AN ABSTRACT OF THE THESIS OF

Djuhartini Tandjo for the degree of Master of Science in Food Science and Technology presented on May 30, 1996. Title: Influence of Extractives on the Chemical Analysis of Switchgrass.

Abstract approved: _______________________________________

Michael H. Penner

This thesis summarizes an investigation into the need for removing extractives from herbaceous biomass feedstocks prior to their chemical characterization. Switchgrass (*Panicum virgatum*), was used in this study as a representative herbaceous biomass feedstock. The influence of extractives on the chemical analysis of switchgrass was done by comparing the composition of native switchgrass and solvent-extracted switchgrass preparations. Solvent-extracted switchgrass was prepared by extracting the native feedstock with either 95% ethanol, hot water or sequentially with ethanol and then water. Each of the feedstocks was analyzed for glycans, Klason lignin, acid soluble lignin, protein, ash, acetic acid and uronic acids. The results demonstrate that the extractives in native switchgrass significantly interfere with the analysis of Klason lignin. The lignin content of the feedstock was overestimated if the extractives were not removed prior to the analysis. The extractives in switchgrass did not affect glycan analyses. However, some soluble sugars are removed from the feedstock during the solvent extraction process. Total extractives removed by ethanol, water and ethanol/water amounted to

9.74%, 16.42%, and 19.11% of the feedstocks total solids, respectively. These amounts of extractives increased Klason lignin values by 4%, 4.5%, and 6.5% (on a wt. percent of total solids), respectively. Most of the extractives in switchgrass are water-soluble and approximately one-quarter of these extractives measure as Klason Lignin. The removal of the water soluble extractives from the feedstock improved the mass closure values for the feedstock's summative analysis. Successive ethanol and hot water extraction has successfully removed most of the extractives in switchgrass yielding 100.4% mass balance. The recommended approach for the analysis of herbaceous biomass feedstocks will include sequential 95% ethanol and hot water extraction followed by chemical analysis on both the preextracted substrate and the extractives obtained from that substrate.

Influence of Extractives on the Chemical Analysis of Switchgrass

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Djuhartini Tandjo, Author

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INFLUENCE OF EXTRACTIVES ON THE CHEMICAL ANALYSIS OF SWITCHGRASS

1. INTRODUCTION

The National Renewable Energy Laboratory (NREL), with the support from the US Department of Energy (DOE), has embarked on a major program to develop the technology for converting lignocellulosic biomass to fuel ethanol. This process is commonly known as bioconversion which refers to the transformation of one substance to another by means of living organisms, or by products derived from living organisms.

Lignocellulosic substrates studied for their potential as biomass feedstocks include wood, agricultural residues, herbaceous crops, municipal wastes and paper. It has been reported that approximately 300 million tons of agricultural residues are generated annually in the United States (Clausen and Gaddy, 1983). Selected herbaceous crops, such as switchgrass, are currently under study as potential “energy crops” to be grown specifically for the production of fuel ethanol (McLaughlin *et al.*, 1992). The selection of switchgrass as a potential feedstock is due to its promising benefits in providing high yields with relatively low nutrients and water input, thus resulting in high net energy yields. An acre of switchgrass will yield 20.6 times the energy required for it's production (Downing *et al.*, 1995). Switchgrass is also high in cellulose and hemicellulose, making it a suitable energy crop.

The process of making ethanol from lignocellulosic biomass involves several steps: biomass production, pretreatment, enzyme production, enzyme hydrolysis, fermentation,

distillation and by-product processing. A complete discussion of each of these steps is beyond the scope of this thesis. The steps of primary relevance to the biomass-to-ethanol research being done in our laboratory are those that directly pertain to the conversion of cellulose and hemicellulose to ethanol. Cellulose and hemicellulose, when acid or enzymatically hydrolyzed, will yield glucose and xylose, respectively. These two sugars can each be fermented to produce ethanol. The cellulose component of native feedstocks is present in highly crystalline forms and it is encrusted by a hemicellulose-lignin matrix. These structural properties greatly hinder the enzymatic accessibility of cellulose. Previous research has shown that the removal of hemicellulose and the condensation of lignin, both of which occur upon treatment of feedstocks with dilute acid, generates pores in the matrix which makes the cellulose more accessible to enzymes (Knappert and Grethlein, 1981). Dilute acid treatments such as this, referred to as "pretreatments", result in prehydrolyzed solid residues consisting mainly of cellulose and lignin. A prehydolysate liquid consisting primarily of xylose and xylose oligomers is also generated during this pretreatment. The effectiveness of an acid pretreatment in terms of increasing the accessibility of the feedstock's cellulose fraction is directly related to the degree of removal of hemicellulose from the feedstock (Grohmann *et al.*, 1985). The potential ethanol yield from a feedstock is the sum of the fermentable glucose in the solid residue and the fermentable xylose in the liquid phase.

With the present surplus of many agricultural products and the simultaneous decrease in fossil fuel reserves, considerable research effort has been devoted to the bioconversion of agricultural residues. Routine analyses must be carried out on all

potential "fuel" residues in order to characterize each native and acid pretreated feedstock. That makes it necessary to develop simple and accurate analytical methods. The components of primary interest in these feedstocks are cellulose, hemicellulose and lignin, since they are the dominant macrocomponents of lignocellulosic feedstocks. The amount of cellulose and hemicellulose in a feedstock are a good estimate the amount of potentially fermentable glucose and xylose in the feedstock, respectively. The most common methods for quantitatively measuring the glycan (polysaccharide) content of a feedstock involve a "two-stage acid hydrolysis" of the polymer followed by the quantitative measurement of the resulting monosaccharides by either gas or liquid chromatography (Kaar *et al.*, 1991a). The insoluble residue under the "two stage acid hydrolysis" conditions is Klason lignin.

Standard methods for wood analysis require solvent extractions prior to macrocomponent analyses in order to remove wood extractives that are not considered part of the structural components of wood and/or which may interfere with some analyses. The term "extractives" refers to a group of low molecular weight chemicals such as fatty acids, waxes, polysaccharide gums, resins, phenolic substances and free sugars. These compounds are more or less soluble in neutral solvents. Some extractives, such as catechol tannin and lignan, will polymerize or condense with lignin under the conditions employed for lignin analysis and, thus, lead to erroneous increases in lignin values (Ritter and Barbour, 1935, and Norman, 1937). Extraction with ethanol is recommended to remove tannins from those woods which are high in catechol tannins (Smelstorius, 1971, Browning, 1967). A considerable amount of work has been done on the properties of wood extractives and the relative importance of removing these extractives prior to the

analysis of other wood components. However, experimental data on the importance of extractives in the analysis of herbaceous feedstocks is limited.

The aim of the study summarized in this thesis was to investigate the interference, if any, of extractives on the chemical analysis of switchgrass. The primary purpose of this study is to determine whether extraction is necessary prior to the chemical analysis of herbaceous feedstocks. This information is important in order to increase the accuracy, decrease the time commitment and lower the overall cost of the chemically characterizing these feedstocks. Extraction procedures are laborious and time consuming. Traditionally, successive extractions with ethanol/ethanol-benzene/hot water have been used to prepare extractive-free wood for analysis (ASTM D1105, 1993). Recent work has shown that an 80% ethanol solvent, combined with ultrasonic treatment, was effective at removing both hydrophilic and lipophilic extractives from a variety of plant sources (Theander, 1991). This finding, along with the report by Schell *et al.* (1992), led the National Renewable Energy Laboratory to adopt a "standard method" for removal of extractives; the method calls for a 24 hr extraction with 95% ethanol in a soxhlet type extractor (NREL LAP-010, 1994). This study tested/compared the benefits of using this NREL "standard" procedure, compared to a hot water extraction procedure, prior to the analysis of switchgrass feedstocks.

2. LITERATURE REVIEW

2.1 BIOMASS AS AN ALTERNATIVE FUEL

Biomass is defined as all organic matter except fossil fuels: that is, all agricultural crops and forest materials, animal products, microbial cell mass. It also includes by-products of agriculture or agricultural processing that are renewable on year-to-year basis (Hiler and Stout, 1985). Lignocellulosic biomass includes plant materials whose principal components are cellulose, hemicellulose and lignin.

Biomass, besides being a source of food, feed and fiber, is also a potential source of fuel. The simplest method by which biomass can be converted to energy is through direct combustion. However, a liquid fuel is need to reduce petroleum imports. Thus, the U.S. government has funded research aimed at converting biomass into a liquid fuel, ethanol, which can serve as an alternative to gasoline and diesel fuel.

The National Renewable Energy Laboratory (NREL), through the support of the U.S. Department of Energy (DOE) has initiated a Biofuels Program. The purpose of this program is to develop the technology of enzymatically converting lignocellulosic biomass to fuel ethanol as an alternative to fossil fuel.

Current sources of energy are obtained primarily from fossil fuels. With an increasing demand for energy, particularly petroleum, the United States has shifted to importing a large quantity of crude oil. By 1979 about one-half of the petroleum used in the United States was imported (Hall, 1981). Prior to the OPEC oil embargo of 1973, the world price of oil was $3/barrel. Since then, the oil price has rocketed, increasing ten fold

in ten years (Stout, 1985). The era of cheap energy has passed and economists foresee increasing energy prices in the future. Thus, all energy consumers will have to cope with higher energy prices and uncertain supplies in the future. Although many factors contribute to the U.S. trade deficit, the portion contributed by energy is the largest. A trade deficit over a long period undoubtedly feeds inflation, which further depreciates the value of the dollar on world markets (Hall, 1981)

Another reason for developing renewable energy sources is that the amount of fossil fuel reserves is limited and is non-renewable. The rate at which fossil fuels are formed is so slow compared to the rate of consumption that the supply must be considered finite. Petroleum and gas supplies may be nearing the end of their lifespan as experts estimate they will run out in less than 100 years (Hall, 1981). World-wide annual consumption of the non-renewable energy sources (coal, oil, gas and petrochemicals) is about five billion tons, while the formation of these sources is negligible (Robertson, 1977).

For these reasons, extensive research and development programs have been undertaken to assess the potential of biomass as an alternative energy source. Energy consumption at present levels must eventually depend on renewable or inexhaustible sources.

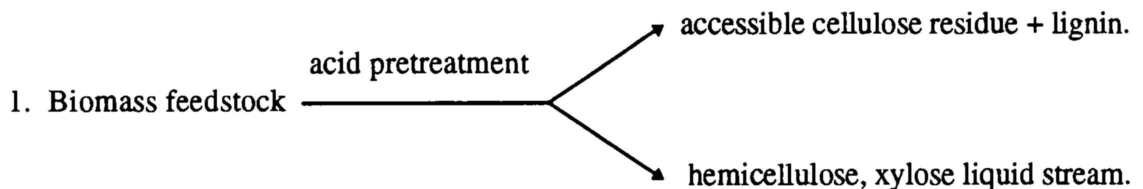
Biomass has several advantages as a source of liquid transport fuel. Biomass represents an enormous quantity of renewable energy resources which are globally available and at present are overproduced and underutilized. The formation of renewable biomass in the world is approximately 200 billion tons annually, of which only two billion

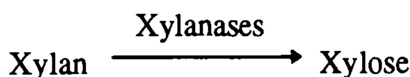
tons are used as food, fiber and building materials (Robertson, 1977, Clausen and Gaddy, 1983). Thus, a huge surplus of 198 billion tons annually remains unused. In 1977, the world's population used only about 7% of the annually produced biomass (Robertson, 1977), leaving the vast majority of this abundant renewable resource to be exploited.

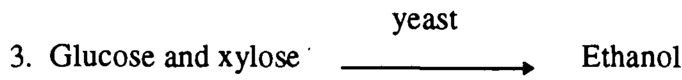
Fuel ethanol's primary environmental advantage over gasoline is that it burns cleaner while producing a similar energy release. Moreover, burning new biomass contributes no new carbon dioxide to the atmosphere because carbon dioxide is returned to the cycle of new biomass growth. On the other hand, burning fossil fuel uses "old" biomass and converts it into "new" carbon dioxide that contributes to the greenhouse effect.

2.2 Bioconversion Process

The conversion of lignocellulosic biomass to ethanol involves three general steps: pretreatment of biomass, enzymatic hydrolysis to release sugars and fermentation of sugars to ethanol. The scheme of the above processes can be summarized as follows:







2.2.1 Dilute-Acid Pretreatment

Biomass feedstocks need to be "pretreated" prior to the enzymatic saccharification of their cellulose component. Dilute-acid pretreatments, employing sulfuric acid at high temperatures, dissolve the hemicellulose component of the feedstock, leaving behind a solid residue whose principle components are cellulose and lignin. The primary purpose of the pretreatment is to increase the susceptibility of the cellulose fraction of the feedstock to enzymatic saccharification. Cellulose fibers in native lignocellulosic feedstocks are encased in a lignin-hemicellulose matrix which forms a shield around the fiber. This shield is a physical barrier which prevents cellulolytic enzymes from gaining access to the cellulose. The resistance of cellulose fibers to enzymatic degradation is further compounded by the crystallinity of cellulose microfibrils and the relatively small pores in untreated biomass (Grohmann, 1985). A successful pretreatment will largely counter many of these factors, making the cellulose readily susceptible to enzymatic degradation.

The lignin-hemicellulose shield can be degraded either by acid or base catalysts. The drawback of using alkali is that it is neutralized by carboxylic acids and phenolics in the substrate, thus demanding a large amount of alkali in the process. Obviously, this operation would not be cost effective for large scale ethanol production (Saddler, 1993).

Acid catalysts, on the other hand, are effective at lower concentrations. Sulfuric acid is most commonly used in this process (Grohmann, 1985). Acids can be neutralized

by the basic cations present in ash, but this does not occur to any large extent because of the low amounts of ash in woody materials (Baker *et al.*, 1975). Even herbaceous feedstocks which contain high amount of ash do not pose a large problem since most of the ash is acidic silica (Grohmann, 1985). Pretreatments with dilute sulfuric acid at high temperature (1% acid at 160°C and above) were found to effectively increase the enzymatic susceptibility of the cellulose in several feedstocks (Knappert and Grethlein, 1981, Grethlein, 1983, and Grohmann *et al.*, 1985). This process breaks glycosidic linkages in hemicellulose, lignin-hemicellulose linkages and, perhaps, lignin-lignin bonds. The result is the dissolution of a major part of the hemicellulose component of the feedstock and an increase in the porosity of the feedstock.

The efficiency of the pretreatment step, i.e., the pretreatment that yields maximum amenable cellulose, is directly related to the amount of hemicellulose removed from the prehydrolyzed solid residues (Grohmann, 1985). The extent of the pretreatment is dependent upon acid concentration, temperature and time. An optimization study of the dilute acid pretreatment of switchgrass has been done here at Oregon State University (Fenske, 1994, and Chung, 1996). Pretreatments at high temperatures, 170-180°C, and short times, < 1 min, were found to be more effective than treatments at lower temperatures, 140-160°C, and longer times, > 2 min.

The liquid stream (prehydrolyzate liquid) resulting from the pretreatment consists mainly of fermentable xylose and xylose oligomers, and small amounts of furfural. Furfural is the degradation product of xylose, which when present at high levels, is toxic to microorganisms (Clausen and Gaddy, 1983). The overall objective of a pretreatment is

to optimize the enzymatic susceptibility of the cellulose, to optimize the amount of xylose in the prehydrolysate liquid, and to minimize furfural formation. To accomplish this, it is important to have good analytical methods that can be readily used to measure the critical chemical constituents of the system.

2.2.2 Simultaneous Saccharification and Fermentation (SSF)

Following the acid pretreatment, the cellulose is hydrolyzed to glucose which is then fermented by yeast to ethanol. The hydrolysis and fermentation can occur either in two separate vessels, SHF (Separate Hydrolysis and Fermentation) or in the same vessel, SSF (Simultaneous Saccharification and Fermentation). The latter was found to be more efficient (Wright, 1988).

The advantage of SSF is the success in overcoming product inhibition that occur in SHF. As glucose is liberated and accumulates, even at low concentration, it will inhibit the activity of cellulase enzymes. In SSF, as soon as sugars are formed, they are fermented by the microorganism and, thus, reaction mixtures do not accumulate inhibitory glucose (Saddler, 1993). Another advantage of SSF is that it requires less enzyme than SHF, making the conversion process cheaper; the cost production of ethanol went down from $2.66/gal. with SHF to $1.78/gal. with SSF - a savings of $0.88/gal (Wright, 1988).

2.2.3 Economic Feasibility

The development of biomass conversion technology at NREL has been successful, This process has reached the pilot scale and is heading to the commercialization of

biomass ethanol. Recent improvements have reduced the cost of biomass ethanol from $4.63/gal in 1980 to a current price of about $1.22/gal for ethanol produced at a 2000 ton per day plant (Mielenz, *et al.*, 1995). Much of the cost savings have resulted from introducing superior cellulolytic enzymes - attributable to a savings of about $1.75/gal. Improved process designs, such as changing from SHF to SSF, have also contributed to cost savings. Finally, a new unit operation, pentose fermentation by yeast will further reduce the estimated cost by about $0.95/gal. (Mielenz, *et al.*, 1995).

2.3 COMPOSITION OF LIGNOCELLULOSIC BIOMASS

The major structural components of lignified cell walls are cellulose, hemicellulose and lignin. The typical composition is about 35-40% cellulose, 20-25% hemicellulose and 20-25% lignin (Lewin and Goldstein, 1991). Monosaccharides commonly present in structural polysaccharides are aldohexoses (D-glucose, D-galactose, D-mannose), aldopentose (D-arabinose, D-xylose), and uronic acids (D-galacturonic acid, D-glucuronic acid and its 4-O-methyl ether). Minor amounts of L-rhamnose and L-fucose may be present in the cell walls. Non-cellulosic structural polysaccharides may contain small amounts of O-acetyl groups.

Approximately, 5% of the material in cell walls consists of minor, non-structural components that are classified as extractives and mineral substances. Examples of the extractives are waxes, fats, gums, tannins, and soluble sugars. Important mineral elements in cell walls are Ca, P, and Si. Protein in plants exist in small amounts as glycoproteins rich in hydroxyproline, arabinose and galactose (Jung *et al.*, 1993).

Herbaceous crops contain much more minerals, proteins, soluble sugars and other water soluble extractives than do hardwoods (Torget *et al.*, 1990). A low content of pectic substances seems also to be present in grasses. This group of amorphous polysaccharides consist of polygalacturonic acids, rhamnose, arabinose and galactose. Starch content in lignified cell wall is negligible.

Plant cell walls consist of two phases, a microfibrillar phase and a matrix phase. The microfibrills are made up of cellulose, the cellulose being paracrystalline and having a degree of polymerization of from 5,000 to 10,000. The matrix of the cell wall is amorphous. It consists of a variety of polymers, including hemicellulose, protein, and lignin.

2.3.1 Cellulose

Cellulose is a linear polymer composed of anhydro-D-glucopyranose units linked by β-1,4 glycosidic bonds. The degree of polymerization is between 5,000 to 10,000 (Brett and Waldron, 1990). These long molecules are hydrogen bonded to their neighboring linear polymers forming a crystalline lattice resulting in a structure with very high tensile strength. However, cellulose is paracrystalline, having both crystalline and amorphous regions. The amorphous regions are primarily on the surface of the microfibrils, only occasionally interrupting the central crystalline core (Sjostrom, 1993). Because of the high degree of H-bonding and crystalline structure, cellulose is quite unreactive. It is insoluble in water or in common solvents. It dissolves in strong acids (72% H_2SO_4, 85% H_3PO_4, and 41% HCl) or in alkaline copper solutions. Cellulose swells

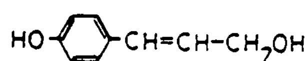
but does not dissolve in NaOH or KOH. Cellulose is "saccharified" to glucose when incubated in warm solutions containing cellulase and beta-glucosidase enzymes (Wayman and Parekh, 1990).

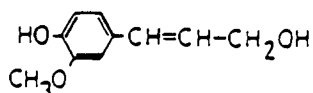
2.3.2 Hemicellulose

In contrast to cellulose, hemicelluloses are branched and heterogeneous polysaccharides. They are smaller than cellulose (degree of polymerization is between 150 to 200 molecules) and do not form crystalline regions. Like cellulose, most hemicelluloses function as supporting material in the cell walls. The composition of hemicelluloses vary greatly among cell types and species. They consist of glucose, mannose, galactose, xylose, arabinose and small portions of rhamnose, galacturonic acid, glucuronic and 4-O-methylglucuronic acids. Hemicelluloses consist of β-1,4 xylan backbone with branches of O-methylglucuronic acid glycosidically linked at C2, arabinose at C2 or C3 and acetyl esters at C2 or C3. However, the distribution of the side chain components do not follow any regular pattern. The predominant sugars bound to the side chains are L-arabinose (Sjostrom, 1993). Hemicelluloses are insoluble in neutral organic solvents and water. They are readily hydrolyzed by hot dilute mineral acids and are dissolved by hot dilute alkalis or cold 5% sodium hydroxide solutions (Browning, 1967, Brett and Waldron, 1990).

2.3.3 Lignin

Lignin is the major non-carbohydrate portion of lignified cell walls. It is located mostly in the middle lamella as well as primary and secondary walls, acting like a glue to hold the cellulose fibers together. Lignin is a very complex molecule made up of phenyl-propane like units. The precursors of lignin are the three aromatic alcohols, coumaryl, coniferyl and sinapyl alcohols (Figure 1). These precursors are linked together through a variety of covalent carbon-carbon and carbon-oxygen bonds. There is no regular pattern in the coupling of the monomeric units. The degree of polymerization ranges between 100-1,000 subunits (Brett and Waldron, 1990) and polymerization can occur as long as the active precursors and space are available in the cell wall. This results in a very strong, hydrophobic network which surrounds the other cell wall components and hold them in place.





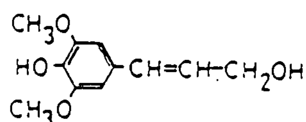


Figure 1. Precursors of lignin.
Adapted from Brett and Waldron (1990).

The components in cell walls do not stand by themselves but are interlinked with one another forming an extremely complicated network (Figure 2, 3 and 4). Cellulose is covalently and hydrogen bonded to hemicellulose, anchoring the microfibrils to the matrix system. Hemicelluloses in the matrix are hydrogen bonded with one another and cellulose. Lignin is believed to be linked covalently to hemicellulose through ester bonds with uronic acid residues and also via phenolic acids (Jung *et al.*, 1990). The encrusting and linking of cellulose and hemicellulose by lignin give mechanical strength and hydrophobicity to the cell walls, as well as a decrease accessibility for the cellulolytic enzymes. Protein in the cell wall is covalently linked to cellulose and noncovalently associated with lignin through hydrophobic forces (Brett and Waldron, 1990).

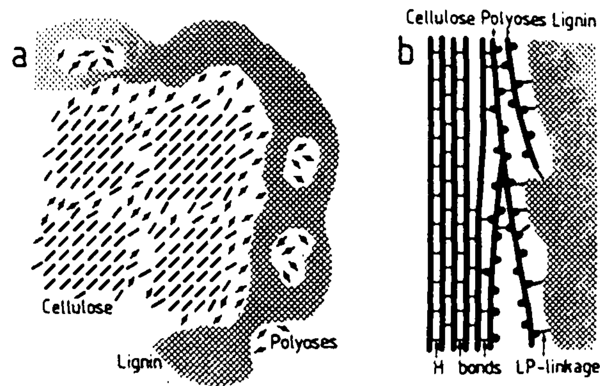


Figure 2. Schematic diagram for the ultrastructural arrangement of cellulose, hemicellulose (polyoses) and lignin in the wood cell wall.
(a) transverse section; (b) longitudinal section. Adapted from Higuchi (1985).

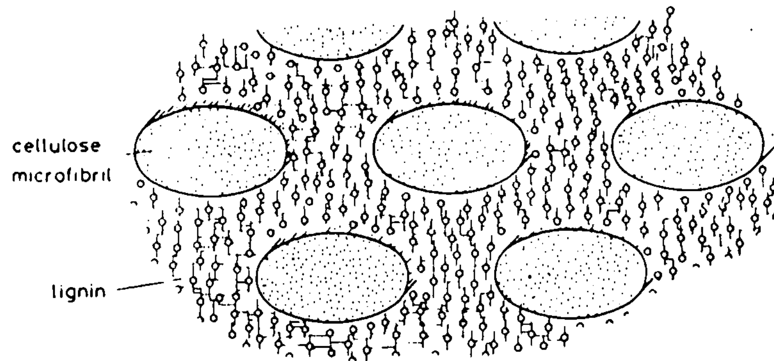


Figure 3. Cell wall model showing interaction of lignin with cellulose microfibrils. Adapted from Brett and Waldron (1990).

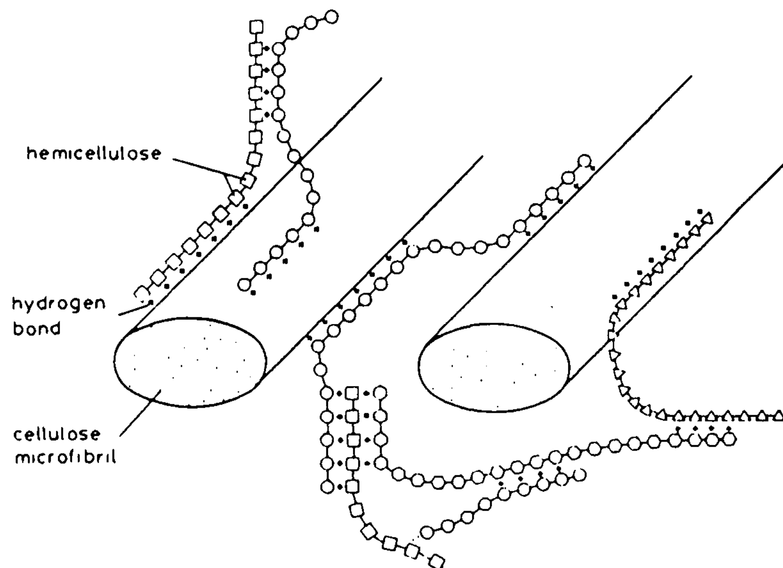


Figure 4. Cell wall model illustrating the hydrogen-bond interaction of hemicellulosic polysaccharides with each other and with cellulose microfibrils. Adapted from Brett and Waldron (1990).

2.3.4 *Extractives*

Extractives or extraneous components are those substances which are removed from lignocellulose cell walls by extraction with neutral solvents. These materials are extraparietal substances which are deposited after cell wall formation and are not considered essential structural components of the cell wall (Soltes, 1983). They may be located within a cell wall, but they are not chemically attached to it. The largest amounts are found in cell lumens but they may also be present in the cell wall matrix. The quantities of extractives in tissues vary widely depending on plant species, family and tissues and within tissues, in addition the components are extraordinarily diverse (Hillis, 1985). Because of this diversity, no generalized procedures for isolation and determination of individual compounds can be established. Several researchers have presented a thorough discussion on the chemical classification, biosynthesis, isolation and quantification of extractives (Sjostrom, 1993, Lewin and Goldstein, 1991, Browning, 1967, Rowe and Conner, 1979, and Hillis, 1962).

Extractives generally have relatively low molecular weights and can therefore be removed with water or neutral organic solvents. However, when they occur in polymerized state, they are nolonger soluble in neutral solvents (Smelstorius, 1971, Rowe and Conner, 1979). The partially or wholly insoluble constituents include crystalline inclusions such as silica, calcium oxalate, salts of organic acids, starch granules, proteins, and inorganic materials.

Extractives can be classified as follows (Lewin and Goldstein, 1991):

1. Volatile materials, e.g., terpenoids and related compounds.

2. Non-volatile resinous extractives, e.g., resin acids, fatty acids and unsaponifiable substances.
3. Phenolic extractives, e.g., stilbenes, lignans, tannins, flavonoids.
4. Soluble carbohydrates and other polar extractives, e.g., monosaccharides, sucrose, arabinogalactans, pectins, cyclitols, and low molecular weight carboxylic acids.

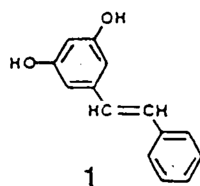
Terpenoids are a group of compounds derived from a family of hydrocarbons known as terpenes. These are acyclic and cylic hydrocarbons whose molecular formulas are some multiple of C_5H_8 which are characterized by the presence of the 2-methylbutane carbon skeleton as a repeating unit. Most of the hydrocarbons are unsaturated (Hillis, 1962). The term "resin" is often used as a collective name for the lipophilic extractives (except phenolic substances) soluble in non-polar organic solvents but insoluble in water. The most abundant resin acids are abietic acid and piramic acid. The unsaponified materials are neutral, water-insoluble hydrocarbons, alcohols, and aldehydes, e.g., sterols. Fatty acids are usually occur in esterified form. The predominanting unsaturated fatty acids are linoleic acids and the saturated are mainly palmitic acids (Sjostrom, 1993).

The term "lignan" is to be distinguished from lignin. Lignans are formed by oxidative coupling of two phenyl propane (C_6C_3) units. Flavonoids are compounds which have a typical tricyclic, $C_6C_3C_6$, carbon skeleton. Catechin is an important flavonol precursor of condensed tannins. Tannins can be classified as "hydrolyzable" or "condensed" tannins. Hydrolyzable tannins yield gallic acid, ellagic acids and sugars when they are hydrolyzed. Condensed tannins are polymers of flavonoids which will polymerize upon acid treatment, yielding insoluble materials. Tannins and lignans are thought to

interfere with lignin determinations because they are often insoluble in acids (Ritter and Barbour, 1935, and Browning, 1967). Tannins may also act as inhibitors of specific enzymes, like cellulases. They are generally complexed with protein, having lesser interactions with cell wall carbohydrates (Robbins *et al.*, 1991). Figure 6 shows the structures of some phenolic extractives. A summary of lignocellulosic biomass components is presented in Figure 7.

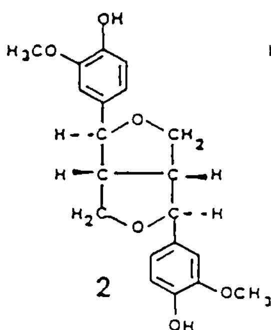
Although several studies on the interference of extractives on wood analyses have been done (Ritter and Barbour, 1935, Browning, 1967, Smelstorius, 1971, Saddler, 1990), only a few studies have been done with herbaceous substrates (Butler and Bailey, 1973, and Jung *et al.*, 1993). Further discussion of the interference of extractives on chemical analysis and their removal is presented in the following section.

STILBENES



1

LIGNANS



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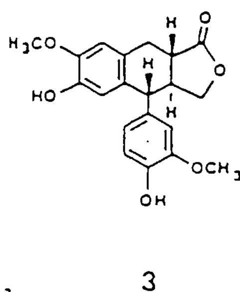

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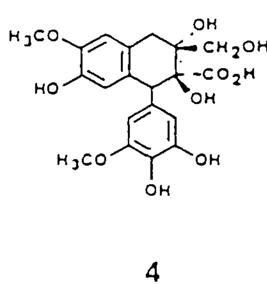

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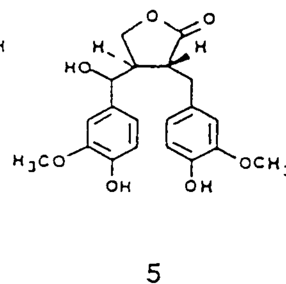

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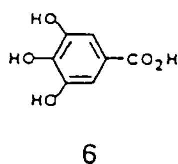
TANNINS



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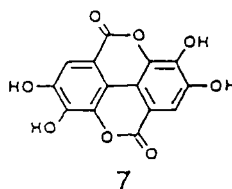

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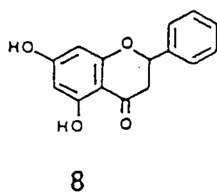
FLAVONOIDS



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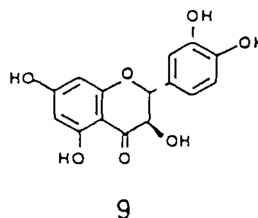

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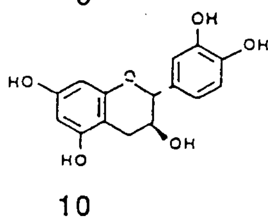

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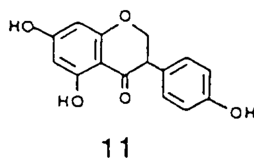

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Figure 5. Phenolic extractives and related constituents.
Adapted from Sjostrom, 1993.
(1) pinosylvin, (2) pinoresinol, (3) conidendrin, (4) plicatic acid, (5) hydroxymatairesinol, (6) gallic acid, (7) ellagic acid, (8) chrysin, (9)taxifolin, (10) catechin, (11) genistein.

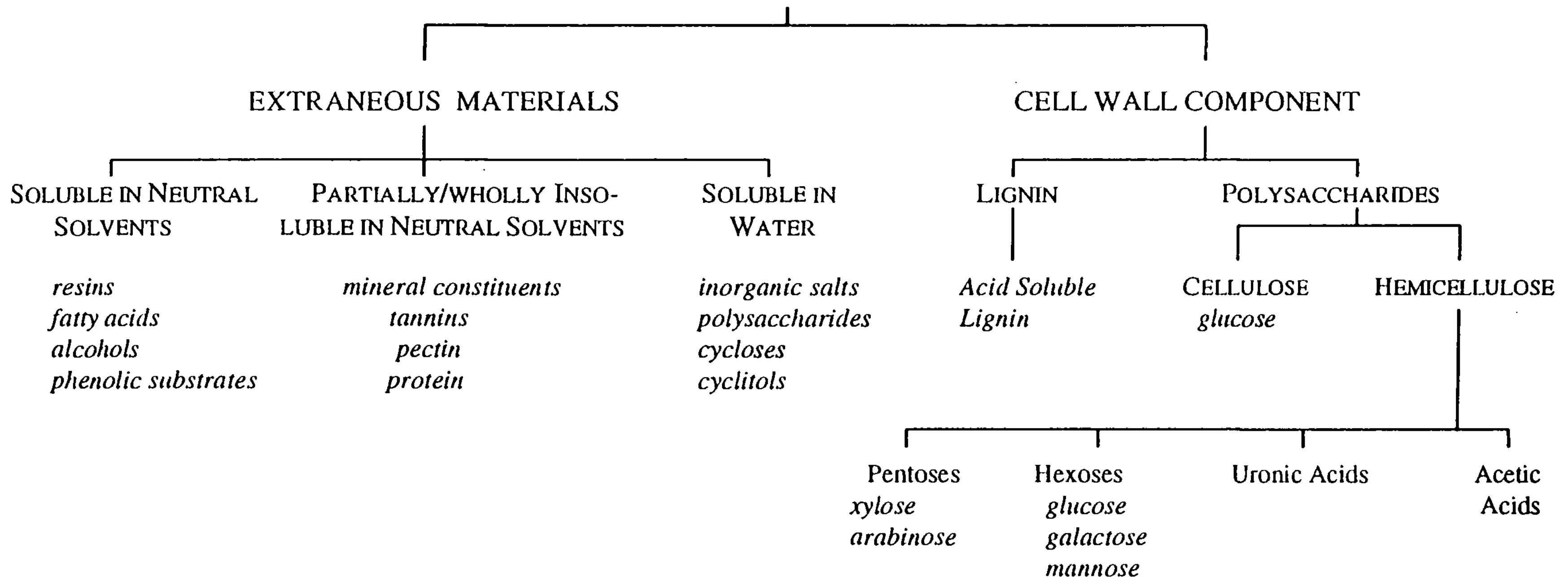

Figure 6. Composition of Lignocellulosic Biomass.
Adapted from Browning, 1967.

2.4 Chemical Analysis of Herbaceous Biomass

The main difficulty in the chemical analysis of lignified cell walls arises from the extreme chemical heterogeneity of the cell wall. The different components of the cell wall interfere with each others respective analyses. A typical characterization of a biomass feedstock involves the macrocomponent analysis of the feedstock; which includes polysaccharides, lignin, acid soluble lignin, protein, ash and organic acids. The primary sugars in lignocellulosic biomass are glucose, xylose, galactose, arabinose and some mannose. Quantification of other sugars such as rhamnose and fucose, starch and pectic substances are of less importance since they are present in negligible amounts (Lindgren *et al.*, 1980). Several methods have been published for the compositional analysis of woody materials (Kaar *et al.*, 1991a and Moore and Johnson, 1967) and forage cell wall (Theander and Westerlund, 1993 and Van Soest, 1963). Standard methods for wood analysis are well described in Technical Association of the Pulp and Paper Industries (TAPPI, 1979).

Traditional wood analysis requires delignification using acid chlorite and treatment with 10% NaOH for isolation of holocellulose, which is subsequently fractionated into cellulose and hemicellulose (Wise and Ratliff, 1947). This method does not permit a quantitative recovery of polysaccharides and was too time-consuming for routine application (Smelstorius, 1975). Van Soest (1963) has introduced the use of detergents in the analysis of fibrous feeds. One detergent-based procedure is an acid detergent fiber (ADF) method using a heat treatment with 0.5 M sulfuric acid containing cetyltrimethyl-ammonium bromide. Another procedure is a neutral detergent fiber (NDF) method which

employs a hot neutral solution of a sodium lauryl sulfate. The ADF residue contains mainly cellulose and lignin. The NDF residue is considered to contain all of the feedstocks cellulose, hemicellulose and lignin, however, some fraction of the hemicelluloses are lost during this treatment.

In the field of biomass conversion, the approach most commonly used for carbohydrate analysis involves complete acid hydrolysis of the polysaccharides. This protocol involves solubilization of the polysaccharides in strong mineral acid, often 72% sulfuric acid, followed by hydrolysis in a more dilute, often 4% acid, at high temperature (Kaar *et al.*, 1991a). This method is based on the early discovery by Peter Klason that the carbohydrates of wood dissolve readily at room temperature in 72% sulfuric acid, leaving behind a dark insoluble material which is known as Klason lignin. The hydrolysate liquid, after the "two stage" hydrolysis, contains monosaccharides produced from cellulose and hemicellulose: glucose, xylose, galactose, arabinose and mannose. During the hydrolysis, some of the hexosans and pentosans are degraded producing hydroxymethyl furfural and furfural, respectively. The amount of these degradation products may be quantified and included in the calculation of glucan and xylan (Kaar *et al.*, 1991a). Some of the lignin is degraded during the acid hydrolysis, this lignin is measured as acid soluble lignin. Uronic and acetic acids are also generated during the hydrolysis due to their cleavage from the hemicellulose. Some of these organic acids are volatile, therefore, a well-sealed container is used to retain those components for subsequent analysis.

The traditional instrument for carbohydrate quantification has been gas chromatography due to its high sensitivity and rapid separation of all carbohydrates

(Moore, 1967, Theander, 1991, and Johnson *et al.,* 1995). The disadvantage of GC is that the sugars must be derivatized. The development of High Pressure Liquid Chromatography (HPLC) has made this an ideal tool for carbohydrate analysis, since no derivatization and little sample preparation is necessary. A common HPLC system column used for carbohydrate analysis includes an Aminex HPX-87P column coupled to a refractive index detector. Aminex carbohydrate columns separate compounds using a combination of size exclusion and ligand exchange mechanisms. For monosaccharide separations, ligand exchange is the primary mechanism. Ligand exchange involves the binding of hydroxyl groups of the sugars with the counter-ion (Pb^{++}) of the resin. The binding ability is affected by the spatial orientation of the carbohydrate's hydroxyl groups. Stronger binding, which results in longer retention times, will occur in sugars which can favorably complex three adjacent hydroxyls to the lead counter-ion compare to those sugars which complex the lead with only two hydroxyls (Bio Rad, Bulletin 1928).

The standard method for wood analysis as described in the Technical Association of the Pulp and Paper Industry (TAPPI, T 264 om-88, 1988) requires solvents extractions prior to the chemical analyses. The extraction removes materials which are not part of the wood structure or which may interfere with some analyses. Wood extractives which are soluble in neutral solvents are generally not considered as part of the wood substances. These materials should be removed before any chemical analysis of wood substances, except where the extraction process and subsequent washing could interfere with certain chemical analysis.

Table 1. Composition of Native and Ethanol Extracted Sepco Rice Straw. (All results are expressed on dry-weight basis).

Components	Native	Ethanol (% extractive-free)	Ethanol (% unextracted)
Glucan	*33.29*	*36.65*	*32.89*
Xylan	*16.6*	*19.39*	*17.40*
Galactan	*0.70*	*0.70*	*0.63*
Arabinan	*3.37*	*3.67*	*3.29*
Mannan	*0*	*0.32*	*0.29*
Total Glycan	53.96 (1.08)	60.73 (0.22)	54.5 (0.20)
Klason Lignin	11.80 (0.05)	10.98 (0.2)	9.85 (0.18)
Acid soluble lignin	2.05 (0.00)	2.09 (0.04)	1.88 (0.04)
Ash	18.85 (0.41)	18.28 (0.27)	16.41 (0.24)
Protein	2.68	2.55	2.29
Starch	4.49 (0.10)	1.62 (0.02)	1.45 (---)
Extractives	n/a (---)	n/a (---)	10.25 (0.16)
Total	93.83	96.25	96.63

Adapted from NREL, 1995 (unpublished data).
Values in parentheses are standard deviation of the means.

Table 1 shows the compositional analysis data for both native, untreated Sepco rice straw and those extracted with 95% ethanol following the standard procedure of

NREL (NREL LAP-010, 1984). These unpublished data were obtained from NREL In-House Chemical Analysis & Testing (CAT) Task Analytical Report, 1995. Values in the second and third columns were presented in terms of the weight percent of the component on a dry weight basis. Values in the forth column are presented as weight percent of the native "unextracted" sample. Comparison between the values of native and extracted samples (calculated in unextracted basis) indicated that the levels of Klason lignin, acid soluble lignin, ash, and starch were significantly reduced in the ethanol pre-extracted sample while the carbohydrates were not effected. Protein was slightly reduced but without a measure of its standard deviation, no conclusion can be drawn. An important contribution of extraction was that it improved mass closure.

2.4.1 Interference of Extractives on the Chemical Analysis of Lignocellulosic Substrates.

The analysis of all cell wall components seems to be influenced by the presence of the other components that they are interlinked with. An example of this phenomenon is the influence of extractives on Klason lignin determination. The high content of extractives in herbaceous biomass complicates lignin analysis (Torget *et al.,* 1990). The interference of extractives on the analysis of lignin has been reported by several researchers (Norman, 1937, Ritter and Barbour, 1935, and Smelstorius, 1971). It was found that some components of the extractives could polymerized during acid treatment and were erroneously measured as lignin (Browning, 1967). Fats, resins, lignans and low molecular weight lignin (Brauns native lignin) would remain wholly or partially in lignin if

they were not removed before lignin analysis. Tannins, which are polyphenolic compounds, will condense with lignin in the harsh acid environment used for this assay (Smelstorius, 1967).

Removal of extractives from substrates was found to lower the level of klason lignin in the extractives-free wood. Ritter and Barbour (1935) have shown that the reduction of KL, after extraction with 95% ethanol, ethanol-benzene and hot water, was not due to removal of lignin by the extraction solvents, instead it was due to the removal of contaminants (extractives) that condensed with lignin. The compounds present in woods that have been found to condense with lignin during acid treatment are catechol tannins, tannic acids, lignan, and protein (Ritter, 1935, and Saddler, 1993).

Substrates that contain much starch and soluble sugars will also impose some interference in the determination of cellulose. These compounds will be hydrolyzed along with cellulose and will contribute to the calculation of cellulose content since this calculation is generally based on the amount of glucose in the hydrolysate. The low starch content in lignocellulosic substrates lessens this concern relative to cellulose.

Although time consuming, the removal of extractives has been recommended prior to chemical analysis of wood materials (Saddler, 1993). The extent of extractives interference in lignocellulose chemical analysis depends on the amount and types of extractives present in the substrate. Since the amount and types of extractives in each plant species varies significantly, evaluating each substrate separately is necessary.

2.4.2 Solvents Extraction

By definition, the extractives are those substances which are removed by extraction with neutral solvents. They have been classified as materials which are (1) volatile with steam, (2) soluble in ethyl ether, (3) soluble in ethanol, and (4) soluble in water (Browning, 1967). The standard method for extractives removal from wood has been soxhlet extraction with ethanol-benzene, 95% ethanol and hot water (TAPPI, 264 om-88, 1988). The process involves successive soxhlet extraction with ethanol-benzene for 6-8 hours followed by 95% ethanol for 4 hours and hot water for another hour. However, due to the diversity of the extractives, choice of extraction methods and solvents is dependent upon the substrate and the purpose of the investigation. It is important to keep in mind that no single solvent is capable of removing all the extractives from the substrate. Successive extraction with neutral organic solvents has been shown to improve the solubilization of extractives (Saddler, 1993).

2.4.2.1 Water soluble extractives

Inorganic salts, sugars, gums, starches, coloring matter and tannins are water-soluble extractives. Some of the materials soluble in water are more or less soluble in many organic solvents. In determination of water soluble extractives, cold water (23°C), hot water (100°C) or a combination of both are utilized. Cold water removes tannins, gums, sugars, and coloring matter in lignocellulosic materials, while hot water procedures may also remove starches (TAPPI T12m, 1979).

2.4.2.2 Organic solvents soluble extractives

Commonly used extraction solvents are petroleum ether, ethyl ether, acetone, carbontetrachloride, ethylene dichloride, 95% ethanol and benzene-ethanol. Petroleum ether or benzene is used to remove fats, waxes, resinous materials and sterols. Acetone, alcohol and water dissolve phenolic substances including coloring materials, tannins and stilbenes. A study comparing different solvent extractions on ponderosa pine found that ethylene dichloride and ethanol-benzene were best for removing resin acids, whereas acetone and ethylene dichloride were best for dissolving fatty acids and benzene was best for esters (Anderson, 1946). The mixture of ethanol-benzene appears to provide complete removal of extractives in pulp, such as waxes, fats, resins, catechol tannins, sterols, nonvolatile hydrocarbons, salts and low molecular weight sugars (TAPPI T 204 os-76, 1979). This solvent mixture is widely used to remove extractives prior to chemical analysis, in particular klason lignin analysis (Kaar *et al.*, 1991a, Madan 1983, Norman 1937, Ritter and Barbour, 1935, and Smelstorius, 1971). However, due to its toxicity, use of benzene as an extraction solvent has recently been avoided.

A study aimed at finding cheaper, less toxic and less flammable solvents was done by Theander (1991). He reported extraction with 80% ethanol with simultaneous ultrasonic extraction or Ultra-Turrax treatment was as effective in removing hydrophilic and lipophilic extractives as various types of conventional extraction procedures using more toxic and expensive solvents. Some types of polymeric extractives, such as tannins, might need other treatments for effective removal. This procedure was modified by Schell

(1992) who used 95% ethanol and a soxhlet extraction system. NREL has recently adapted this protocol as the standard method for the determination of extractives in biomass feedstocks (NREL, LAP-010, 1994). An obvious advantage of this procedure versus the ethanol-benzene procedure is that it uses a less toxic solvent. An advantage of this procedure compared to hot water extraction is that ethanol dissolves catechol tannins which are incompletely dissolved in hot water (Ritter and Barbour, 1935).

Some extractives are also soluble in alkaline solutions, however, much of the cell wall materials are also removed. Extractions with dilute acids removes carbohydrates which may interfere with lignin analysis but some lignin might be lost during the treatment (Browning, 1967). Thus, alkaline and acid treatments are rarely used to remove extractives prior to chemical analysis.

Smelstorius (1971) did a comparative analysis study of *Pinus radiata* wood prepared from various solvent extractions. The milled wood was extracted successively in a soxhlet extractor with benzene-ethanol mixture, diethy-ether and 0.1N NaOH. Another portion of the wood was extracted with boiling 0.1N NaOH and $NaClO_2$/HOAc without pre-extraction with organic solvents. The results showed that each extraction method reduced the amount of lignin measured but to different extents. Different solvents removed different kinds of extractives, some of which condensed with lignin but others did not. In the ethanol-benzene extraction, lignin was reduced by 11% of the dry weight of lignin. Approximately 3% of the substrate weight that can condense with lignin were removed. Pinus radiata wood contains 0.3% of ether soluble substances which are not soluble in benzene-ethanol mixture. This ether soluble extractives do not seem to effect

the total lignin value. Boiling with 0.1N NaOH failed to remove 0.5% of substrate weight which were soluble in organic solvent. These results suggest that extraction with 0.1N NaOH alone, without organic solvent, is sufficient to remove extractives that interfere with lignin analysis. However, it has a serious drawback in that carbohydrates are also solubilized.

3. EXPERIMENTAL METHODS

Switchgrass (*Panicum virgatum*) used in this study was supplied by the National Renewable Energy Laboratory (NREL), Golden, CO. It had been milled to pass 40 mesh screen. The sample was stored at room temperature prior to analysis. Standards of glucose, xylose, galactose, arabinose, mannose, glucuronic and galacturonic acids for chromatographic analysis were obtained from Sigma.

Analytical methods for the determination of moisture, carbohydrate, lignin and ash are described in the 'Chemical Analysis and Testing (CAT) Standard Procedure' provided by NREL.

3.1 CHEMICAL ANALYSIS

3.1.1 Total Solids/Moisture

Total solids were determined in each analysis unless the samples had been dried at 105°C prior to analysis. Approximately one gram of sample (weighed to the nearest 0.1 mg) was placed in a pre-weighed aluminum foil weighing dish and dried to constant weight in a 105°C convection oven. The sample was cooled to room temperature in a desiccator and was reweighed (CAT NO. 001).

CALCULATION:

$$\%\ \text{Moisture at } 105\ ^{\circ}\text{C} = \left[\, 1 - \frac{(\text{wt. dried sample plus dish - wt. dish})}{\text{wt. undried sample}} \right] \times 100\ \%$$

$$\% \text{ Total solids} = \frac{(\text{wt. dried sample plus dish - wt. dish})}{\text{wt. undried sample}} \times 100\ \%$$

3.1.2 Carbohydrate

The NREL Standard procedure for carbohydrate analysis (CAT No. 002) was modified in this study based on previous findings in our laboratory. The modified protocol was found to be more reproducible (Fenske, 1995). Wet samples were dried at 45 °C prior to analysis.

Approximately 0.3 g of sample weighed to the nearest 0.1 mg was placed in a glass test tube. Three mls of 72% H_2SO_4 were added and the tube was placed in a 30 °C water bath for two hours with periodic stirring. These steps were repeated with 0.3 g of a sugar mixture which included glucose, xylose, galactose, arabinose, and mannose. The sample and sugars were then transferred to Pyrex™ screw cap bottles. The acid slurry was diluted to 4 % acid by addition of 84 ml of deionized distilled water, then autoclaved for one hour at 121 °C. The samples were cooled before transferring to 100 ml volumetric flasks and the liquid was brought to volume with deionized distilled water. The purpose of cooling is to prevent loss of volatile compounds. Approximately 20 ml of samples was poured into beakers and neutralized with calcium carbonate to pH 6, measured by pH paper. Following neutralization, samples stood for 45 minutes to allow complete precipitation and were then passed through 0.22 μm filter membranes (Millipore) into HPLC autosampler vials. A set of sugar standards, concentration ranging from 0.025 to 4.0 mg/ml, consisting of all the sugars analyzed were prepared in Milli-Q grade water.

Samples were analyzed using HPLC with a Biorad Aminex HPX-87P column at 85°C and refractive index detection. The mobile phase was distilled/deoinized water at a flow rate of 0.6 ml/min. The injection volume was fifty μL.

CALCULATION:

$$\% \text{ Sugar recovered} = \frac{\text{conc. detected by HPLC, mg/ml}}{\text{known conc. of sugar before hydrolysis, mg/ml}} \times 100\%$$

$$\text{Corrected sugar conc., mg/ml} = \frac{\text{sugar conc. obtained by HPLC, mg/ml}}{\% \text{ sugar recovered} / 100}$$

$$\% \text{ Glycan} = \frac{\text{corrected sugar conc. x } 100 \text{ x } 1\text{g}/1000 \text{ mg}}{\text{sample dry weight, g}} \times \frac{Mw_{sugar} - MW_{water}}{MW_{sugar}}$$

3.1.3 Klason Lignin

Approximately one gram of sample, dried at 105°C, was treated with 15 ml of 72% ($^{w}/_{w}$) H_2SO_4 at room temperature for two hours. The sample was stirred periodically to ensure complete mixing and wetting. It was then diluted to 3% acid by the addition of 560 ml of double distilled water. The sample was boiled for four hours under refluxing conditions and then cooled to room temperature. The solution was vacuum filtered through a predried and weighed 50 ml medium porosity, sintered glass. The filtrate was saved for acid-soluble lignin analysis. The crucible and contents were dried overnight at 105°C and weighed. In order to correct for acid-insoluble ash, the crucible containing the

dried residue was ashed at 500°C. The ashed crucible with residue was cooled in a desiccator and weighed to the nearest 0.1 mg (CAT NO. 003).

CALCULATION:

$$\% \text{ Klason Lignin} = \frac{\text{wt. crucible plus acid insoluble residue - wt. crucible plus ash}}{\text{initial sample weight}} \times 100$$

3.1.4 Acid Soluble Lignin

Acid soluble lignin (ASL) was determined by measuring the absorbance of the filtrate from the Klason Lignin analysis at 205 nm (CAT NO. 004). A 3% ($^{w}/_{w}$) sulfuric acid solution was used as the reference blank. All samples were diluted with water to make the absorbance value fall between 0.2 and 0.8 absorbance units. A low wavelength was chosen for this analysis in order to avoid interference from furfural and non-lignin polyphenols, both of which absorb at higher wavelengths (Smelstorius, 1974).

CALCULATION:

$$\% \text{ ASL} = \frac{\dfrac{A}{b \times a} \times df \times V}{\dfrac{1000 \text{ ml}}{1L} \times W} \times 100$$

where: A = absorbance at 205 nm
df = dilution factor
b = cell path length of 1 cm
a = absorptivity value of 110 L/g cm
V = Volume of filtrate
W = initial sample weight in grams

3.1.5 Ash

Approximately one gram of a 105°C dried sample was weighed to the nearest 0.1 mg in a pre-weighed ashing crucible. The crucible and contents were ashed at 500°C overnight until the residue turned gray or white. The sample was cooled to room temperature in a desiccator and weighed to the nearest 0.1 mg (CAT No. 005).

CALCULATION:

$$\% \text{ Ash} = \frac{\text{wt. ashed residue plus container - wt. ashed container}}{\text{Wt. 105°C dried sample}} \times 100$$

3.1.6 Protein

Protein in this study was quantified using a micro-Kjeldahl method adapted from the Official Method of Analysis, AOAC, 15th edition, 960.52, 1990 (Penner and Suttiprasit, 1991). This method measures the amount of organic nitrogen in the sample and then this value is converted to percentage of crude protein.

Approximately 40-50 mg of sample was weighed to the nearest 0.1 mg on glassine paper. The paper and samples were put in a 30 ml Kjeldahl flask along with 4 mm glass beads, 2.4 ml of concentrated sulfuric acid and 1 Kjeldahl digestion pellet (Kelmate MT-Auto, VWR). During digestion, carbohydrates were oxidized to CO_2 and H_2O and the protein nitrogen was reduced and transformed to ammonium sulfate, $(NH_4)_2SO_4$. The sample was digested on a micro-Kjeldahl digestion unit until the solution become clear, indicating that the carbon had been oxidized to CO_2. Then the sample was cooled to room

temperature and approximately 10 ml of distilled water were added to dissolve the white precipitate.

The digested sample was transferred to a Labconco Micro Still distillation unit and heated to boil. Five mls of 60% NaOH (w/v) were added to the boiling sample followed by addition of another five ml of 60% NaOH + 10 % $Na_2S_2O_3$. The liberated NH_3 was trapped in the form of NH_4^+ in a receiving flask containing 2.5 ml of saturated boric acid (H_3BO_3) and 1-2 drops of methyl red-bromocresol green indicator. The indicator was prepared by mixing one part of 0.2% methyl red solution with five part of 0.2 % bromocresol green. Distillation was continued until approximately 25 ml of distillate was collected. At the completion of distillation, the borate ion is titrated with standardized 0.01N HCl to a gray or colorless endpoint. A blank sample was digested alongside the samples to correct for any nitrogen contaminants present in the reagents chemicals.

CALCULATION:

$$\% \text{ Nitrogen} = \frac{(\text{ml HCl - ml blank}) \times \text{HCl normality} \times 14.007}{\text{dry weight of sample, mg}} \times 100 \%$$

% Crude protein = % Nitrogen x 6.25 (correction factor for animal feed)

(AOAC 988.05, 1990).

3.1.7 Acetic Acid Determination

Biomass samples were hydrolyzed using the two-stage acid hydrolysis procedure described for carbohydrate analyses. At the end of the autoclave period, the sample was cooled in ice bath and vacuum filtered through Whatman No. 1 filter paper. The filtrate

was transferred to a 100 ml volumetric flask and brought to volume with double distilled water. The sample was filtered through a 0.22 μm filter into an autosampler vial and analyzed via HPLC using Biorad Aminex HPX-87H column, and an RI detector. The column temperature was 65°C, the mobile phase was 0.005M sulfuric acid, and the flow rate was 0.6 ml/min. A set of standards covering a concentration range from 0.025 to 1.0 mg/ml was included in each analysis to calibrate the HPLC.

CALCULATION:

$$\% \text{ Acetyl} = \frac{\text{Acid conc., mg/ml} \times 100 \text{ ml} \times 1\text{g}/1000 \text{ mg}}{\text{sample dry weight, g}} \times \frac{Mw_{acetic} - MW_{water}}{Mw_{acetic\ acid}}$$

3.1.8 Uronic Acid Determination

Samples were prepared as described for acetic acid determinations. Approximately 20 ml of the filtered hydrolysate that had been diluted to 100 ml was neutralized with $CaCO_3$ to a pH of about 2.5, measured by pH paper. The purpose of neutralization is to precipitate the sulfate which, if present in large amounts, can interfere with the integration of the glucuronic acid peak. Samples were then filtered through a 0.22 μm filter and analyzed using Biorad Aminex HPX-87H, and RI detection. The chromatographic conditions were the same as those described for the acetic acid determination. External standards containing glucose, glucuronic and galacturonic acids covering a concentration range of 0.025 to 1.0 mg/ml were used.

CALCULATION:

$$\% \text{ Uronic Acid} = \frac{\text{Acid conc., mg/ml x 100 ml x 1g/1000 mg}}{\text{sample dry weight, g}}$$

3.1.9 Extraction

3.1.9.1 Ethanol Extraction

The following procedure is described in the "Standard Method for the Determination of Extractives in Biomass" (NREL, 1984). Extraction was done in a conventional Soxhlet extractor. Approximately 7.0 g of sample, weighed to the nearest 0.1 mg, was transferred to a 33 x 80 mm cellulose medium porosity thimble (Whatman CAT No. 2800338). A plug of glass wool was placed on top of the sample to prevent sample loss during extraction. The extraction solvent was 95% ethanol. Approximately 160 ml of solvent was poured in a weighed round bottom flask containing boiling chips. Sample was extracted for 20 hours, resulting in a total of 100 - 120 solvent exchanges. When extraction was completed, the extracted biomass was transferred to a beaker and dried at 45°C. This sample was kept in a sealed bottle at room temperature for further analyses. Ethanol in the flask was evaporated to dryness using a rotary evaporator. The flask was heated to 35°C in a water bath during evaporation. The flask containing the extractives was dried at 45°C for 24 hours and weighed to nearest 0.1 mg.

CALCULATION:

$$\% \text{ Ethanol extractives} = \frac{\text{wt. flask plus residue - wt. flask}}{\text{sample dry weight}} \times 100$$

3.9.1.2 Hot Water Extraction

Hot water extractions were done following the procedure described in the Standard T 1 os-59 of Technical Association of Pulp and Paper Association. A known amount of native, unextracted switchgrass (about five grams) was placed in a 500 ml flask containing 250 ml of double distilled water. The flask was heated at 100°C for 3 hours under a refluxing conditions. At the completion of the extraction, the contents were cooled and filtered using a 500 ml sintered glass crucible. The solid materials were dried at 45°C and stored at room temperature for subsequent analysis. The filtrate was transferred to a pre-weighed beaker and heated on a steam bath until all of the solvent was evaporated. The beaker was dried in 45°C oven for 24 hours and weighed.

CALCULATION:

$$\% \text{ Water extractive} = \frac{\text{wt. beaker plus residue - wt. beaker}}{\text{sample dry weight}} \times 100$$

3.1.9.3 Ethanol and Hot Water Extraction

Five grams of dried, ethanol pre-extracted biomass was extracted with water following the hot water extraction procedure described above.

% Total extractives = % ethanol extractives + % hot water extractives.

3.2 Statistical Analysis

One way analysis of variance was conducted on the results from the carbohydrate and lignin analyses. There were four treatment groups: unextracted, ethanol, hot water and sequential ethanol/hot water extraction. The analysis is based on Least Significant Different (LSD) Multiple Comparison using Statgraphic 7.0.

The mean values for the individual components of the native feedstock are based on the following number of independent analyses: glycan, 10; KL, 4; ASL, 4; ash, 3; protein, 4; acetyl group, 3; and uronic acid, 2. Mean values for the glycans, KL, ASL and ash of ethanol extracted switchgrass are based on the analysis of four independent ethanol extracted preparations. Protein, uronic acid, and acetyl group values are based on the analysis of two of the ethanol extracted preparations. Mean values for all of the individual components in hot water and sequential ethanol and hot water extracted switchgrass are based on the analysis of two independent hot water and ethanol/hot water extracted preparations.

4. RESULTS AND DISCUSSION

4.1 COMPOSITION OF NATIVE SWITCHGRASS

The average composition and corresponding statistical parameters (SEM) for native switchgrass are summarized in Table 2. All values for carbohydrates are reported as percent homopolymers (glycan) in the substrate, e.g. glucose as glucan. During acid hydrolysis, the glycosidic linkages in the polysaccharides are cleaved, resulting in monomeric sugars, which are then quantified by HPLC. The monomeric sugars contain extra water molecules which are not present in the polymers. The stoichiometric factor used in back calculating monomers to anhydromonomers is the ratio: $(MW_{compound} - MW_{water}) / MW_{compound}$. The same rationale is applied in calculating the amount of *acetic anhydride* from acetic acid which is bound to the hemicellulose. The correction factor in this calculations is 43/60, which is obtained by taking the difference between the MW of acetic acid and water and then dividing by the MW of acetyl groups. The carbohydrate values in Table 2 have been corrected for the loss of sugars due to degradation reactions. Treatment of biomass with sulfuric acid will degrade some of the hexosans and pentosans to 2-hydroxymethyl furfural and furfuraldehyde, respectively. For accurate determination of the total glycan, correction factors for the degradative losses must be incorporated. The way of doing this is to hydrolyze known amount of the pure sugars along with the biomass sample and then analyze via HPLC. The percent sugar recovered in the standard sample is used to correct for the sugar degradation which occurred in the biomass sample. We are aware that this adjustment may overestimate the degradation of the

polysaccharides in biomass because monomeric sugars are degraded faster than polymeric carbohydrates. Correction factors for xylose degradation ranged from 10-12%, reflecting that amount of loss during the acid hydrolysis.

Table 2. Composition of Native Switchgrass
(All results are expressed as percentage of oven-dry feedstock).

Component	**Native Switchgrass**
Total Glycan	56.85 (0.51)
Glucan	*31.30*
Xylan	*20.56*
Galactan	*1.86*
Arabinan	*3.13*
Mannan	*not detected*
Klason Lignin	21.37 (0.26)
Acid Soluble Lignin (ASL)	3.37 (0.10)
Ash	7.10 (0.11)
Protein	3.90 (0.20)
Uronic Acids	1.92 (0.22)
Acetyl group	1.87 (0.03)
Extractives	not applicable
Total:	96.38

Values in parentheses are standard errors of the mean.

The total polysaccharide, or glycan, content of the native switchgrass feedstock used in this study was 57%. Glucans and xylans made up roughly 90% of the total carbohydrate. Only small amounts of galactose and arabinose were present and mannose was not detected. The relatively high xylan and arabinan content in SG indicates that xylan is the major component of hemicellulose while the predominant sugars on the side chains are arabinose. These results agree with Coughan and Hazlewood (1992), who reported that hemicellulose from grasses contain large portion of L-arabinose units. The other components of hemicellulose are uronic and acetic acids, each contributing about 2% of the total feedstock dry weight. The chromatogram of carbohydrate analysis is shown in Figure 6(a). Compounds are listed in the order of their elution time.

Kaar *et al.* (1991a) have developed methods for organic acids (acetic, glucuronic and galacturonic acids) analysis by HPLC using "Polypore" HPX-87H column and Refractive Index detection. The advantages of this method as opposed to the traditional colorimetric, CO_2 adsorption and mass spectroscopy methods is that it is simpler and it is more accurate. The colorimetric and decarboxylation methods could not be used to differentiate between uronic acids and were insensitive to the presence of monosaccharides that might be bonded to a uronic acid moiety (Kaar *et al.*, 1991a). In this study, we developed an assay for uronic acids based on Kaar's protocol, but using a different column that was available in our laboratory. We simplified Kaar's method by eliminating the need to remove glucose prior to the application of the sample to the column.

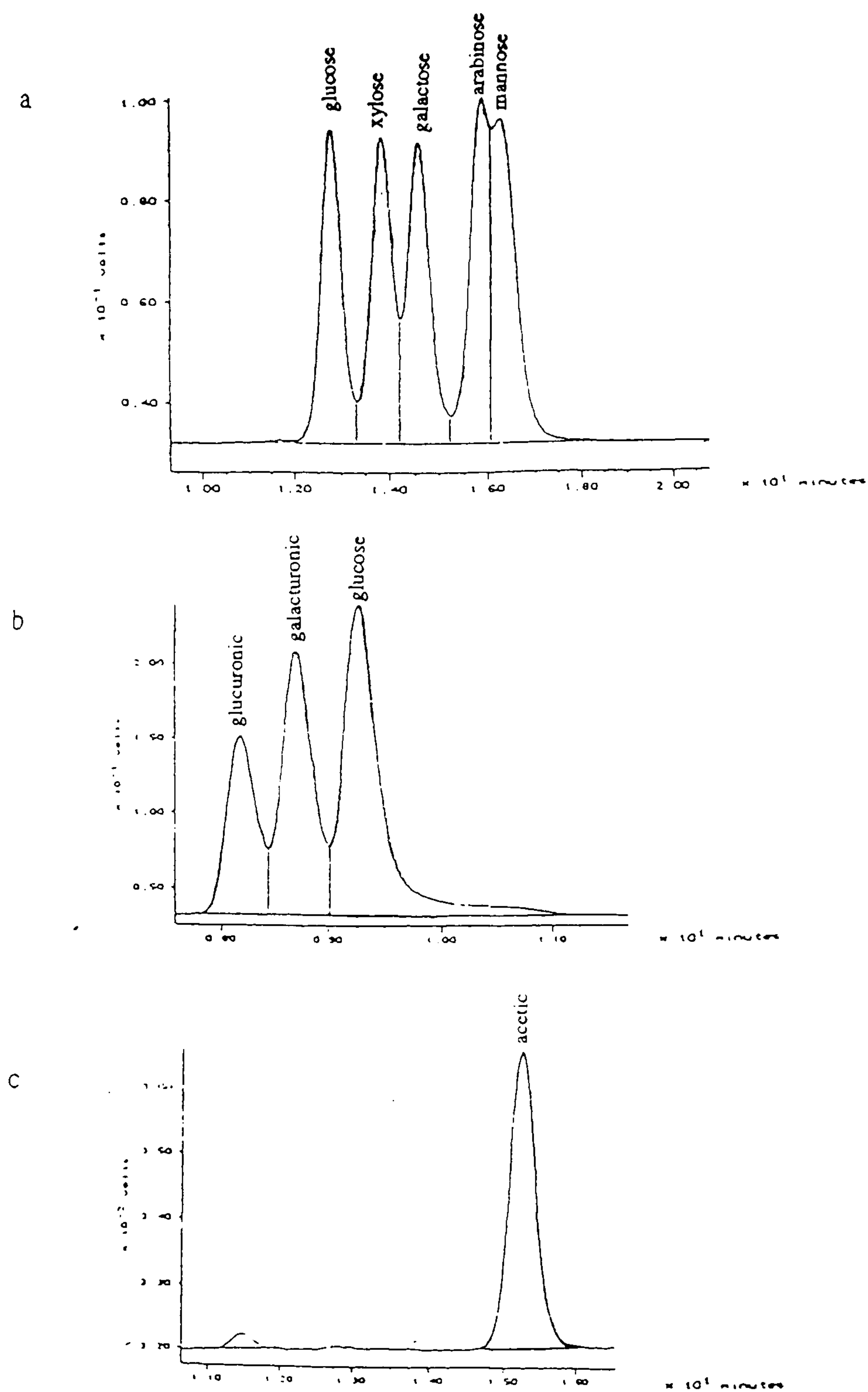

Figure 7. HPLC Chromatograms.
(a) Sugars, glucose, xylose, galactose, arabinose, and mannose, (b) glucuronic, galacturonic acids and glucose, and (c) acetic acid standards.

The Aminex HPX-87H column used in our laboratory can separate glucuronic and galacturonic acids without having interference from glucose or other compounds when operated at 65°C, 0.6 ml/min using 0.005 M H_2SO_4, (Figure 6b). This new method was not tested for the quantification of 4-O-Me-glucuronic acid. Our approach greatly simplifies the uronic acid determinations because the removal of neutral sugars prior to HPLC analysis was quite time consuming.

The Klason lignin (KL) content of the native SG feedstock averaged approximately 21%. This value has been corrected for ash which will partially condense with lignin. It represents the total "acid insoluble" material in the sample, consisting of lignin and also phenolic compounds that condensed with lignin (Torget *et al.*, 1990). In addition, sugar degradation products such as furfural, have also been known to measure as lignin. Errors in the KL determination which relate to sugar degradation are significant if the time of contact with 72% sulfuric acid is prolonged or if the temperature used for digestion is above 20°C (Norman, 1937). The amount of acid soluble lignin (ASL) in the native SG was approximately 3% of the total dry weight of the feedstock. ASL is related to the amount of lignin degraded during KL acid treatments, thus the ASL content of a hydrolysate increases with the severity of hydrolytic conditions (Kaar and Brink, 1991b). The acid soluble lignin value which was quantified by spectrometric measurement at 205 nm, will also include the polyphenolic extractives which absorbed at 203 - 205 nm (Smelstorius, 1974). Considerable amounts of ash (7.1%) and protein (3.9%) were also present in SG. The failure to obtain a mass closure of 100% in the analysis of native switchgrass is assumed to be because extractives were not included in this analysis.

4.2 Role of Extraction on Chemical Analysis

Investigation of the influence of extraction on the chemical analysis of switchgrass (SG) was done by comparing the composition of native SG with ethanol and water extracted SG. Utilization of ethanol is based on the current finding that ethanol is very effective in removing lipophilic and hydrophilic extractives (Theander, 1991, Schell *et al.*, 1992 and NREL LAP-010, 1994). Water extraction and sequential ethanol-water extractions were employed in an attempt to solubilize as much extractives as possible in order to obtained extractive-free samples, and ultimately improve mass balance. The average composition of the three extracted SG preparations and the statistical analysis are summarized in the Table 3. The values in the "Native Switchgrass" column are the same as those in Table 2, they were included merely to make the comparison between treatments easier. The relative composition of the individual glycans is presented in Table 4. All values in these tables are reported in terms of the weight of the measured component per unit weight of original (native) substrate. This is commonly referred to the "unextracted" basis. The composition of ethanol extracted SG is also presented in Table 5 on an "extractive-free" basis. Values for "unextracted" components are obtained by multiplying each of the measured extractive-free components by (100 - % extractives) / 100. This adjustment is important because expressing the results on an extractive-free basis does not allow direct comparison between the composition of extracted and unextracted samples, nor among the extracted samples themselves. This is due to the fact that removal of any component will change the percentage of all other components in the sample, even though these other components were not affected by the extraction.

Table 3. Compositional Analysis of Solvent-extracted Switchgrass.
(All results are expressed as percentage of unextracted oven-dry feedstock).

Component	Native Switchgrass	95 % Ethanol	Hot water	95 % Ethanol and hot water
Total Glycan	56.85^{b} (0.51)	54.49^{a} (0.36)	54.44^{a} (0.44)	55.61^{ab} (0.93)
Klason Lignin	21.37^{d} (0.26)	17.85^{c} (0.28)	16.87^{b} (0.04)	14.93^{a} (0.12)
Acid Soluble Lignin	3.37^{c} (0.10)	2.39^{b} (0.03)	1.83^{a} (0.07)	1.74^{a} (0.12)
Ash	7.13^{c} (0.11)	5.72^{b} (0.13)	2.85^{a} (0.1)	2.45^{a} (0.07)
Protein	3.90^{c} (0.20)	3.69^{bc} (0.30)	2.83^{a} (0.08)	3.11^{ab} (0.08)
Uronic Acids	1.92^{b} (0.10)	1.46^{a} (0.11)	1.50^{a} (0.03)	1.45^{a} (0.02)
Acetyl group	1.87^{a} (0.03)	1.76^{a} (0.06)	1.80^{a} (0.05)	1.94^{a} (0.06)
Extractives	na	9.74^{a} (0.05)	16.42^{b} (0.23)	19.11^{c} (0.11)
Total:	96.38	97.1	98.67	100.3

na = not applicable
Values in parentheses are standard error of the means
Mean values in the same horizontal row with same superscript letters were not significantly different ($P < 0.05$).

Table 4. Carbohydrate Analysis of Solvent-extracted of Switchgrass.
(All results are expressed as percentage of unextracted oven-dry feedstock).

Component	Native Switchgrass	95 % Ethanol	Hot water	95 % Ethanol and hot water
Glucan	31.30[b] (0.46)	29.88[a] (0.25)	29.5[a] (0.24)	29.71[a] (0.40)
Xylan	20.56[b] (0.16)	19.88[a] (0.16)	21.58[c] (0.35)	23.45[d] (0.80)
Galactan	1.86[b] (0.12)	1.38[a] (0.02)	1.63[ab] (0.11)	nd
Arabinan	3.13[c] (0.06)	3.35[c] (0.09)	1.73[a] (0.09)	2.45[b] (0.20)
Mannan	nd	nd	nd	nd
Total Glycan	56.85[b]	54.49[a]	54.44[a]	55.61[ab]

nd = not detected
values in parentheses are standard error of the means
Mean values in the same horizontal row with same superscript letters were not significantly different ($P < 0.05$).

Table 5. Compositional Analysis of Extractive-free Switchgrass.
(All results are expressed as percentage of extractive-free oven-dry feedstock).

Component	95% Ethanol	Hot Water	95 %Ethanol and Hot Water
Total Glycan	60.37 (1.26)	65.16 (1.06)	68.75 (1.93)
Glucan	*33.10*	*35.30*	*36.75*
Xylan	*22.03*	*25.83*	*28.99*
Galactan	*1.53*	*1.95*	*nd*
Arabinan	*3.71*	*2.08*	*3.03*
Mannan	*nd*	*nd*	*nd*
Klason Lignin	19.78 (0.92)	20.25 (0.13)	18.46 (0.23)
Acid soluble lignin	2.64 (0.11)	2.20 (0.18)	2.15 (0.23)
Ash	6.34 (0.49)	3.43 (0.31)	3.03 (0.13)
Protein	4.08 (0.75)	3.42 (0.2)	3.84 (0.1)
Uronic Acids	1.62 (0.17)	1.80 (0.04)	1.79 (0.03)
Acetyl groups	1.95 (0.1)	2.18 (0.15)	2.40 (0.12)
Total	96.78	98.44	100.4

nd = not detected
Values in parenthesis are standard deviation of the means

For this reason, we will focus our discussion on the data as presented in Table 3. The data presented in Table 5 is more commonly used in comparative studies of the chemical analysis of different lignocellulosic materials (Kaar and Brink, 1991c). The results of this experiment show that extractives influence the results of macrocomponent analyses done on switchgrass. The extractives content of SG varied depending on the solvent used for extraction; 9.7% of the dry matter being extracted with ethanol, 16.4% of the dry matter being extracted with water and 19% being extracted by the sequential treatment. Clearly, the "extractives" content is dependent on the conditions used for extractions and thus, any such values must be explicitly defined. Statistical analyses of the macrocomponent results from the different treatments show that the ethanol extracted samples measure significantly lower in KL, total carbohydrate, acid soluble lignin, ash and uronic acids compared to the unextracted, native samples (F-test, p-value <0.05). Approximately 17% of the total material measuring as KL in the native feedstock was extracted with ethanol and approximately 21% was extracted with water. The sequential extraction removed 30% of the material measuring as KL in the native feedstock.

Total carbohydrates in the three extracted sample preparations were reduced by approximately 4% with either ethanol or water extraction. Amount of carbohydrate in the sequentially extracted samples was not significantly different from that in the native feedstock, although, a 2% reduction in total carbohydrate was observed (Table 3). The glucan content of all of the extracted samples was approximately 5% less than that of the native feedstock (Table 4), which is statistically significant at $p < 0.05$. The xylan content of all of the samples were different, approximately 3% of xylan was extracted with ethanol

while the other two extraction procedures appeared to overestimate xylan content relative to the native SG. The xylan content of the hot water extracted samples was only slightly above the native SG but the overestimation in the ethanol-water extraction sample was larger. The high xylan values may be the result of the overlapping of galactan and xylan peaks in some of the analyses. The retention time of xylose is within one minute of that for galactose. When xylose is present in relatively large amounts, the small galactose peak is not separated from xylose. The ethanol-water extractives residues were analyzed for carbohydrate in order to verify this phenomena (Table 6). The data show that the amount of galactan removed from native SG due to sequential extraction with ethanol and then water was 0.4% of the native SG dry weight. The total galactan in the original native SG was 1.86% - thus, there is approximately 1.5% galactan left in the ethanol-water extracted residue that did not show up in the chromatogram. Presumably that material coeluted with the relatively large xylose peak and was measured as xylose. The relative loss of glucan and xylan is low compared to the loss of galactan and arabinan (Table 4).

Table 6. Composition of Water Extractives in an Ethanol Preextracted Sample.

Components	**Weight percent in ethanol/water extractives**	**Weight percent in native SG**	**Total lost (%) per unit weight of native SG***
TOTAL GLYCAN	20.32	0.19	2.2
glucan	13.13	0.12	1.7
xylan	1.39	0.01	0.01
galactan	3.35	0.03	0.4
arabinan	2.45	0.02	0.06
mannan	not detected	not detected	not detected

* Loss from sequential ethanol then hot water extractions.

The extracted sugars are likely to originate from the hemicellulose side chains rather than from cellulose polymers. One consideration is the report by Grohmann *et al.* (1984) which showed that the rate of cleavage of β1-4 linkages in soluble β-glucans is only about one fifth the rate of cleavage of similar linkages in β-xylans. The fact that ethanol and water remove sugars is not unexpected, since these same solvents are used to extract low molecular weight hydrophilic extractives including soluble sugars. The removal of water or ethanol soluble glucans from a feedstock prior to acid hydrolysis means that the resulting glucan value will more accurately reflect the cellulose content of the feedstock. This is because cellulose is not soluble in the extracting solvents (Wayman and Parekh, 1990). However, the decreased values for "total glycans" (particularly glucans) in extracted feedstocks means that the analysis of the pre-extracted feedstock will underestimate the total amount of glucose equivalents available in the original biomass sample.

The acid soluble lignin, ash and uronic acids contents of the extracted SG also followed the same trend as that for KL and carbohydrate, decreasing by 20 to 30% (Table 3). There were no differences in protein or acetyl groups of the ethanol extracted feedstock. The solubility of protein is low in organic solvents. The complete removal of protein requires more harsh treatments, such as with acid detergent solution (Robertson and Van Soest, 1981). This was not done in this study since these treatments also remove hemicellulose.

The ethanol extractives were analyzed in order to verify that the observed reduction in the specific components was actually due to their being extracted. The data

are presented in Table 7. The values in column 2 are presented in terms of weight of the measured component per unit weight of extractives and those in column 3 are calculated as weight of the measured component per unit weight of the original (native) feedstock. The correction factor used was (% extractives/100).

Ethanol extractives were comprised of approximately 40% acid insoluble materials which corresponded to approximately 4% of the dry matter in the native feedstock. This is in agreement with the amount of KL lost from the ethanol extracted sample (3.5% of the native feedstock dry weight). The acid insoluble material was measured using the procedure for klason lignin. These insoluble materials were a mixture of extractable KL, if any, and condensed extractives which measured as KL. In this study, we will assume that there is no loss of KL due to extraction. This assumption is based on the findings by Ritter and Barbour (1935) who have shown that the reduction of KL, after extraction with 95% ethanol, ethanol-benzene and hot water, was not due to the removal of lignin by the extracting solvents, but instead it was due to the removal of contaminants (extractives) that condensed with lignin. Some workers have reported that Brauns native lignin was solubilized in ethanol, but its removal required 8 to 10 days of extraction, which was much longer than our 20-hour extraction time (Browning, 1967, and Obst and Kork, 1988). 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 which 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 (Smelstorius 1971, Ritter and Barbour, 1935, and Norman, 1937).

Table 7. Compositional Analysis for Ethanol Extractives of Switchgrass. (All results are expressed on the basis of oven-dry extractives)

Component	**% w/w in extractives**	**% w/w in native switchgrass**
Total Glycan	20.7	2.01
Glucan	*16.35*	*1.59*
Xylan	*nd*	*nd*
Galactan	*3.9*	*0.38*
Arabinan	*0.45*	*0.04*
Mannan	*nd*	*nd*
Acid Soluble Lignin	3.61	0.35
Ash	12.15	1.18
Protein	0.60	0.06
Acid insoluble matter (measure as KL)	40.67	3.96
Total:	77.73	7.56

The total glycan content in the extractives was 20%, which corresponded to approximately 2% of the feedstock total solids. The predominant carbohydrate extracted was glucan, which amounted to 16.3% of the extractives dry matter. This again, agreed with the loss of total carbohydrate and glucan in the ethanol extracted sample - 2% and 1% of the feedstock weight, respectively. Protein content in the ethanol extractives was less than 1% which was expected because protein is partially or wholly insoluble in organic solvents. All of the recovery values in the extractives are in agreement with those

that were lost from the pre-extracted SG, except ASL. The loss of ASL in the ethanol extracted sample was 1.5% of the native dry weight and that recovered in the extractives corresponded to 0.35% only. The reason for the low recovery is not clear. However, it points out that there is plausible interference of the non-lignin polyphenols on the analysis of ASL (Smelstorius, 1974). This is true because ASL is generated from the degradation of KL which is thought to not exist in the extractives. The satisfactory recovery of the components confirm that the observed differences in the composition of native and extracted SG are due to the actual extraction of analytes as interfering impurities.

4.2.1 Effect of Extractives on Klason Lignin and Carbohydrate Analysis

The reduction in the KL content of extracted SG can have two possible reasons, (1) under-estimation due to the removal of KL during extraction, or (2) over-estimation of KL in native SG due to condensation of extractives with lignin. The phenomenon of the extractives measuring as KL was investigated in this study by directly mixing KL with extractives prior to analysis. Extractives were collected by ethanol extracting the native SG and then dried at 45°C. Klason Lignin was prepared by acid hydrolyzing the native SG and the preparation was dried at 105°C. Klason lignin determination on a model system consisting of only switchgrass KL and switchgrass extractives clearly demonstrated the over-estimation of KL due to the presence of extractives (Table 8). This finding is in agreement with observations on the analysis of wood by Norman (1937), Ritter and Barbour (1935), and Smelstorius (1974). The amount of over-estimation is shown to be a function of the amount of extractives present (row 2 versus 3, Table 8). The values in the

Table 8 assume that the purified extractives contain no lignin - the basis of this assumption has been explained in the previous section. It is assumed that the extractive preparation does not undergo significant chemical and physical change due to the purification procedure. This phenomena was tested by ethanol extracting the extractives preparation. The result indicated that 95% of the extractives are still soluble in the ethanol solvent used in their original extraction. The presence of endogenous extractives in the native switchgrass did not effect the analysis of the supplemented KL (Table 8). This conclusion is based on the observation that the percent recovery of the supplemented lignin was essentially the same (approximately 90%) when the supplemented KL was added to either the native or the extracted switchgrass. The inability to recover 100% of purified KL upon reklasonation is consistent with previous reports (Kaar and Brink, 1991b).

The influence of extractives on glucan and xylan analyses is of interest in our study because these carbohydrate fractions are important in the biomass-to-ethanol processes. The effect of extractives on glycan analysis was tested in an experiment analogous to that just described for KL. In this case, pure glucose and xylose was used as the model substrates and the amount of glucose and xylose equivalents in the extractives was accounted for in the calculation. In contrast to the effect of extractives on the KL analysis, the presence of extractives did not effect the analysis of supplemented glucose (Table 9) and xylose (Table 10). In the presence of extractives, the recovery of supplemented glucose is from 99-100%. The recovery of supplemented xylose was slightly over 100%, which may be due to the overlapping of galactose peak with xylose.

The extractives consist of 4% galactose, all of which were not separated from the xylose peak.

Table 8. Influence of Extractives on Klason Lignin (KL) Determination.

Sample	A Theoretical Total KL (g)	B Measured Total KL (g)	Percent Recovery of Theoretical Total KL	Percent Recovery of Added KL
Extractives	na[1]	na[1]	na	na
0.0883 g Extractives + 0.0813 g KL	0.0813[2]	0.1006	124	124
0.4598 g Extractives + 0.0924 g KL	0.0924[2]	0.2436	264	264
1 g Switchgrass (SG)	0.2133[3]	0.2133	na	na
1.0549 g SG + 0.0773 g KL	0.3023[4]	0.2935	97.1	88.6
1.0287 g SG + 0.0795 g KL	0.2990[4]	0.2916	97.5	90.7
1 g Extracted SG	0.2363[4]	0.1931	81.7	na
0.6917 g Extracted SG + 0.0669 g KL	0.2303[4]	0.1947	84.5	91.5
0.7255 g Extracted SG + 0.0627 g KL	0.2341[4]	0.1983	84.7	93.0

[1] Assume no Klason Lignin in extractives.
[2] Amount of supplemented KL.
[3] Percent Klason Lignin in native Switchgrass.
[4] Theoretical values based on KL in native SG plus the supplemented KL.

Table 9. Influence of Extractives on Glucose Determination.

Sample	A Theoretical Total glucose (g)	B Measured Total glucose (g)	Percent Recovery of Theoretical Total glucose
1 g Extractives	0.1817[1]	0.1817	na
0.275 g Extractives + 0.1022 g glucose	0.1522[2]	0.1506	98.9
0.2745 g Extractives + 0.1031 g glucose	0.1530[2]	0.1536	100.4
1 g Switchgrass (SG)	0.3478[3]	0.3478	na
0.3006 g SG + 0.1012 g glucose	0.2057[4]	0.1983	96.4
0.3008 g SG + 0.1011 g glucose	0.2057[4]	0.1988	96.6
1 g Extracted SG	0.3853[4]	0.3648	94.7
0.3008 g Extracted SG+ 0.1033 g glucose	0.2192[4]	0.2108	96.2
0.3094 g Extracted SG+ 0.1124 g glucose	0.2316[4]	0.2187	94.4

[1] Amount of glucose in extractives.
[2] Glucose in extractive plus the supplemented glucose.
[3] Weight percent of glucose in native Switchgrass.
[4] Theoretical values based on glucose in native SG plus the supplemented glucose

Table 10. Influence of extractives on Xylose Determination.

Sample	A Theoretical Total xylose (g)	B Measured Total xylose (g)	Percent Recovery of Theoretical Total xylose
1 g Extractives	0[1]	0	na
0.275 g Extractives + 0.1004 g xylose	0.1004[2]	0.1014	101
0.2745 g Extractives + 0.1041 g xylose	0.1041[2]	0.1070	103
1 g Switchgrass (SG)	0.2336[3]	0.2336	na
0.3006 g SG + 0.1007 g xylose	0.1709[4]	0.1755	102.7
0.3008 g SG + 0.1011 g xylose	0.1714[4]	0.1786	104.2
1 g Extracted SG	0.259[4]	0.2614	100.9
0.3008 g Extracted SG + 0.1031 g xylose	0.1809[4]	0.1883	104.1
0.3094 g Extracted SG + 0.1063 g xylose	0.1864[4]	0.1883	101.0

[1] Amount of xylose in extractives.
[2] Xylose in extractive plus the supplemented xylose.
[3] Weight percent of xylose in native Switchgrass.
[4] Theoretical values based on xylose in native SG plus the supplemented xylose

4.2.2 Comparison of Extraction Solvents

The significant over-estimation of KL when analyzing native SG means that it is necessary to prepare an extractive-free substrate. A comparison of extraction methods and solvents was done to evaluate the effectiveness of solvents in removing extractives. The solvents used were 95% ethanol and hot water. Individual and sequential extractions were done using those solvents.

The total mass balance value for the native switchgrass feedstock was 96% (Table 3). The other 4% which was not accounted for by the analysis included in this study was presumably extractives. This portion of extractives must be those that were dissolved during the klason lignin determination and were not accounted for in the ASL determination. The other fraction of the extractives that condensed in acid was included in the value estimating klason lignin. Extraction with ethanol, even though it removed 9.74% of total dry matter, did not give 100% mass balance closure. The lack of improvement in mass closure can be rationalized based on the data in Table 6, which shows that a very high percentage of the ethanol extractives are measured by the assays which are included in the mass closure. Thus, these compounds are measured either as "extractives" or as an individual component (for example, "glucan"). This result indicates that most of the extractives removed were compound which would be detected in one of the assays. Analysis of the extractives shows that approximately 3-4% of the native switchgrass dry matter that could condense with lignin had been removed by ethanol extraction. The inability to get a 100% mass balance closure with the ethanol extracted

feedstock indicates that ethanol is not sufficient to remove all of the components that are not accounted for in the summative analysis.

Hot water extraction removed 16.4% of native switchgrass dry weight, 27% of which interfere with lignin analysis. Statistical analysis shows a significant difference in the measurement of Klason lignin in the unextracted, ethanol and hot water extracted SG (F-test, p-value < 0.05). This result showed that hot water extraction, compared to ethanol extraction, removed more of the extraneous matter that measured as KL. A significantly higher amount of protein was removed by hot water extraction. Some proteins have been shown to be insoluble under KL assay conditions and, thus, measure as KL (Norman, 1937, Browning, 1967, and Torget, 1990). In terms of the native feedstock, approximately 4.5% of the total solids measuring as KL were extracted with water. Water extractions removed about the same amount of carbohydrate from the native feedstock as did the ethanol extractions. Water extraction removed greater amounts of ash, protein and compounds soluble in KL reagents. The total mass balance resulting from the analysis of water extracted samples was 98.67%. The improvement in mass balance indicates that water extractives, compared to ethanol extractives, contained more materials that were not analyzed by the assays performed in obtaining the mass closure of the native substrate.

Successive ethanol-hot water extractions removed most or all of the extractives interfering with the chemical analysis of switchgrass, resulting in 100% mass balance. Total extractives removed were 19.11% of the dry matter in native switchgrass. The result also suggests that each solvent removes some portion of extractives that are not

soluble in the other. Water removed an additional 9.37% of extractives that were not soluble in ethanol. On the other hand, ethanol removed an additional 2.69% that were not soluble in water. Extractives that are soluble in both water and ethanol amounted to approximately 7.05% of the dry weight of native SG (calculated from data in Table 11). It appears that sequential extractions with ethanol and water were adequate to obtain an extractive-free switchgrass.

Table 11. Comparison of the Effectiveness of Extraction Methods and Solvents.

	95 % Ethanol	**Hot Water**	**95% Ethanol/ Hot Water**
Extractives removed (% total weight)	9.74	16.42	19.11
Extractives not removed (% total weight)	9.37	2.69	----
Extractives measure as KL **% of substrate wt.** **% of extractives wt.**	 3.5 36.0	 4.5 27.4	 6.5 34.0
Components Removed	Low molecular weight sugars, ASL, ash and uronic acids.	Low molecular wt. sugars, ash ASL, protein, uronic acids.	Low molecular wt. sugars, ASL, ash, protein, and uronic acids.
Mass Balance	**97.1**	**98.7**	**100.4**

The level of klason lignin is reduced from 21% in the native substrate to 15% in the ethanol/water extracted substrate, so approximately 6% of the original dry matter that measure as KL was removed. Statistical analyses show that the values of klason lignin are

different among all the extracted and unextracted samples (F-test, p-value < 0.05). As would be expected, the sequential ethanol-water extraction removed a larger amount of extractives which would measure as KL.

The summary of the amount of extractives removed by different extraction methods is presented in Table 11.

4.3 ETHANOL EXTRACTION ON DILUTE-ACID PRETREATED SAMPLE

In the conversion of biomass to ethanol the feedstock is treated with dilute acid prior to enzyme saccharification. These "pretreated feedstocks" must be analyzed for their carbohydrate content in order to determine the efficiency of the pretreatment and to estimate the theoretical amount of glucan available for fermentation. Thus, it is of interest to see if extractives influence carbohydrate analyses of acid pretreated feedstocks. Table 12 shows the effect of solvent preextraction on SG pretreated at 180°C, 1.2% acid, 1 min and 140°C, 0.6% acid, 3 minutes. The high temperature treatment is considered as the harsh treatment and the low temperature as the mild one. All values in Table 12 were calculated back to an original, untreated and unextracted basis.

Ethanol extraction of samples from both pretreatment conditions removed approximately 12% and 17% of the 140°C and 180°C pretreated solids, respectively, which correspond to approximately 10% of the solids in the original feedstock. This value is not expected to include the low molecular weight sugars which should have been solubilized during the pretreatment. This indicates that greater amount of materials are soluble in ethanol after the acid pretreatment process.

Table 12. Chemical Analysis of the Pretreated Switchgrass for Both Unextracted and Preextracted Preparations. (All results are expressed as percentage of untreated and unextracted oven-dry feedstock).

Components	Native Switchgrass	180 °C, 1.2 % acid, 1 minute		140 °C, 0.6 % acid, 3 minutes	
		Unextracted	Ethanol Extracted	Unextracted	Ethanol Extracted
Glucan	31.3	28.27	27.42	29.66	29.06
Xylan	20.56	1.98	1.54	15.36	15.67
Galactan	1.86	0.17	0.23	1.31	not detected
Arabinan	3.13	0.11	not detected	0.66	0.47
Mannan	not detected	not detected	not detected	not detected	not detected
Klason Lignin	21.37	19.53	11.86	20.91	15.44
Acid Soluble Lignin	3.37	0.585	0.31	2.20	1.43
Ash	7.1	not determined	3.18	not determined	4.06
Extractives	not applicable	not applicable	9.86	not applicable	9.97

This portion of the extractives has no significant effect on glucan analysis (t-test, p-value > 0.05). Loss of xylan in the 180°C pretreated sample during extraction can be explained by the increasing solubility of the hemicellulose due to harsh acid treatment as shown by the low xylan remaining in the solid.

The level of KL in the pretreated SG is slightly lower than that in the native sample which can be explained by the hydrolysis of klason lignin (Browning, 1967). In both the harsh and mild treatments, the klason lignin content was significantly reduced when ethanol extraction was employed. Apparently, most of the extractives that would be measured as Klason lignin were not removed during the pretreatment. The amount of KL in the 180°C pretreated sample after ethanol extraction was reduced by approximately 40% which was much higher than any other observation. This might be due to structural changes in KL after being subjected to the relatively harsh acid pretreatment (Grohmann *et al.*, 1985), which makes it more susceptible to ethanol dissolution. In general, solvent pre-extractions are not done prior to the analysis of pretreated feedstocks.

It is clear that solvent extraction will influence subsequent analysis of switchgrass. The usage of an extraction step prior to chemical analysis seems to be dependent upon what type of data one is trying to obtain. For the accurate determination of cellulose content, pre-extraction seems to be necessary. However, if the purpose of the study is to obtain the theoretical amount of glucan available for fermentation in the biomass conversion process (Carrasco, *et al.*, 1994), then direct analysis of the native feedstock seems more appropriate. A conservative approach for the analysis of switchgrass used in the biomass conversion study would include a solvent extraction step followed by the chemical analysis of both the preextracted sample and its extractives. The benefits of this

approach, compared to direct analysis on native substrate, are that it provides more accurate estimates of the true lignin and cellulose contents. This approach, compared to the analysis of pre-extracted substrate alone, provides a more accurate value for the total theoretical carbohydrate that is available for fermentation.

5. CONCLUSION

The results clearly show that extractives significantly interfere with klason lignin analysis. Some compounds in extractives such as protein, lignans, catechol tannins and tannic acids condense with lignin during the KL assay and, thus, increase in the measured klason lignin content of the feedstock. For accurate estimation of klason lignin, extraction must be employed to remove extractives. Our results show that 95% ethanol extraction alone is not adequate to remove the extractives that interfere with lignin determination. High amounts of extractives removed by hot water extraction suggest that switchgrass contains relatively large amount of water soluble extractives. This is in agreement with the comments of Torget *et al.* (1990) who indicated that herbaceous substrates consist of high amounts of water soluble extractives. Hot water extraction improved mass balance from 96% in native SG to 99%. Sequential extraction with 95% ethanol followed by hot water was found to sufficiently remove the extractives in native switchgrass. This extraction further improved mass balance to 100%.

Carbohydrate analysis on the other hand is not affected by the presence of extractives. The reduction of total carbohydrate observed after solvent extraction is due to solubilization of monomeric, oligomeric sugars or polymeric carbohydrates. The loss of sugars through extraction will under-estimate the amount of total glycan in the sample. Thus, in quantifying total glycan, sample extraction with 95% ethanol or hot water is not recommended. However, removal of extractives is necessary for estimating cellulose in

order to prevent overestimation by the soluble sugars which are not part of the cellulose chains.

The relative merit of extracting a lignocellulosic feedstock prior to chemical analysis is dependent upon the purpose of the analysis. If the objective is to obtain an accurate measure of true lignin and cellulose content, then extraction is necessary. However, direct analysis on the native substrate is better for estimating the total amount of carbohydrate available for fermentation since preextraction will result in an underestimation of the overall carbohydrate content. A conservative approach for the analysis of herbaceous biomass feedstocks will include a sequential 95% ethanol-hot water extraction followed by chemical analysis on both the pre-extracted samples and the extractives obtained from the substrates.

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