Influence of Extractives on the Analysis of Herbaceous Biomass†

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The consequences of extracting switchgrass, corn stover, and fescue feedstocks with either 95% ethanol or hot water prior to the chemical characterization of the feedstock have been determined. Glycans (glucan and xylan), Klason lignin, acid soluble lignin, uronic acids, acetyl groups, protein, and ash analyses were done on each feedstock. Extraction with 95% ethanol or hot water significantly reduced the measured Klason lignin values of all the feedstocks. Ethanol extractions reduced the apparent glucan content of two of the feedstocks, fescue and switchgrass. Extractions with hot water reduced the apparent glucan content of all the feedstocks. The apparent content of other components in the feedstock, such as ash and protein, was also reduced as a result of doing solvent extractions. The results indicate that more accurate estimates of the true lignin and cellulose content of a feedstock are obtained if extractives are removed from the feedstock prior to the analysis. However, in some cases the analysis of extracted feedstocks resulted in lower values for the total carbohydrate content of those feedstocks. This parameter is critical when evaluating these feedstocks for biomass-to-ethanol processes.

Keywords: *Corn stover; switchgrass; fescue; cellulose; lignin; extractives.*

Herbaceous agricultural residues represent a major source of lignocellulosic material with considerable potential for use in biomass-to-ethanol, renewable energy schemes. It is estimated that there are 300 million tons of this material generated annually in the United States (Schell et al., 1992). Selected herbaceous crops, such as switchgrass, are currently under study as potential "energy crops" to be grown specifically for the production of fuel ethanol (Torget et al., 1990; Downing et al., 1995; Lynd et al., 1991).

The widespread interest in the use of these feedstocks for ethanol production has increased the demand for simple, accurate analytical methods that are particularly suited for this area of research. The primary components of interest for biomass-to-ethanol schemes are cellulose, hemicellulose, and lignin (Johnson et al., 1995; Zhang et al., 1995). The cellulose fraction represents the majority of the potentially fermentable glucan available in the feedstock. The hemicellulose fraction contains the majority of the potentially fermentable xylan available in the feedstock. Lignin is the recalcitrant component of the feedstock, which is most generally negatively correlated with the enzymatic accessibility of the feedstocks cellulose component (Jung et al., 1992). Many analytical approaches are available for the analysis of these macrocomponets (Karr and Brink, 1991a; Karr et al., 1991; American Society for Testing and Materials, 1993). The approach most commonly used in biomass-to-ethanol research for the quantification of neutral polysaccharides (primarily cellulose and hemicellulose) involves the complete acid hydrolysis of the carbohydrate fractions followed by quantitative measurement of the resulting sugars in the hydrolysate by either liquid or gas chromatography. The amount of glucose recovered is then adjusted for polymerization and reported as glucan. In a similar way the amount of xylose recovered in the hydrolysate is reported as xylan. The lignin content of the feedstock is most generally based on the gravimetric method of Klason (72% sulfuric acid method), or one of its permutations, which separates lignin based on its insolubility in sulfuric acid (American Society for Testing and Materials, 1993).

The above analytical approaches have been widely used for the characterization of woody species (Moore and DeLuca, 1967; Theander, 1991; Norman, 1937). It was noted over 60 years ago that extracting wood with solvents prior to the analysis of lignin by the method of Klason improved the accuracy of the assay (Ritter and Barbour, 1935; Norman, 1937; Ritter et al., 1932). Solvent treating/extracting wood removes extraneous compounds that may decompose and subsequently associate with lignin during the course of the assay. Traditionally, nonpolar solvents are used to extract extraneous lipophilic compounds and water is used to extract extraneous hydrophilic compounds. The standard extraction protocol currently accepted by the American Society for Testing and Materials (1993) and the Technical Association of the Pulp and Paper Industry (TAPPI, 1988) calls for sequentially pretreating wood with ethanol–benzene, 95% ethanol, and hot water. This extraction scheme is recommended as a means of preparing woody samples for further analyses.

Extraction protocols using 80% ethanol as the sole extracting solvent were recently published (Theander, 1991). These extraction protocols were developed for the quantitative removal of hydrophilic and lipophilic extractives prior to the analysis of the starch and fiber components of a wide range of plant materials. A slightly modified extraction protocol using 95% ethanol was then reported for use with herbaceous biomass-to-ethanol feedstocks (Schell et al., 1992). This approach, extracting for more than 20 h with 95% ethanol in a Soxhlet-type apparatus, was recently adopted as a standard method by the National Renewable Energy Laboratory (1994). The different extraction methods are all designed to remove nonfiber components from the feedstock prior to subsequent analyses. An obvious

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† Oregon State University Agricultural Experiment Station Technical Publication No. 10 989.

S0021-8561(96)00401-3 CCC: $14.00

advantage of extracting with 95% ethanol, versus the other "standard methods" mentioned above, is that ethanol is a relatively nontoxic solvent. The other advantages and limitations of using this extraction scheme prior to the chemical characterization of feedstocks intended for biomass-to-ethanol processing have not been discussed. In this paper we present data that provides a quantitative understanding of the consequences of using 95% ethanol to remove extractives prior to the macrocomponent analysis of herbaceous feedstocks. Three representative feedstocks were included in the study: switchgrass, fescue straw, and corn stover. The results obtained from analyzing the ethanol-extracted feedstocks are compared with those obtained when the same analyses are applied to hot water-extracted feedstocks and native feedstocks. The results are interpreted with specific reference to the utilility of these extraction schemes when evaluating herbaceous feedstocks for biomass-to-ethanol conversion.

MATERIALS AND METHODS

Switchgrass (*Panicum virgatum*) and corn stover feedstocks were provided by the National Renewable Energy Laboratory (NREL), Golden, CO. Corn stover is used here in reference to the combined stalks and cobs remaining in the field following harvesting of the kernels from the cobs. The fescue feedstock was obtained from the Oregon Department of Energy. All feedstocks were milled to pass a 40 mesh screen and stored at room temperature prior to analysis. Sugar standards were obtained from Sigma.

Chemical Analyses. *Percent solids/moisture* was calculated from the weight loss of a preweighed sample dried to constant weight at 105 °C in a convection oven.

Neutral carbohydrates, uronic acids, and acetic acid were determined by first hydrolyzing the feedstock in H_2SO_4 (Theander and Westerlund, 1986) and then quantitatively measuring the resulting monosaccharides and acids. Following hydrolysis, components were separated and quantified via HPLC using Biorad Aminex HPX-87P (neutral sugars) and HPX-87H (acids) columns coupled with a Waters Model 710 differential refractive index detector. Water and 0.005 M H_2SO_4 were the mobile phases for neutral sugar and acid columns, respectively. Standard sugar solutions were included in all procedures to account for sugar degradation during the hydrolysis step. *Klason lignin* (KL) was defined as the ash-free insoluble residue resulting from the two-stage acid hydrolysis procedure (72% H_2SO_4/20 °C; 3% H_2SO_4/reflux) used for polysaccharides (NREL, 1992b). *Acid soluble lignin* (ASL) was calculated from the absorbance at 205 nm of the liquid phase resulting from the Klason lignin assay; an absorptivity of 110 L/g cm was used to convert absorbance readings to mass values (Karr et al., 1991). *Ash* was defined as the inorganic material remaining following complete combustion of the feedstock in a muffle furnace at 525 °C. *Protein* was estimated by a micro-Kjeldahl method (AOAC, 1990) using a nitrogen-to-protein conversion factor of 6.25. A more detailed account of each of these analytical methods is available (Tandjo, 1996; Thammasouk, 1996).

Extractions. Extractions with 95% ethanol were done according to the standard method as described by the National Renewable Energy Laboratory (NREL, 1994). A 7 g sample of feedstock, weighed to the nearest 0.1 mg, was extracted with 95% ethanol in a medium porosity thimble (Whatman CAT No. 2800338) using conventional Soxhlet glassware (approximately 150 mL per solvent cycle). The total extraction time was approximately 22 h. The reflux rate of the solvent was adjusted to allow for approximately 5 solvent cycles/h, giving a total of 100−120 solvent exchanges over the complete extraction period. The extracted sample residue was dried at 45 °C and kept in a sealed bottle at room temperature prior to analysis. The extracting solvent was removed using a rotary evaporator while keeping the solvent temperature at 35 °C. The resulting extractives residue was subsequently dried in a convection oven at 45 °C to constant weight (approximately 24 h) and weighed to the nearest 0.1 mg. The dried extractives were stored in a desiccator at room temperature. The percent extractives was calculated from the weight of total solids loaded into the extraction thimble and the weight of dried extractives recovered from the extracting solvent.

Hot water extractions were done according to standard method T264 (om-88) of the Technical Association of the Pulp and Paper Industry (TAPPI, 1988). A 5 g sample of feedstock, weighed to the nearest 0.1 mg was extracted in a 500 mL flask containing 250 mL of double-distilled water for 3 h under refluxing conditions. At the completion of the extraction period, the sample plus solvent was cooled and filtered through a medium porosity sintered glass crucible. The solid material was dried to constant weight at 45 °C and stored in a sealed bottle at room temperature for subsequent analyses. The filtrate was transferred to a preweighed beaker and the water evaporated on a steam bath until the filtrate was nearly dry. The beaker with extractives was then dried at 45 °C for 24 h. The percent extractives was calculated from the total solids loaded into the extracting flask and the weight of dried extractives recovered from the extracting solvent.

Statistical Analyses. One-way analysis of variance (ANOVA) was conducted on the results from the carbohydrate and lignin analyses. There were three treatment groups: unextracted, ethanol-extracted, and hot water-extracted. The analysis was based on least significant differences (LSD) multiple comparison. Statistical calculations were done using Statgraphic 7.0 software.

Table 1. Composition of Switchgrass As Determined by the Analysis of Native, Ethanol-Extracted, and Water-Extracted Preparations[a,b] (Results Expressed As a Percentage of the Native, Oven-Dried Feedstock)

component	native substrate	ethanol-extracted	water-extracted
total glycans	56.85[b](0.51)	54.49[a] (0.36)	54.44[a] (0.44)
glucan	31.30[b]	29.88[a]	29.50[a]
xylan	20.56[b]	19.88[a]	21.58[c]
galactan	1.86[b]	1.38[a]	1.63[ab]
arabinan	3.13[b]	3.35[b]	1.73[a]
mannan	bdl[c]	bdl	bdl
Klason lignin	21.37[c](0.26)	17.85[b](0.28)	16.87[a](0.04)
acid soluble lignin	3.37[c](0.10)	2.39[b](0.03)	1.83[a](0.07)
ash	7.10(0.11)	5.72(0.13)	2.85(0.1)
protein	3.90(0.20)	3.69(0.30)	2.83(0.08)
uronic acids	1.92(0.22)	1.46(0.11)	1.50(0.03)
acetyl groups	1.87(0.03)	1.76(0.06)	1.80(0.05)
extractives	na[d]	9.74(0.05)	16.42(0.23)
total:	96.38	97.10	98.54

[a] Values in parentheses are standard error of the means. [b] Mean values with different superscript letters were significantly different from one another ($P < 0.05$). [c] bdl = below detection limit. [d] na = not applicable.

RESULTS

The chemical composition of the switchgrass, fescue, and corn stover feedstocks are presented in Tables 1, 2, and 3, respectively. The values in these tables are presented in terms of the weight of the measured component per unit weight of original (native) feedstock. The composition of each of the extracted feedstocks is also presented in Table 4 on an "extractives-free" basis. We will focus our discussion on the data presented in Tables 1−3, since it provides a summative measure of feedstock composition in terms of the actual feedstock to be used in biomass-to-ethanol processes. The data as presented in Table 4 are more commonly used in comparative studies of the chemical composition of different lignocellulosic materials (Karr and Brink, 1991b). The macrocomponent composition of each feedstock was determined following three distinct sample preparation protocols. The three protocols were (a) no

Table 2. Composition of Fescue As Determined by the Analysis of Native, Ethanol-Extracted, and Water-Extracted Preparations[a,b] (Results Expressed As a Percentage of the Native, Oven-Dried Feedstock)

component	native substrate	ethanol-extracted	water-extracted
total glycans	60.57[a](1.03)	53.27[b](0.93)	50.16[b](0.98)
glucan	35.84[a]	31.33[b]	28.40[b]
xylan	20.41[a]	19.21[a]	19.27[a]
galactan	0.96[a]	1.41[a]	0.00[a]
arabinan	2.61[a]	1.32[a]	2.14[a]
mannan	0.75[a]	bdl[c]	0.35[a]
Klason lignin	18.18[a](0.16)	15.54[b](0.57)	15.38[b](0.18)
acid soluble lignin	2.86[a](0.05)	1.97[b](0.03)	1.43[c](0.08)
ash	6.67(0.06)	4.66(0.30)	1.14(0.16)
protein	6.75(0.03)	5.42(0.12)	3.50(0.04)
uronic acids	2.87(0.34)	2.06(0.13)	1.62(0.00)
acetyl groups	1.40(0.06)	1.59(0.21)	1.30(0.01)
extractives	na[d]	16.12(0.42)	25.40(1.10)
total:	99.30	100.63	99.93

[a] Values in parentheses are standard error of the means. [b] Mean values with different superscript letters were significantly different from one another ($P < 0.05$). [c] bdl = below detection limit. [d] na = not applicable.

Table 3. Composition of Corn Stover As Determined by the Analysis of Native, Ethanol-Extracted, and Water-Extracted Preparations[a,b] (Results Expressed As a Percentage of the Native, Oven-Dried Feedstock)

component	native substrate	ethanol-extracted	water-extracted
total glycans	58.99[a](0.16)	58.18[a,b](0.46)	52.83[b](0.23)
glucan	35.25[a]	34.27[a,b]	31.79[b]
xylan	19.71[a]	19.78[a]	17.52[a]
galactan	1.36[a]	1.32[a]	0.98[a]
arabinan	2.67[a]	2.81[a]	2.54[a]
mannan	bdl[c]	bdl	bdl
Klason lignin	18.60[a](0.22)	16.56[b](0.09)	15.67[b](0.31)
acid soluble lignin	2.49[a](0.10)	1.71[b](0.14)	1.18[b](0.01)
ash	10.58(0.26)	9.23(1.00)	5.38(0.29)
protein	4.19(0.19)	4.48(0.32)	2.81(0.15)
uronic acids	1.97(0.25)	2.09(0.01)	1.62(0.11)
acetyl groups	1.47(0.04)	0.25(0.01)	0.62(0.11)
extractives	na[4]	4.88(0.04)	17.18(0.16)
total:	98.29	97.38	97.29

[a] Values in parentheses are standard error of the means. [b] Mean values with different superscript letters were significantly different from one another ($P < 0.05$). [c] bdl = below detection limit. [d] na = not applicable.

extraction—analyzed as the native feedstock, (b) feedstock analyzed following extraction with 95% ethanol, and (c) feedstock analyzed following extraction with hot water. The macrocomponent composition of the three feedstocks is similar. Each preparation is roughly 60% carbohydrate, the glucan and xylan fractions making up over 90% of total carbohydrate. The KL content of the samples averaged approximately 19%. The next highest component in each case is ash, with the ash content of the corn stover feedstock (10.6%) being higher than that of either fescue (6.7%) or switchgrass (7.1%).

Compositional analyses on the switchgrass feedstock are summarized in Table 1. The extractives content of the this feedstock differed by approximately 1.7-fold depending on the solvent used for extraction; 9.7% of the dry matter was extracted with ethanol compared to 16.4% extracted with water. Clearly, the "extractives" content of the feedstock is dependent on the conditions used for extraction, and thus any such values must be explicitly defined. The extractives content of a feedstock is, with respect to biomass-to-ethanol schemes, of less interest than the content of the structural components. Thus, the primary reason for doing the extractions is to improve the accuracy of subsequent analyses. The extraction protocols used in this study significantly altered the measured KL values for switchgrass. Approximately 17% of the KL measured in the native sample was extracted with ethanol, and roughly 21% could be extracted with water. In a subsequent experiment we found that over 30% of the KL in the original feedstock could be extracted by the sequential extraction of the feedstock with ethanol and then water. It is presumed that the lower KL values associated with the analysis of extracted feedstocks more accurately reflect the true lignin content of the native material. The rational for this assumption is that the higher KL value for the native feedstock is attributable to the condensation/precipitation of extractives under the harsh acidic conditions used for the measurement of KL (Browning, 1967; Smelstorius, 1971; Wise and Ratliff, 1941). The phenomenon of extractives measuring as KL was demonstrated in this study when the extracted solids themselves were subjected to macrocomponent analyses (Table 5). Approximately 40% of the total solids extracted from switchgrass with ethanol measured as KL. The value of 40% may be a minimum estimate of the actual percentage of extractives that measure as KL when the extractives are an endogenous part of the lignocellulosic cell structure. In this experiment with purified extractives only those compounds that become insoluble under KL assay conditions in the absence of core lignin will be measured, while those compounds that directly condense with core lignin will not be detected (Ritter and Barbour, 1935; Norman, 1937; Browning, 1967; Smelstorius, 1971).

The carbohydrate fractions of a feedstock are of obvious importance in biomass-to-ethanol processes. The extractives obtained from the ethanol extraction of switchgrass were nearly 21% neutral carbohydrates (Table 5), with glucose being the predominant sugar in the hydrolysate resulting from the acid treatment of dried extractives. The glucan content of the extractives was 16.3% and the total extractives from switchgrass accounted for 9.7% of total solids, which indicates that the amount of glucan extracted by the ethanol treatment corresponded to approximately 1.6% of the dry matter in the native feedstock. The fact that the extraction procedure removes carbohydrate (particularly glucans) from the feedstock means that the analysis of the extracted feedstock will underestimate the total amount of glucose equivalents available in the original biomass sample. However, the glucan content obtained from measuring the extracted feedstock is likely to be a more accurate measure of the actual cellulose content of the native feedstock, since cellulose is not soluble in the extracting solvents (Wayman and Parekh, 1990).

The compositional analyses done on the fescue feedstock are summarized in Table 2. The extractives content of this feedstock was 1.5−1.7-fold higher than that of the switchgrass feedstock. The trend of solvent power was the same for the two feedstocks; water extracted 25.4% of total solids compared to 16.1% of total solids extracted with ethanol. The removal of either the ethanol-soluble extractives or the water-soluble extractives resulted in significantly lower measured KL values. As explained above, the lower values presumably are more representative of the true lignin content of this feedstock. The total carbohydrate and glucan values were also significantly lower for the extracted samples. The results indicate that greater than 10% of the total carbohydrate in this feedstock is

Table 4. Composition of Extractive-Free Switchgrass, Corn Stover, and Fescue[a] (Results Expressed As a Percentage of the Extractive-Free, Oven-Dried Feedstocks)

	switchgrass		corn stover		fescue	
component	ethanol-extracted	water-extracted	ethanol-extracted	water-extracted	ethanol-extracted	water-extracted
total glycans	60.37(1.26)	65.16(1.06)	60.05(0.92)	63.80(0.32)	63.72(1.32)	67.24(1.38)
glucan	33.10	35.30	35.58	38.38	37.35	38.10
xylan	22.03	25.83	20.25	21.17	22.90	25.84
galactan	1.53	1.95	1.35	1.18	1.89	bdl
arabinan	3.71	2.08	2.87	3.07	1.58	2.87
mannan	bdl[b]	bdl	bdl	bdl	bdl	0.43
Klason lignin	19.78(0.92)	20.25(0.13)	17.42(0.19)	18.92(0.53)	18.53(0.83)	20.62(0.09)
acid soluble lignin	2.64(0.11)	2.20(0.18)	1.81(0.30)	1.40(0.02)	2.35(0.04)	1.92(0.11)
ash	6.34(0.49)	3.43(0.31)	9.70(2.10)	6.50(0.49)	5.55(0.54)	1.54(0.28)
protein	4.08(0.75)	3.42(0.2)	4.72(0.47)	3.39(0.23)	6.46(0.16)	4.78(0.05)
uronic acids	1.62(0.17)	1.80(0.04)	2.20(0.02)	1.96(0.18)	2.46(0.17)	2.21(0.02)
acetyl groups	1.95(0.1)	2.18(0.15)	0.26(0.02)	0.75(0.20)	1.90(0.36)	1.77(0.01)
total:	96.78	98.44	96.16	96.72	100.97	100.08

[a] Values in parentheses are standard deviations of the means. [b] bdl = below detection limit.

Table 5. Composition of Ethanol Extractives from Switchgrass, Corn Stover, and Fescue (Results Expressed As a Percentage of the Oven-Dried Extractives)

component	switchgrass extractives	corn stover extractives	fescue extractives
total glycans	20.70(0.20)	2.85(0.20)	18.37(1.19)
glucan	16.35	2.08	17.72
xylan	bdl[c]	0.32	bdl
galactan	3.90	0.21	0.61
arabinan	0.45	0.09	bdl
mannan	bdl	0.15	0.04
Klason lignin[a]	40.67(0.76)	48.29(0.07)	15.76(0.28)
acid soluble lignin[b]	3.61(0.06)	5.71(0.07)	5.52(0.08)
ash	12.15(0.24)	33.67(0.78)	12.54(1.35)
protein	0.60(0.08)	5.14(0.18)	6.38(0.56)
total:	77.73	95.66	58.57

[a] Extractives measuring as Klason lignin. [b] Extractives measuring as acid soluble lignin. [c] bdl = below detection limit.

ethanol and/or water soluble. Thus, these carbohydrates are not included in estimates of the feedstock's potentially available carbohydrate if the estimate is based on the analysis of extracted samples.

The compositional analyses done on the corn stover feedstock are summarized in Table 3. The ethanol extractable solids in the cornstover feedstock were relatively low, approximately 2- and 3-fold lower than the corresponding values for the switchgrass and fescue feedstocks, respectively. In contrast, the level of water-extractable solids in the corn stover was essentially the same as that in the switchgrass and only 30% lower than that in the fescue. Removal of extractives from the corn stover prior to KL measurements resulted in significantly lower KL values, as observed for the other feedstocks. The level of KL in the two extracted feedstocks was not significantly different, indicating that although the total amount of solids extracted with ethanol was lower than that extracted with water, the amount of extractives that measure as KL was essentially the same with the two solvents. There was no significant difference in the total carbohydrate and glucan content of the native and ethanol-extracted preparations. This result is supported by our direct analysis of the ethanol extractives (Table 5), which showed that the extractives themselves are very low in total carbohydrate (only 2.8%). This is in contrast to the ethanol extractives from the other two feedstocks, which were 20.7% and 18.4% carbohydrate. The water-extracted corn stover was significantly lower in carbohydrate than the native feedstock. This indicates that the much higher level of water extractives (17.2%) compared to ethanol extractives (4.9%) in corn stover is at least partially due to the extraction of water soluble neutral polysaccharides that are insoluble in ethanol. The ash values for the three corn stover preparations follow a trend similar to that of the carbohydrates, i.e., relatively lower amounts of ash are extracted with ethanol compared to water.

The removal and quantification of extractives did not have a large effect on the mass balance closures for these feedstocks (see "total", Tables 1−3). A small improvement in mass closure was observed for the extracted switchgrass samples, while the mass balance of the fescue and corn stover preparations remained essentially the same. In all cases the mass balance closures were above 96%. The lack of improvement in mass closure for the switchgrass and cornstover samples can be rationalized based on the data of Table 5, which show that a very high percentage of the "extractives" for these feedstocks is measured by the assays that are included in the mass closure. Thus, these compounds are measured either as "extractives" or as individual components (for instance "glucan"). However, only about 60% of the extractives from fescue could be detected by these same assays (Table 5). A possible explanation for the fescue result is that the endogenous and isolated extractives have different analytical properties. In this regard we tested for changes in the solublility properties of the ethanol-soluble extractives. We could detect only minor differences in the endogenous and isolated extractives; 95% of the isolated extractives could be reextracted into the original ethanol solvent.

The influence of extractives on KL, glucan, and xylan determinations was also tested by directly mixing these analytes with isolated extractives prior to their analysis. KL determinations on a model system containing only switchgrass KL and switchgrass extractives clearly demonstrated the overestimation of KL due to the presence of extractives (Table 6). The amount of overestimation is shown to be a function of the amount of extractives added (row 2 versus row 3, Table 6). The numbers in the table assume that the purified extractives contain no lignin, which is the basic premise for using these solvents prior to KL determinations. The presence of endogenous extractives in native switchgrass did not affect the analysis of the supplemental KL that had been added to the system. This conclusion is based on the observation that the percent recovery of the supplemental lignin was essentially the same when the supplemental KL was added to either the native or

Table 6. Influence of Extractives on Klason Lignin (KL) Determination of Switchgrass (SG)

sample	*A* theoretical total KL (g)	*B* measured total KL (g)	percent recovery of *A*	percent recovery of added KL
extractives	na[a,b]	na	na	na
0.0883 g extractives + 0.0813 g KL	0.0813[c]	0.1006	124	124
0.4598 g extractives + 0.0924 g KL	0.0924[c]	0.2436	264	264
1 g native SG	0.2133[d]	0.2133	na	na
1.0549 g SG + 0.0773 g KL	0.3023[e]	0.2935	97.1	88.6
1.0287 g SG + 0.0795 g KL	0.2990[e]	0.2916	97.5	90.7
1 g extracted SG	0.2363[e]	0.1931	81.7	na
0.6917 g extracted SG + 0.0669 g KL	0.2303[e]	0.1947	84.5	91.5
0.7255 g extracted SG + 0.0627 g KL	0.2341[e]	0.1983	84.7	93.0

[a] Assume no true lignin in extractives. [b] na = not applicable. [c] Amount of supplemented KL [d] Value based on measured KL content of native switchgrass. [e] Theoretical values based on KL in native switchgrass plus supplemented KL

Table 7. Selectivity of Ethanol, Water, and Sequential Ethanol/Water Extractions of Switchgrass

	ethanol extraction	water extraction	sequential ethanol/water extraction
extractives removed (% of native feedstock solids)	9.74	16.42	19.11
relative selectivity of extraction protocols (% of native feedstock solids)	2.69[a]	9.37[b]	na[c]
extractives measuring as KL (% of native feedstock solids)	3.5	4.5	6.5
extractives measuring as KL (% of extracted solids)	36.0	27.4	34.0

[a] Percent of native feedstock solids extracted in ethanol that were not extracted in water. [b] Percent of native feedstock solids extracted in water that were not extracted in ethanol. [c] na = not applicable.

the extracted switchgrass preparation. The percent recovery of the supplemental KL was appoximtely 90% in both cases. The inability to recover 100% of purified KL upon reklasonation is consistent with previous reports (Karr et al., 1991). In contrast to the effect of extractives on the analysis of KL, the presence of extractives did not affect the analysis of supplemental glucose or xylose (data not shown). The data presented in Table 6, based on the analysis of switchgrass, are supported by similar experiments with corn stover (Thammasouk, 1996).

The switchgrass feedstock was also extracted sequentially with 95% ethanol and hot water in an experiment to evaluate the selectivity of the two extraction protocols. The results of this experiment (Table 7) indicate that 9.4% of the feedstocks total solids were insoluble in 95% ethanol but readily extracted with hot water. Similarly, approximatley 2.7% of the feedstock was not extracted in hot water but was extracted by the 95% ethanol treatment. This experiment indicates that each of the extraction protocols is at least partially selective with respect to the components removed from the feedstocks.

DISCUSSION

The data in this paper demonstrate that solvent extractions of a herbaceous feedstock will impact subsequent analyses of the macrocomponents of that feedstock. Extraction of feedstocks with ethanol (95%) prior to KL determinations resulted in lower KL values, which is assumed to present a more accurate value for the true lignin content of the feedstock. The ethanol extractions were shown to lower subsequent KL values by removing solvent soluble components that become insoluble during the exteme acid conditions used in KL determinations. Ethanol extraction was also shown to lower the apparent total carbohydrate and cellulose (glucan) values of two of the feedstocks. Considering the switchgrass and fescue feedstocks, solvent extractions lowered the apparent cellulose content by extracting noncellulosic, glucose-containing compounds. These compounds are considered "noncellulosic" based on the insolubility of cellulose in ethanol. The extracted "glucans" are most likely low molecular weight sugars that are soluble in ethanol/water solvents (Theander, 1991). The lower cellulose values determined on extracted feedstocks would appear to more accurately reflect the true cellulose content of the feedstock.

The water extractions were included in this study in order to compare the most simple solvent (water) versus the solvent in question (95% ethanol). When considering the analysis of the two major macrocomponents, lignin and cellulose, water seemed to be as good a solvent for preextraction as ethanol. The lignin values determined following preextraction with water and ethanol were not significantly different for the fescue and corn stover feedstocks. The apparent lignin content of water-extracted switchgrass was slightly lower than that of ethanol-extracted switchgrass. The cellulose content of the switchgrass and fescue feedstocks were not significantly different when analyzed after either water or ethanol extractions. The cellulose value for corn stover was significantly lower when the water-extracted sample was analyzed compared to the ethanol-extracted sample. By use of the same rationale as described above, the water-extracted sample is expected to provide a more accurate estimate of the cellulose content of the corn stover feedstock. The measured xylan content of these feedstocks would be expected to be lower after water extractions compared to ethanol extractions because of the higher solubility of most neutral polysaccharides in water. This was found to be the case only with the cornstover feedstock, which was much higher in water-soluble extractives than in ethanol-soluble extractives.

It is clear that solvent extractions will influence subsequent analyses of herbaceous feedstocks, but it is not so clear which solvents should be used on which feedstocks. The advantage of using a solvent extraction

step prior to an analysis seems to depend on the type of data sought. In this study we have evaluated native feedstocks that have not undergone a pretreatment to increase the enzymatic susceptibility of their cellulose fraction; pretreatments of this nature are necessary for optimum performance in biomass-to-ethanol processes. It is not at all clear what happens to the extractives fraction of native feedstocks during one of these pretreatments. One of the most promising pretreatment schemes involves exposing the native feedstock to dilute acid (0.4−1.2% sulfuric acid) at high temperatures (100−260 °C) (Grohmann et al., 1985; Torget et al., 1991; Schell et al., 1992). To our knowledge, the extent to which extractives condense with lignin and/or become insoluble under these conditions has not been published. Hence, it is possible that KL values for the native feedstocks measured in the presence of extractives will have some relevance to the KL values for the pretreated feedstocks. It is reasonable to assume that more sophisticated approaches to lignin characterization (Jung and Himmelsbach, 1989) will be necessary when evaluating the fate of extractives in biomass-to-ethanol pretreatments.

A typical approach for glucan (or xylan) analyses is to hydrolzye the feedstock and then quantify the glucose (or xylose) in the hydrolysate. We have shown in this paper that such hydrolysates resulting from extracted feedstocks are likely to have significantly less glucan than the corresponding hydrolysates resulting from native feedstocks. If an analyst is interested in the cellulose content of a feedstock, then the correct approach would be to analyze the extracted feedstock. However, if the point of the analysis is to determine the total amount of glucan theoretically available for fermentation in a biomass-to-ethanol process (Carrasco et al., 1994), then the analysis should be done on the native feedstock.

A conservative approach for the analysis of these feedstocks is to include a solvent extraction step followed by the macrocomponent analysis of both the extracted feedstock and the extractives obtained from that feedstock. This approach, compared to the analysis of the corresponding unextracted feedstock, will provide a more accurate estimate of the true lignin and cellulose content of the feedstock. This approach, compared to the analysis of only extracted feedstocks, will provide a more accurate value for the amount of carbohydrate that is theoretically available for microbial fermentation to ethanol. Analysis of both the extracted feedstock and the extractives will obviously mean an increase in the total number of assays required, although it will not require new methodology, since the assays traditionally used for macrocomponent analyses of feedstocks can be readily applied to the analysis of the extractives fraction.

LITERATURE CITED

American Society for Testing and Materials. D-1106-84. Standard Test Method for Acid-Insoluble Lignin. In *1993 Annual Book of ASTM Standards*; American Society for Testing and Materials: Philadelphia, PA**,** 1993.

Association of Official Analytical Chemists. 960.52. Official methods of analysis. Microchemical determination of nitrogen micro-Kjeldahl method. In *Official Methods of Analysis of the Association of the Official Analytical Chemists*; Association of Official Analytical Chemists, Inc.: Arlington, VA, 1990.

Browning, B. L. Determination of lignin. In *Methods of Wood Chemistry*. Interscience Publishers: New York, 1967; Vol. 2, pp 785−823.

Carrasco, J. E.; Faiz, Ma C; Navarro, A.; Sariano, P.; Saez, F.; Martinez, J. M. Effects of dilute acid and steam explosion pretreatment on the cellulose structure and kinetics of cellulosic fraction hydrolysis by dilute acids in lignocellulosic materials. *Appl. Biochem. Biotechnol.* **1994,** *45/46*, 23−34**.**

Downing, M.; McLaughlin, S.; Walsh, M. Energy, economic, and environmental implications of production of grasses as biomass feedstocks. In *Second Biomass Conference of the Americas: Energy, Environment, Agriculture, and Industry Proceedings*; National Renewable Energy Laboratory: Golden, CO, 1995**.**

Grohmann, K.; Torget, R.; Himmel, M.; Optimization of dilute acid pretreatment of biomass. *Biotechnol. Bioeng. Symp.* **1985,** *15*, 59−80.

Johnson, D. K.; Ashley, P. A.; Deutch, S. P.; Davis, M.; Sennel, J. A.; Yiselogel, A. Compositional variability in herbaceous energy crops. In *Second Biomass Conference of the Americas: Energy, Environment, Agriculture, and Industry Proceedings*; National Renewable Energy Laboratory: Golden, CO, 1995.

Jung, H. G.; Himmelsbach, D. S. Isolation and characterization of wheat straw lignin. *J. Agric. Food Chem.* **1989**, *37*, 81−87.

Jung, H. G.; Valdez, F. R.; Hatfield, R. D.; Blanchette, R. A. Cell wall composition and degradability of forage stems following chemical and biological delignification. *J. Sci. Food Agric.* **1992**, *58*, 347−355.

Karr, W. E.; Brink, D. L. Summative analysis of nine common North American woods. *J. Wood Chem. Technol.* **1991a,** *11*, 479−494.

Karr, W. E.; Brink, D. L. The complete analysis of wood polysaccharides using HPLC. *J. Wood Chem. Technol.* **1991b,** *11*, 447−463.

Karr, W. E.; Cool, L. G.; Merriman, M. M.; Brink, L. Simplified analysis of acid soluble lignin. *J. Wood Chem. Technol.* **1991,** *11*, 465−477.

Lynd, L. R.; Cushman, J. H.; Nichols, R. J.; Wyman, C. E. Fuel ethanol from cellulosic biomass. *Science* **1991**, *251*, 1318−1323.

Moore, R. N.; DeLuca, T. H. Procedures for the chemical analysis of wood and wood products. *U.S. Forest Products Labatory*; U.S. Department of Agriculture: Madison, WI, 1967.

Norman, A. G. The determination of Lignin. *Biochem. J.* **1937**, *31*, 1567−1574.

NREL. Determination of Klason lignin in biomass. Chemical analysis and Testing Task Laboratory Analytical Procedure No. 003; National Renewable Energy Laboratory: Golden, CO, 1992a.

NREL. Two stage sulfuric acid hydrolysis for determination of carbohydrates. Chemical analysis and Testing Task Laboratory Analytical Procedure No. 002; National Renewable Energy Laboratory: Golden, CO, 1992b.

NREL. Standard method for the determination of extractives in biomass. Chemical analysis and Testing Task Laboratory Analytical Procedure No. 010; National Renewable Energy Laboratory: Golden, CO, 1994.

Ritter, G. J.; Barbour, J. H. Effect of pretreatments of wood on the lignin determination. *Ind. Eng. Chem.* **1935**, *7*, 238−240.

Ritter, G. J.; Seborg, R. M.; Mitchell, R. L. Factors affecting quantitative determination of lignin by 72% sulfuric acid method. *Ind. Eng. Chem.* **1932**, *4*, 202−204.

Schell, D. J.; Walter, P. J.; Johnson, D. K. Dilute sulfuric acid pretreatment of corn stover at high solids concentrations. *Appl. Biochem. Biotechnol.* **1992**, *34/35*, 659−665.

Smelstorius, J. A. Chemical composition of wood of Australian grown *Pinus radiata* D. Don. *Holzforschung* **1971**, *25*, 33−39.

Tandjo, D. Influence of extractives on the chemical analysis of switchgrass. Master of Science Thesis, Oregon State University, Corvallis, Oregon, 1996.

TAPPI. T264 om-88. Preparation of wood for chemical analysis. In *Tappi Test Methods*; Technical Association of the Pulp and Paper Industry: Atlanta, GA, 1988.

Thammasouk, K. The role of solvent extraction in the chemical characterization of corn stover feedstock. Master of Science Thesis, Oregon State University, Corvallis, Oregon, 1996.
Theander, O. Chemical analysis of lignocellulosic materials. *Anim. Feed Sci. Technol.* **1991**, *32*, 35−44.
Theander, O.; Westerlund, E. Studies on dietary fiber (3). Improved procedure for analysis of dietary fiber. *J. Agric. Food Chem.* **1986**, *34*, 330−336.
Torget, R.; Werdene, P.; Himmel, M.; Grohmann, K. Dilute acid pretreatment of short rotation woody and herbaceous crops. *Appl. Biochem. Biotechnol.* **1990**, *24/25*, 115−126.
Torget, R.; Walter, P.; Himmel, M.; Grohmann, K. Dilute acid pretreatment of corn residues and short rotation woody crops. *Appl. Biochem. Biotechnol.* **1991**, *28/29*, 75−86.
Wayman, M.; Parekh, S. R. Wood. In *Biotechnology of Biomass Conversion*; Open University Press: Stony Stratford, Milton Keynes, U.K., 1990; pp 41−72.
Wise, L. E.; Ratliff, E. K. Summative analysis of Quebracho Woods. *Trop. Woods* **1941**, *91*, 40−45.
Zhang, M.; Eddy, C.; Deanda, K; Finkelstein, M.; Picataggio, S. Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. *Science* **1995**, *267*, 240−243.

Received for review June 7, 1996. Revised manuscript received November 12, 1996. Accepted November 13, 1996.[⊗] This study was funded in part by the National Renewable Energy Laboratory, Golden, Colorado.

JF960401R

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1997.