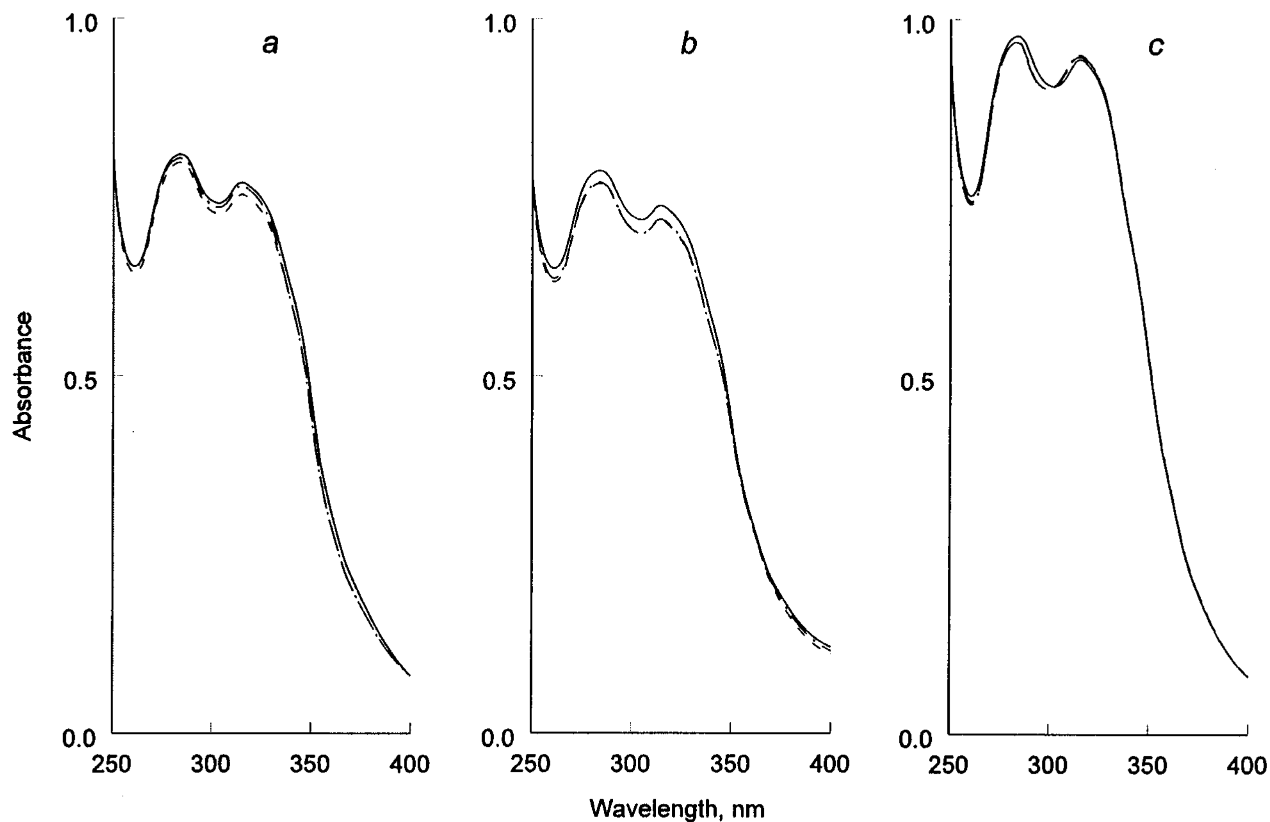

Figure 3. Ultraviolet spectra of cell wall preparations from wheat whole-crop silage treated according to the triethylene glycol soluble lignin procedure; —, - - -, and —•— lines indicate that each cell wall preparation was analyzed in triplicate. (a) Hot water−organic solvent fiber; (b) ethanol−benzene residue; (c) neutral detergent fiber.

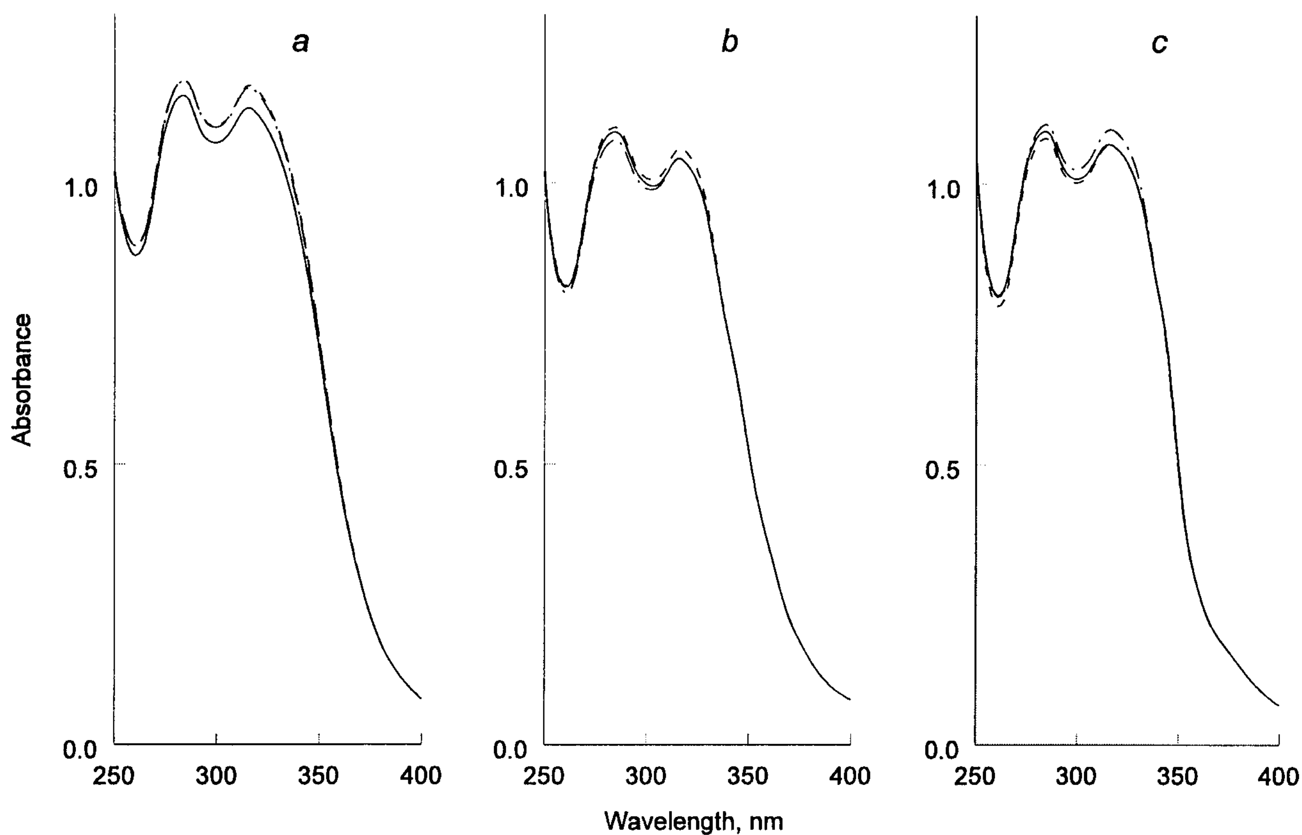

Figure 4. Ultraviolet spectra of cell wall preparations from wheat stems treated according to the triethylene glycol soluble lignin procedure; —, - - -, and —•— lines indicate that each cell wall preparation was analyzed in triplicate. (a) Hot water−organic solvent fiber; (b) ethanol−benzene residue; (c) neutral detergent fiber.

was obviously only caused by the solvent. Hence absorbance at 280 nm does not seem a correct measure for lignin in the ABSL procedure, because lignin concentrations are underestimated. Reading absorbances

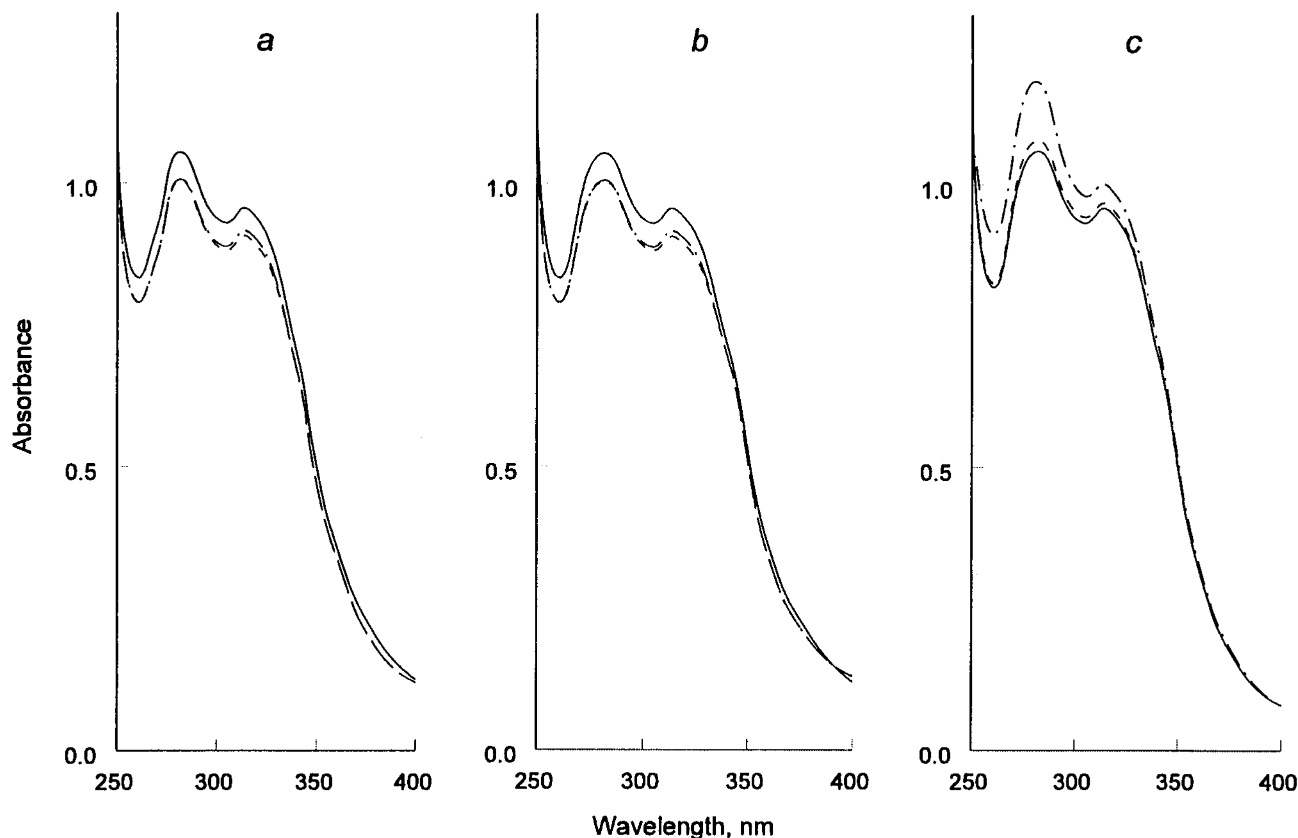

Figure 5. Ultraviolet spectra of cell wall preparations from wheat straw treated according to the triethylene glycol soluble lignin procedure; —, - - -, and —•— lines indicate that each cell wall preparation was analyzed in triplicate. (a) Hot water–organic solvent fiber; (b) ethanol–benzene residue; (c) neutral detergent fiber.

at 270 nm would thus result in more reliable data. Unless ultraviolet spectra of forage materials are known, we recommend that spectra of cell wall materials be recorded to minimize the risk of false estimations of lignin concentrations. Hitherto existing work, however, used the absorbance at 280 nm to quantify lignin concentrations from absorbance readings. For the ease of comparison, therefore, the following comparison of lignin concentrations is based on values derived from the absorbance at 280 nm.

Least squares means of lignin concentrations in whole-crop silage, stems, and straw as related to solvent, method of cell wall preparation, and standard together with the results of analysis of variance are reported in Tables 4–6. Irrespective of method of cell wall preparation, all lignin concentrations that were determined spectrophotometrically in whole-crop silage (Table 4), stem (Table 5), and straw (Table 6) were lower than the corresponding Klason lignin values (Table 3; Klason lignin expressed as g/kg of dry matter of the original material). The discrepancies can be due either to incomplete recovery of lignin in acetyl bromide and(or) triethylene glycol (Reeves, 1993) or to contamination of Klason lignin residues with N-containing compounds (Hatfield *et al.*, 1994). The CP content of the three cell wall preparations on which Klason lignin was determined generally was low (≤31 g/kg of cell wall dry matter). Therefore, it seems unlikely that protein contamination did greatly bias Klason lignin values, although the use of 6.25 as the N to CP conversion factor is questionable in heavily pre-extracted cell wall materials (Reeves, 1993).

Across solvents and standards used for the spectrophotometric lignin assays, lignin contents in the three wheat materials was always higher with HWO than with EBR and NDF with the only exception that lignin content in EBR was greater than in HWO when calculations were based on triethylene glycol and REEVES in whole-crop silage (Table 4). In wheat stems (Table 5) and straw (Table 6) lignin concentrations were higher with REEVES than HIGUCHI and, averaged over these two standards, higher with triethylene glycol than with acetyl bromide. Conflicting observations were obtained with WIND. This standard gave lignin concentrations that were lower than or equal to those

Table 4. Lignin Concentrations (g/kg of Dry Matter) in Winter Wheat Whole-Crop Silage As Related to Solvent, Method of Cell Wall Preparation, and Standard Used for Spectrophotometric Analysis

solvent[a]	cell wall preparation[b]	standard HIGUCHI	REEVES	WIND	SEM[c]
TEG	HWO	50.3	61.0	65.3	0.13
	EBR	46.9	71.1	40.1	
	NDF	39.9	58.2	33.6	
AcBr	HWO	26.7	28.1	63.6	
	EBR	23.0	24.2	56.1	
	NDF	22.7	24.7	57.2	

effect	*P*-level
solvent (SO)	<0.001
cell wall preparation (CWP)	<0.001
standard (ST)	<0.001
SO × CWP	<0.001
SO × ST	<0.001
CWP × ST	<0.001
SO × CWP × ST	<0.001

[a] Solvents: TEG, triethylene glycol; AcBr, acetyl bromide. [b] Cell wall preparations: HWO, hot water–organic solvent fiber; EBR, ethanol–benzene residue; NDF, neutral detergent fiber. [c] Standard error of the mean.

Table 5. Lignin Concentrations (g/kg of Dry Matter) in Winter Wheat Stems As Related to Solvent, Method of Cell Wall Preparation, and Standard Used for Spectrophotometric Analysis

solvent[a]	cell wall preparation[b]	standard HIGUCHI	REEVES	WIND	SEM[c]
TEG	HWO	74.3	95.6	96.7	0.05
	EBR	63.6	88.7	80.5	
	NDF	64.6	91.5	84.2	
AcBr	HWO	46.0	50.5	120.2	
	EBR	40.6	43.3	105.4	
	NDF	42.9	46.2	112.3	

effect	P-level
solvent (SO)	<0.001
cell wall preparation (CWP)	<0.001
standard (ST)	<0.001
SO × CWP	<0.001
SO × ST	<0.001
CWP × ST	<0.001
SO × CWP × ST	<0.001

[a] Solvents: TEG, triethylene glycol; AcBr, acetyl bromide. [b] Cell wall preparations: HWO, hot water−organic solvent fiber; EBR, ethanol−benzene residue; NDF, neutral detergent fiber. [c] Standard error of the mean.

Table 6. Lignin Concentrations (g/kg of Dry Matter) in Winter Wheat Straw As Related to Solvent, Method of Cell Wall Preparation, and Standard Used for Spectrophotometric Analysis

solvent[a]	cell wall preparation[b]	standard HIGUCHI	REEVES	WIND	SEM[c]
TEG	HWO	82.8	119.3	71.5	0.14
	EBR	66.8	96.2	56.4	
	NDF	82.1	116.0	68.0	
AcBr	HWO	50.1	57.9	139.4	
	EBR	35.4	42.3	111.6	
	NDF	44.5	52.3	127.9	

effect	P-level
Solvent (SO)	< 0.001
Cell wall preparation (CWP)	< 0.001
Standard (ST)	<0.001
SO × CWP	0.012
SO × ST	<0.001
CWP × ST	0.051
SO × CWP × ST	<0.001

[a] Solvents: TEG, triethylene glycol; AcBr, acetyl bromide. [b] Cell wall preparations: HWO, hot water−organic solvent fiber; EBR, ethanol−benzene residue; NDF, neutral detergent fiber. [c] Standard error of the mean.

derived from REEVES when triethylene glycol was the solvent. When acetyl bromide was used, however, WIND yielded more than double the amount of lignin in wheat stems and straw than REEVES. There are no obvious reasons for this strong interaction between solvent and standard but different solubilities of the whole lignin standard (WIND) versus the phenolic monomer standards (HIGUCHI, REEVES) in the two different solvents might at least partly have contributed to the interaction.

The present work has indicated that due to strong interactions among cell wall preparations, solvents, and standards used for quantifying absorbance readings at 280 nm, it is not possible to indicate one single procedure as being superior to others in characterizing true concentration of lignin. Our data suggest that NDF best recovered total cell wall material in whole-crop silage, whereas HWO was slightly superior to NDF in stem and straw. The least appropriate method of cell wall preparation in all three wheat materials was EBR due to low recovery and health risks. Triethylene glycol has advantages over acetyl bromide because handling is much easier and less harmful to people and the environment. Acetyl bromide treatment resulted in shifting of spectra to the shorter wavelengths. When considered across cell wall preparations and standards, absorbances of wheat materials in triethylene glycol were more consistent. According to previous work on monomeric composition of grass lignins, a mixture of phenolic aldehydes and acids should best mimic lignin and phenolic acids adjacent to lignin and cell wall carbohydrates. Consequently, lignin concentrations related to absorbance readings based on standard REEVES are suggested as being close to the true situation in the wheat materials under consideration.

ABBREVIATIONS USED

ABSL, acetyl bromide soluble lignin; ADL, acid detergent lignin; CP, crude protein (N × 6.25); EBR, ethanol−benzene residue; HIGUCHI, lignin standard based on phenolic aldehydes according to Higuchi *et al.* (1967); HWO, hot water−organic solvent fiber; NDF, neutral detergent fiber; REEVES, lignin standard based on phenolic aldehydes and acids according to Reeves (1985) and Mason *et al.* (1988); TGSL, triethylene glycol soluble lignin; WIND, lignin standard based on washed alkali-lignin, Indulin.

The following abbreviations are only used in Table 3 (KLDM) or in Tables 4−6: AcBr, acetyl bromide; CWP, cell wall preparation; KLDM, Klason lignin expressed as g/kg of dry matter of the original material; SO, solvent; ST, standard; TEG, triethylene glycol.

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