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Title: The Role of Solvent Extraction in the Chemical Characterization of Corn Stover Feedstock

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The consequences of extracting corn stover feedstock with either 95% ethanol or hot water prior to the chemical analysis of the macrocomponents of that feedstock have been determined. Reports by others have recommended the removal of extraneous substance by solvent extraction prior to chemical analyses (Browning, 1967; TAPPI, 1988). The 95% ethanol extraction evaluated in this study is currently the "standard" method recommended by the National Renewable Energy Laboratory, Golden, Co. Hot water extractions were tested as a simple, less time consuming and less expensive alternative to ethanol extractions. Compositional analyses involved the quantification of glycans, Klason lignin, acid soluble lignin, ash, protein, acetic acid, and uronic acids.

The summative analysis of native, ethanol extracted and water extracted feedstocks were all in the range of 97 to 98%. Ethanol extractions removed 4.9% of the feedstock dry weight, compared to 17.2% of the dry matter being extracted with hot

water. The extractives obtained via ethanol had negligible amounts of glycans. In contrast, the water extracted solids contained nearly 10% of the native feedstock total glucan. Pre-extracting the feedstock with ethanol had little effect, relative to the native feedstock, on the quantification of glycan components. In contrast, the water extracted feedstock measured significantly lower in total glucans and total glycans than the native feedstock. The lower values associated with the water extraction were due to the actual extraction of glucans from the feedstock, and not due to analytical interferences associated with the extractives. Ethanol and water extracted feedstocks measured significantly lower in Klason lignin than the corresponding native feedstock. This was presumably due to the removal of Klason lignin impurities present in the native feedstock, and not due to the extraction of lignin itself

The combined results from this study indicate that an informative approach to the analysis of corn stover feedstock would include the pre-extraction of the feedstock with hot water prior to further analyses. The appropriate macrocomponent analyses should then be done on both the extracted feedstock and the "extractives" obtained from that feedstock. Analysis of the extracted feedstock, as compared to the native feedstock, would provide more accurate estimates of the cellulose and lignin content of the feedstock. The summative analysis of both the extracted solids and the extractives will provide a reliable estimate of the total amount of carbohydrate potentially available in the feedstock for microbial fermentation to ethanol.

The Role of Solvent Extraction in the Chemical Characterization of Corn Stover Feedstock

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Major Professor, representing Food Science and Technology

Chair of Department of Food Science and Technology

Dean of Graduate School

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.

Khamphet Thammasouk, Author

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INTRODUCTION

The oil crisis of the 1970s was one of the principal events that spurred several countries to take up the search for alternative or renewable energy resources. That search is continued today partly because of the crisis and partly because of the realization that the supply of fossil fuel is finite. Other reasons are based on environmental and economic concerns. In 1980 the Japanese government established an agency, Research Association for Petroleum Alternatives Development, aimed at developing technologies for the production of liquid fuels. The agency has studied rice straw and cane sugar bagasse as raw materials for biomass conversion (Miyakawa et al., 1986). In the U.S., extensive biomass conversion studies have also been done, by agencies such the National Renewable Energy Laboratory (NREL), the Fuels and Chemicals Research and Engineering Division, and the Solar Fuels Research Division.

A potential source of lignocellulosic material for use in biomass-to-ethanol schemes is agricultural residue. Enormous amounts of agricultural residue are produced in the U.S. and it is not utilized to its full potential (Grohmann, et al., 1984). It is estimated that up to 300 million tons of residues are produced each year, about one half of which is from corn stover (Schell, et al., 1992) Corn stover is the stalks and cobs obtained from the residues left in the field when kernels are harvested from the cobs (Schell et al., 1992). With such a high volume of "waste", the U.S. could potentially produce up to 15 billion

gallons of ethanol per year, an equivalent of 10% of the current consumption of gasoline (Clausen and Gaddy, 1983).

Extensive interest in the use of corn stover and other herbaceous feedstocks for ethanol production has increased the demand for simple, accurate analytical methods for characterizing biomass. Lignocellulosic biomass consists largely of cellulose, hemicellulose, lignin, and extraneous substances (Browning, 1967; Wayman and Parekh, 1990; Johnson et al., 1995). The cellulose and hemicellulose fractions represent fermentable sugars that can be converted to ethanol. Lignin and degradation products of these fermentable sugars also have uses in other industries. Lignin can be used to make vanillin or be used in animal feeds. Furfural from xylose degradation can be used as solvent in oil-refining (Wayman and Parekh, 1990).

The conversion of biomass into useful products requires the input of energy. Transportation of these materials to the production plant, biomass preparation, utilities, chemicals (acids), yeast and enzyme requirements all add to the cost of the biomass-to-ethanol process. Knowing the precise quantity of each of the components in a biomass feedstock is instrumental in obtaining an accurate assessment of the efficiency of the processes, and in selecting the best feedstocks for ethanol production.

Chemical characterization of the lignocellulosic cell wall is one way to quantify the macrocomponents of interest. Several analytical approaches are available (NREL, 1992b; NREL, 1992C; Karr et al, 1991; Karr and Brink, 1991a, 1991b; TAPPI, 1988). The common approach in biomass-to-ethanol research for quantifying neutral polysaccharides (primarily cellulose and hemicellulose) involves quantitative measurement of the sugars in an acid hydrolysate of the feedstock by either liquid or gas chromatography. The weight

of glucose and xylose recovered is then adjusted for the water of hydrolysis and reported as glucan and xylan, respectively. Lignin content is generally determined based on gravimetric method of Klason (72% sulfuric acid method), or one of its permutations, which isolates lignin as an insoluble residue in strong sulfuric acid (ASTM, 1993). These approaches have been widely used for the characterization of wood species (Norman, 1937; Moore and Johnson, 1967; Theander, 1991).

A mass balance that involves a summation of all the components analyzed separately is often used in biomass characterization. Indirectly, this approach can measure the adequacy of the analytical methods or the characterization process. Because of the complex nature of the cell wall structure, a mass balance of 100% is very difficult to obtain.

A 100% summation is the ideal goal but not simple to achieve because of potential overlapping contamination as well as degradation processes. Extraneous substances that do not contribute to the structural role of the cell wall have been reported to be among the culprits causing difficulties in the characterization of biomass, particularly in lignin determination. The problem becomes especially bad with herbaceous plants which contain relatively high amounts of minerals, proteins, soluble sugars, and tannins that can interfere with the quantification of the components of interest (Torget et al., 1990).

Pre-extraction of these extraneous components has been recommended prior to the analysis of lignin by the method of Klason to improve the accuracy of the assay (Ritter et al., 1932; Browning, 1967; Ritter and Barbour, 1935; TAPPI, 1988). A comparison of the analysis before and after extraction will indicate whether the extractives removed had any effect on the analysis. Traditionally, nonpolar solvents are used to isolate lipophilic

extraneous compounds, while water is used to solubilize hydrophilic compounds. The current accepted standard extraction protocol of the American Society for Testing and Materials (ASTM D1105-84, 1993) and the Technical Association of the Pulp and Paper Industry (1988) involves sequential extraction of wood with ethanol-benzene, 95% ethanol, and hot water. The extraction scheme is recommended as a means of preparing woody samples for subsequent analysis.

Ethanol has been employed in the past as the sole solvent of extraction (Theander, 1991; Schell et al., 1992). Theander (1991) developed a protocol that used only 80% ethanol to quantitatively remove extractives prior to starch and fiber analysis of plant materials. A slight modification of the extraction protocol using 95% ethanol was employed by Schell et al. (1992) in their study of herbaceous biomass-to-ethanol feedstocks. The approach of using 95% ethanol in a soxhlet extractor for 24 hours is now being used as the standard method at the National Renewable Energy Laboratory (NREL, 1994b). One advantage of this approach is that ethanol is relatively nontoxic. Other advantages and limitations of this approach for chemical characterization of feedstock intended for biomass-to-ethanol processing have not been discussed.

In this study, a summative analysis of a sample pre-extracted with 95% ethanol was compared to a summative analysis of the native, unextracted corn stover. Another comparison was made with hot water as the solvent of extraction. This allowed for a three-way summative analysis comparison between: 1. native and ethanol-extracted corn stover; 2. native and hot water-extracted corn stover; and 3. ethanol and water extraction.

Along with these comparisons, high temperature, dilute acid pretreated corn stover was also analyzed for the presence of extractives and their influence on chemical analyses.

Analysis of the extractives from native substrate removed via ethanol extraction was also attempted. This allowed for a more specific classification of compounds removed from the extraction scheme.

LITERATURE REVIEW

Background:

Environmental costs of fossil fuels and the concern over their depletion have broadened the search for alternative energy resources, such as biomass energy. The oil crisis of the 1970s sent a shock throughout the economic world. That historical event affected many nations that relied heavily on fuels from the Middle East. Japan was particularly impacted because at that time 80% of Japan's energy resources were imported (Miyakawa et al. 1986).

In the U.S., voluntary and compulsory measures were introduced as a result (Fried and Schultze, 1975). Industries and utilities already using coal were prohibited from switching to oil. Competing air lines were urged by the Civil Aeronautics Board to coordinate services in order to conserve jet fuel. Government agencies were asked to reduce the lighting and temperatures in buildings, and to lower speeds of government vehicles. A year-round daylight-saving time was legislated in December, 1973 that was to take effect in early 1974. That same year legislators proposed a nationwide speed limit of fifty-five miles per hour that still remained in some parts of the nation over two decades later. The President of the United States urged gas stations to close voluntarily on Sundays in order to discourage pleasure drivings (Fried and Schultze, 1975). Other motivations for turning to biomass as an alternative resource as cited by Wayman and Parekh (1990) include: 1. Environmental pressure to substitute ethanol for the toxic lead as an octane enhancer in gasoline; 2. Ethanol yields a cleaner exhaust when burned than

gasoline; 3. Farm community pressuring to diversify agricultural products, particularly those of surplus crops that pose a problem in Europe and North America; and 4. The quest for a long-term security of motor fuel to decrease dependency on non-renewable fossil fuels or imported petroleum.

Biomass has been shown to be a considerable good source of raw material for gaseous fuels, liquid fuels, and certain petrochemical intermediates (Shah, et al., 1984). As an alternative resource, biomass is a promising choice not only because it is abundant, but also because it has the advantage of being renewable and with careful management, it can provide a sustaining source of energy. More importantly, this renewable resource is cheap (because it has no competing food values), readily available, does not contribute to any long-term increase in atmospheric carbon dioxide (White and Plaskett, 1981), and can solve the problem of waste disposal (Spilda et al., 1991). In addition, a biomass-to-ethanol industry would create jobs. In Canada, it is estimated that for an addition of 10% of ethanol to gasoline, 42,000 permanent jobs can be created (Wayman and Parekh, 1990). Just as important, ethanol from biomass helps countries reduce fossil fuel imports (Wrixon et al., 1993). In Brazil, a large scale production of fuel from sugar cane was established after the 1973 oil supply crisis (White and Plaskett, 1981). At the present, corn grain is the chief biomass source for ethanol production in the American Midwest (Keeney and DeLuca, 1992). Other potential fuel sources from agriculture include oils extracted from seed crops and methane from waste digestion.

Biomass can be defined as a mass of substance produced by the growth of living organisms. This includes plants, animals, as well as microorganisms (Wayman and Parekh, 1990). The term also encompasses agricultural residues, such as straws, corn stover,

sugar cane bagasse, as well as municipal solid waste. Lignocellulosic biomass or cellulosic biomass is a more specific term referring to biomass derived from plant materials, such as forest industries' waste wood, agricultural crops and crop residues and energy plantations of fast growing wood species (Wayman and Parekh, 1990). These materials contain a substantial amount of organic carbon that can be converted to ethanol and other useful chemicals.

Lignocellulosic biomass consists mainly of cellulose, hemicellulose, lignin, and extraneous substances, often referred to as extractives. In woods, about 40-50% of the cell wall consists of cellulose, 20-35% hemicellulose, 15-35% lignin, and 3-10% extractives (Madan et al., 1983).

Cellulose:

Cellulose is the principal component of cell walls of higher plants and is the basic structural constituent of all plant life (Plunguian, 1943). It is a homopolysaccharide consisting of glucose molecules linked by β1,4-glycosidic bonds with a degree of polymerization ranging from 8,000 to 10,000. These glucose molecules, formed via photosynthesis, are linked together to form a long-chain molecule, with each glucose molecule rotated 180° from its neighbor (Tsoumis, 1968). The exact mechanism of cellulose synthesis remains unclear, but it is known that the cellulose molecules associate to form microfibrils that subsequently adhere to each other to form fibers (Tsoumis, 1968). Hydrogen bonds between the molecules of the fibers allow for the formation of crystalline regions where the molecules are arranged in optimal, parallel order. Because of

the tight hydrogen bonds between cellulose molecules, these molecules cannot partake in water-cellulose hydrogen bonds and are therefore, not reactive, and are insoluble in water and other common solvents (Wayman and Parekh, 1990). However, some regions of the cellulose molecules intertwine with other molecules, causing little or no order and allowing the molecules in these areas to hydrogen bond to water (Whistler and Daniel, 1985). Thus, these amorphous regions are more susceptible to cellulase and chemical hydrolysis. Under appropriate conditions, cellulose can be nearly quantitatively hydrolyzed to glucose molecules.

The glucose subunits can be converted to ethanol by cellulase saccharification or acid hydrolysis of the cellulose and subsequent fermentation of the glucose to ethanol by yeast. Saccharification of cellulose to glucose can be achieved by the use of cellulase enzyme systems, such as that produced by the fungus *Trichoderma reesei* (which was initially developed at the US Army Natick Laboratories in Massachusetts) and *Aspergillus niger* (Wayman and Parekh, 1990). Fermentation with *Saccharomyces cerevisiae*, a common brewer's yeast, converts glucose to ethanol. The saccharification and fermentation can take place separately in a separate hydrolysis and fermentation process (SHF) or simultaneously in a process called simultaneous saccharification and fermentation (SSF) (Wayman and Parekh, 1990).

SSF is accomplished by adding a mixture of fungal cellulase enzyme and a culture of yeast to a fermenter containing lignocellulosic substrate. This system provides the advantage that the glucose in the fermenter is rapidly converted to ethanol and does not reach levels which are inhibitory to the enzymes (Wyman et al., 1992). This provides a large benefit because sugars are found to have more of an inhibitory effect than ethanol

(Takagi, 1984; Holtzapple, 1990). Consequently, ethanol production is more rapid. the reaction can be completed in less than 48 hours, and a higher ethanol yield is obtained than when the saccharification and fermentation are carried out in separate systems (Wayman and Parekh, 1990). Another advantage reported by the U.S. Solar Energy Research Institute is that SSF system uses less than 79% of the amount of enzyme required for SHF. Since enzyme costs are one of the chief barriers to the bioconversion process becoming economically feasible, reduction in the enzyme requirement is very important (Wayman and Parekh, 1990). Also of advantage is that less equipment is used in SSF than in SHF because the process takes place in one vessel.

Hemicellulose:

Another prominent component of the cell wall is hemicellulose. Hemicellulose is a heterogenous polysaccharide with a lower degree of polymerization than cellulose, approximately 150 on average (Tsoumis, 1968). It consists of a xylan chain backbone, linked by β1,4-glycosidic bonds. Unlike cellulose, hemicellulose is branched with glucose, arabinose, mannose, galactose, and uronic acid side chains. In deciduous woods O-acetyl-4-O-methyglucurono-β-D-xylan makes up a large part of the hemicellulose. In addition, about 25% of the xylan residues are acetylated. The structure also includes some rhamnose and galacturonic acid. In conifers, glucomannan and arabino-4-O-methylglucurono-β-D-xylan are more prevalent (Wayman and Parekh, 1990). The backbone of grass hemicellulose is similar to that of deciduous woods. Grasses contain a small amount of uronic acids. Compared to hardwoods, the xylan of grasses are more

highly branched and contain more L-arabinofuranosyl units. In addition, hemicellulosic structures in grasses vary considerably, depending on the species and tissues (Coughlan and Hazlewood, 1992).

Some hemicelluloses are readily hydrolyzed to their monomeric sugars, acetic acid, and other compounds at 185 ^{0}C under steam or at a lower temperature under mild acidic condition (Wayman and Parekh, 1990). Studies are being conducted to assess the potential of biomass xylose as a source of ethanol. Saccharification of the xylan backbone can be achieved using xylanase enzyme and xylose fermenting yeast *Pichia stipitis* (Wayman et al., 1987). Other uses of hemicellulose include the conversion of xylose component to xylitol, a sweetener that is found to suppress tooth decay and used largely in chewing gum. Solution of hemicellulose can also be evaporated to form wood molasses, which may be used as a flavoring agent and binder in animal feeds. Furfural from the degradation of xylose can be used as a solvent in oil-refining and a base for furane resins (Wayman and Parekh, 1990).

Lignin:

Lignin or Klason Lignin (lignin isolated with 72% sulfuric acid) contains only carbon, hydrogen, and oxygen and is the major aromatic component of wood. Lignification marks the last stage of cell wall development. Only living cells produce lignin and completion of lignification almost always coincide with the disappearance of the protoplasm or cell death (Tsoumis, 1968). The lignin molecule is a polymer of phenylpropane units, with a degree of polymerization of 450 to 550. It is highly stable in

organic solvents such as ethanol (Wayman and Parekh, 1990). The polymer is formed via an enzyme-catalyzed, free radical, oxidative condensation of three monomeric alcohols: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol. Glucose is the ultimate lignin precursor, and it is formed via the photosynthetic process and later converted to aromatic compounds through the shikimic acid pathway (Wayman and Parekh, 1990). The alcohols are glucosylated or formed as glucosides and transported along with the sap to their destination where they are transformed to lignin by enzymic dehydrogenation (Sjostrom, 1981).

Different structural make ups have allowed for simple classification of lignin (Sjostrom, 1981). For example, "guiacyl lignin" is found in almost all softwoods and is identified as a polymer of coniferyl alcohol. On the other hand, "guiacyl-syringyl lignin" is found more in hardwood and is identified as a polymer of coniferyl and sinapyl alcohols (Sjostrom, 1981).

Along with intermolecular linkages of the phenylpropane units, studies have indicated the existence of linkages between lignin and cell wall carbohydrate. The majority of the phenylpropanes are linked by ether bonds. Only about one third of the units are covalently linked (Sjostrom, 1981). Covalent bonds are also found between lignin and the carbohydrate components of cell walls. In the separation of these lignin-carbohydrate complexes in softwood, it was found that hemicellulose is bound to lignin chiefly through its arabinose, xylose, and galactose constituents (Sjostrom, 1981).

Unlike cellulose and hemicellulose, lignin is not fermentable by yeast. When biomass is hydrolyzed with hot dilute sulfuric acid, lignin can be recovered and converted to coke for use in steel-making. One study showed that pyrolysis and hydrodeoxygenation

of lignin can result in phenolic compounds, methylaryl ethers, and aromatic hydrocarbons that are similar to aromatic compounds found in newer gasolines. A proposal has been made to include nitrated lignin as additive to diesel fuels (Wayman and Parekh, 1990). Thus, although lignin may not be directly converted to a motor fuel, it may still have use in fuel related applications. Also, a lignin boiler can be used to lower energy required in acid-pretreatment of biomass in the biomass-to-ethanol process (Clausen and Gaddy, 1983). Furthermore, the economic potential of alcohol fuel can be greatly enhanced with the application of by-product lignin (Sundstrom and Klei,1982). For example, lignin can be used as plywood and particle-board adhesives and extenders for asphalt in pavement mixture. Other applications of lignin include its use in animal diets where it has been shown to reduce blood cholesterol level and prevent gallstone formation. In addition, lignin can be used to treat gastrointestinal disease in cattle (Wayman and Parekh, 1990). Another use of lignin is in the vanillin production industry.

Evidence has shown that lignin and hemicellulose form a matrix sheath that hinders the enzymic saccharification of cellulose and hence, the bioconversion process (Grohmann et al., 1985). This complex confers upon the native substrate the property to resist enzymic hydrolysis. A pretreatment method using dilute sulfuric acid and heat has been established to hydrolyze the hemicellulose fraction to increase enzyme accessibility to the cellulose. Dilute acid (0.4-1.2% wt/wt) treatment at high temperature (100-260 ^{o}C) was shown to be effective (Grohmann, et al., 1985; Torget et al., 1990, 1991; Schell et al., 1992). Over ninety percent of the hemicellulose fraction can be hydrolyzed to xylose while the cellulose fraction remains essentially intact. The intact cellulose is no longer shielded from the enzyme and is more susceptible to hydrolytic attack (Schell et al., 1992).

The efficiency of enzymic digestibility of cellulose after pretreatment is greatly improved, even without the removal of lignin (Grohmann et al., 1984).

Extractives:

The above mentioned macrocomponents (cellulose, hemicellulose, and lignin) form the majority of cell wall weight, and play a role in the architectural network. In addition to these components, there are other compounds that have little or no role in the cell wall structure.

Extraneous substances are extraparietal compounds not involved in the structural function of the cell wall (Browning, 1967). These compounds are often referred to as extractives because they are isolated by solvent extraction. They consist of varying chemical components, such as gums, fats, waxes, resins, sugars, oils, starches, alkaloids, and tannins (Tsoumis, 1968). Some of the substances classified as extractives are also fungicides that help protect the plant from microbiolgical attack (Sjostrom, 1981).

The common approach to the chemical characterization of wood is a summative analysis where various components are analyzed individually and the results added together (Smelstorius, 1971; Madan et al, 1983). Analytical techniques of the past, such as those of TAPPI standards and others applied by Moore and Johnson (1967) and Smelstorius (1971) had allowed for sufficient accounting of most wood components. However, the mass balance from these techniques may have overestimated or underestimated the total components by as much as 10% (Karr and Brink, 1991b). Other methods involved normalization of the results to obtain a 100% mass balance. While such

methods assured a perfect summation, they assumed that the errors that occur in each analytical technique were the same. Thus, although glucose determination is reliable. it is adjusted by a larger amount when compared to uronic acid that may be present in smaller amount and whose technique of detection less reliable (Karr and Brink, 1991b). Because of more accurate instrumentation, the summative approach has been improved immensely. The vast improvement in detection techniques of carbohydrates and organic acids such as the use of HPLC have allowed for a more accurate summation, but, because of possible overlapping contamination or component degradation, a summation of 100 ± 2% is considered acceptable (Madan et al., 1983). This may have stemmed from reports that extractives can interfere with the characterization of the biomass, especially herbaceous plants (Ritter and Barbour, 1935; Norman, 1937; Browning, 1967). The problems lie mainly in the high content of minerals, proteins, soluble sugars, and other interfering components (Torget et al., 1990). It has been recommended that these components be extracted from the wood prior to chemical analysis, except in cases where the washings during the extraction would interfere with certain chemical analysis (Browning, 1967; TAPPI, 1988).

Quantifying "extractives" is difficult because it includes a wide variety of compounds soluble in different solvents. No one method removes all the extractives from the cell wall structure. As a result, extractives are classified on the basis of their extraction or solubilization from wood with cold, or hot water, or neutral solvents such as alcohol, benzene, acetone, or ether. The proportion of extractives can vary from less than 1 percent of dry wood weight, as in poplar, to more than 10%, as in redwood. This

variation extends between species as well as within a single tree, such as between the leaves and the twigs (Tsoumis, 1968).

Extractives are formed from a variety of biosynthetic pathways. Factors such the environment and age of the tree can also contribute to the variation found among and between species. These extractives are deposited after the formation of the cell wall, with polyphenolic compounds as the major building blocks (Soltes, 1983).

The contents of extractives from oftwood and hardwood are different, both in quantity and quality. Softwoods often contain resin canals or oleoresin which are produced by the epithelial cells surrounding the canal. Resin acids make up approximately 50% of the spruce resin, whereas pine oleoresin contains about 70-80% resin acids. The other 20-30% of softwood resin consists of volatile monoterpenes. Terpenoids and fatty acids esters make up the remaining 20 or 30%. In parenchyma cells, the resin consists mostly of fatty acid esters and sterols. The resins remain encapsulated in the parenchyma cells when wood is pulped. Oleoresin, on the other hand, is released into the pulp liquor. When parenchyma cells die, heartwood or the inner, darker portion of the stem, is formed, and many chemical changes take place. For instance, when parenchyma cells die in pine wood, formation of fungicides and phenolic substances in the heartwood takes place. In fact, one defining characteristic of heartwood is the presence or accumulation of phenolic compounds (Sjostrom, 1981).

Many theories have been proposed to explain the formation of extractives in the heartwood. Frey-Wyssling and Bosshard (1959) believed that the parenchyma cells in the heartwood gradually age, degrade, and become inactive because of the semi-anaerobic conditions that exist in the sapwood zone surrounding the cells. Under such conditions

phenols undergo oxidative polymerization. Another theory suggested by Hillis (1985), proposes that extractives are formed when metabolic activities of living parenchyma cells cease as a result of an accumulation of air in the interior of the stems. This accumulation causes a reduction in the water content, which in turn halts the metabolism of the cells. Another explanation claims that extractives are formed when parenchyma cells die as a result of accumulation of secondary metabolites or biochemical "waste" products in the heartwood (Sjostrom, 1981).

Wood extractives can be classified into three groups: aliphatic compounds; terpenes and terpenoids; and phenolic compounds (Sjostrom, 1981). Aliphatic extractives (fats and waxes) include several groups of compounds, and can be found in abundance in the parenchyma cells. Alkanes and alcohols are relatively stable and lipophilic. The saturated and unsaturated aliphatic fatty acids are often found in the ester form in the parenchyma resin. Fats or glycerol esters are the most important esters in plants (Sjostrom, 1981). The polyunsaturated fatty acids are quite unstable and can undergo addition reactions or oxidation.

Terpenoid compounds include volatile oil (turpentine) and nonvolatile residues (resin acids), or polyprenols. They contain various functional groups such as hydroxyl, carbonyl, carboxyl, and esters. Triterpenoids can be found in hardwood parenchyma resin. Terpenes are compounds formed by the condensation of two or more isoprene units with the elementary formula of $(C_{10}H_{16})_n$. Terpenes can be classified as monoterpenes (n=1), sesquiterpenes $C_{15}H_{24}$ (n=1.5), diterpenes (n=2), triterpenes (n=3), tetraterpenes (n=4), and polyterpenes (n>4). Diterpenes can be found as acyclic, monocyclic, dicyclic, and

tricyclic structure. Many of them are polyunsaturated and can polymerize to form high molecular weight products that are only slightly or no longer soluble (Sjostrom, 1981).

The other group of extractives, phenolics, consists of several classes of compounds that can have fungicidal properties (Sjostrom, 1981). These include hydrolyzable tannins, flavonoids, lignans, stilbenes, and tropolones. The hydrolyzable tannins, compounds that are present in small amounts in woods, yield gallic acids, ellagic acids, and sugars as the main products upon hydrolysis. Flavonoids are polyphenolic compounds represented by chrysin (5,7-dihydroxyflavone) and taxifolin (dihydroquercetin). These compounds can polymerize to form condensed tannins. Lignans are the product of oxidative coupling of two phenylpropane units such as conidendrin, pinoresinol, and syringaresinol. Stilbenes, 1,2-diphenylethylene compounds, contain conjugated double bonds. As a result, they are reactive compounds. Tropolones are identified by unsaturated seven-membered carbon rings. They can be found in decay-resistant conifers. Along with the fungicidal properties, the phenolics also give the woods their natural color. Other compounds, such as taxifolin and pinosylvin, have been found to inhibit delignification during acid sulfite pulping even in low concentrations. Tropolones can form strong complexes with heavy metal ions and cause erosion problems (Sjostrom, 1981).

Many of the extractives are low molecular weight compounds. These are relatively easy to remove by steam distillation or extraction with water or neutral organic solvents. However, in a polymerized state, such as from oxidative polymerization during long-term storage of plant materials, these compounds become unextractable by neutral solvents. These high molecular weight compounds can severely interfere with compositional analysis of biomass, particularly that of lignin. This may explain variations

in compositions of the same species of wood as determined by different laboratories (Smelstorius, 1971). Examples of these high molecular weight compounds include condensed tannins, polymerized phenolic acids, and protein. Although proteins are hydrolyzed to their amino acid components during acid treatment of lignin determination, these amino acids can condense and precipitate with the lignin in solution. Thus, they can cause an overestimation of the lignin. Condensed tannins, which contain high amount of phenolic hydroxyl functions can also precipitate in the presence of acids during lignin determination (Smelstorius, 1971). In one report by Smelstorius (1971), it was found that these phenolic hydroxyl units can be effectively removed via boiling alkali extraction. However, such extraction will result in an underestimation of the lignin because the less resistant fraction of lignin is also removed, as are some carbohydrates.

Extractives are removed from biomass feedstocks for quantification, for characterization and to improve the accuracy of subsequent analyses. To determine the influence of extractives on a particular analysis the analyte in question, i.e. lignin, is assayed prior to and after the extraction (Wise and Ratliff, 1941; Smelstorius, 1971).

To avoid an over- or under-estimation of the composition of the biomass, preextraction of the sample has been recommended prior to analysis (Browning, 1967; TAPPI, 1988). Extraction of nonvolatile extractives can be accomplished using a Soxhlet extractor. It should be noted that no single solvent is capable of solubilizing all extractives. Often successive extractions or extraction using a mixture of solvents is required. Selection of the solvent must be based on the aim of the work. For example, water extraction can remove compounds such as inorganic salts, sugars, polysaccharides,

cyclones, cyclitols, and some phenolic substances. Organic solvents can be used to extract resins, fatty acids, waxes, unsaponifiable substances, pigments, etc. For pine extractives, ethyl ether is the preferred solvent because it gives a complete extraction with no hydrophilic compounds removed. For wood samples containing high amounts of lipids or terpenoids, both ether and benzene are good solvents (Hillis, 1985). For maximum yield of extractives, Lewin and Goldstein (1991) recommended an ethanol-benzene (1:2) mixture. However, one difficulty with organic solvents such as benzene and ethanol is that a significant amount of the solvent is absorbed by the biomass residue. The absorbed solvent is difficult to remove (TAPPI, 1988). Subsequent washing with hot water can remove the absorbed solvent but the small advantage gained can be offset by the loss of water soluble materials (Browning, 1967). Soxhlet extraction utilizing 95% ethanol has been found to be effective as well as being non-toxic in comparison to benzene (NREL, 1994b). Ethanol has been used in the past (Theander, 1991; Schell et al., 1992) and is the standard method used by the National Renewable Energy Laboratory.

Temperature and duration of extraction are crucial factors which affect the amount and type of extractives removed by a given solvent. It is found that the amount of substances soluble in water tends to increase with time, which may be a result of the solution becoming acidic from the hydrolysis and release of the acetyl groups from the biomass (Browning,1967). Thus, the condition becomes similar to that of hydrolysis by dilute acid (Browning, 1967).

METHODS

Summative characterization of corn stover feedstock involved compositional analysis of the substrate. Corn stover is defined by Schell et al., (1992) as consisting of the stalks and cobs obtained as the residues remaining in the field when the kernels are harvested from the cobs. The components analyzed in this study included carbohydrates (glucan, xylan, galactan, arabinan, and mannan), lignin (both Klason lignin and acid-soluble lignin), ash, acetic acid, extractives, uronic acids, and protein. These components demand several analytical assays for their quantification. The following illustrates the procedures used for the determination of each component.

Corn stover feedstock was supplied by the National Renewable Energy Laboratory (NREL), Golden, Colorado. The dilute acid pretreated samples were prepared here at Oregon State University, Corvallis, Oregon. Pretreatment was performed according to the NREL protocol, with a 10% solid content (weight/weight) in a 0.6 L Parr batch reactor equipped with an acid injector and a temperature controller. Typically, 30 g of biomass and 240 g distilled water were added to a Pyrex™ reactor liner. At the end of the reaction, the pretreated substrate was filtered, washed with distilled water, and dried at 45 ^{0}C for 48 hours before chemical analysis.

Solid/Moisture Content Determination:

Almost all analyses required that moisture content be determined to express the results in terms of dry weight basis. NREL's Chemical Analysis and Testing Standard

Procedure #001 was used as the protocol for this determination. A sample of 1 to 2 grams was weighed into a tared, dried aluminum weighing dish and dried to constant weight in a convection oven at 105 ± 3 °C. The sample was placed in a desiccator to cool to room temperature and weighed.

Calculations:

Eq. 1

$$\% \text{ Solids at } 105\ ^{0}C = \frac{(WSD\text{-}WD)_{\text{after drying}}}{(WSD\text{-}WD)_{\text{prior to drying}}} * 100$$

Eq. 2

$$\% \text{ Moisture at } 105\ ^{0}C = 1 - \frac{(WSD\text{-}WD)_{\text{after drying}}}{(WSD\text{-}WD)_{\text{prior to drying}}} * 100$$

where: WSD = weight of sample plus weight of dish

WD = weight of dish.

Solvent Extraction:

Prior to the analysis, a portion of the feedstock was extracted with either ethanol or water. Ethanol extraction was conducted according to the protocol suggested by the NREL procedure No. 010 using Soxhlet extraction. The hot water extraction procedure was adapted from the TAPPI: Standards and Suggested Methods (1932).

95% Ethanol Extraction:

At the time of sample preparation, the moisture content of the sample was determined. Approximately 5.0 g (weighed to the nearest 0.1 mg) of the sample was

added to a cotton cellulose thimble. A plug of glass wool was placed on top of the sample to prevent sample loss during the semi-continuous extraction process. Several boiling chips were placed into a clean, dried round bottom receiving flask. The flask, along with the boiling chips, were weighed to the nearest 0.1 mg and recorded as the tare weight of the flask. At least 160 ml of 95% ethyl alcohol (Sigma ™) was used for the extraction. The ethanol was heated under reflux for 24 hours while adjusting the heating rate such that there were four to five solvent exchanges per hour in the Soxhlet thimble, or approximately 100 to 120 solvent exchanges during the 24 hour period. After the sample was cooled, the thimble was removed and the contents carefully transferred to a Buchner funnel. Residual solvent was removed by vacuum filtration and the sample washed thoroughly with approximately 30 ml of 95% ethanol. The filtrate was combined with the filtrate from the round bottom Soxhlet flask and the ethanol solvent was evaporated under vacuum in a Roto-vap. A water bath of $45 \pm 5\ ^{0}C$ was used to heat the flask to facilitate evaporation. After the solvent was removed, the flask was placed in a $45\ ^{0}C$ oven for 24 hours to dry, cooled in a desiccator, and weighed.

Calculation:

$$\textit{Eq. 3} \quad \%\ \text{Extractives} = \frac{(WRF - WF)}{(TS)} * 100$$

where: WRF = the weight of the residue plus flask

WF = the weight of the flask

TS = total solid or (weight solid*%solid/100).

The extractives remaining in the flask were collected for subsequent chemical analysis.

The solid remaining in the filter was dried at 45 °C oven. Analysis of the different components were done on the dried pre-extracted solid. To correct for the amount of extractives removed and to express the values in terms of unextracted, native material, the components were multiplied by [1-(% extractives/100)] (Karr and Brink, 1991b).

95% Ethanol Extraction with Extractives Added Back:

Corn stover was extracted with 95% ethanol as described above. Instead of evaporating the solvent containing extractives to dryness, the solvent and its content was added back to the extractives-free solid fraction and mixed well. A total of 70 ml of distilled water was added to the sample and the whole content roto-evaporated to near dryness.

The solid was then dried at 45 °C. Analysis of the different components were done on the dried solid.

Hot Water Extraction:

Standard method T1 0S-59 of the Technical Association of Pulp and Paper Association (1932) was used to determine water-soluble extractives in corn stover. A known amount of native corn stover (with moisture content determined at the time of sample preparation) was placed in a 1000 ml Erlenmeyer flask. About 75 ml of distilled water was added per gram of sample. The sample was boiled under reflux for 3 hours, cooled, filtered, and the solid washed with hot distilled water. The filtrate was evaporated to about 10 ml and quantitatively transferred to a tared, clean beaker, placed in a 45 °C

oven, and dried overnight. The residue remaining in the beaker was weighed as water-soluble extractives.

Calculation:

$$\text{Eq. 4} \quad \%\ \text{Extractives} = \frac{(WRB - WB)}{(TS)} * 100$$

where: WRB = weight residue plus weight beaker

WB = weight of beaker

TS as defined in equation 3.

The solid remaining in the filter was dried in a 45 ^{0}C oven. Analysis of the different components were done on the dried, pre-extracted solid. To correct for the amount of extractives removed and to express the values in terms of unextracted, native material, the components were multiplied by [1-(% extractives/100)] (Karr and Brink, 1991b).

Ash Determination in Biomass:

Ash determination was performed using NREL protocol No. 005. The sample was dried at 105 ^{0}C and cooled to room temperature in a dessicator. One to three grams of dry sample were weighed into a tared crucible, which had been flamed and cooled in a dessicator. The sample was ashed in a muffle furnace at 575 ± 25 ^{0}C for a period of 12 to 18 hours until no black residue remained. After ignition the crucible was cooled in a dessicator and weighed as crucible plus ash.

Calculation:

$$\text{Eq. 5} \quad \%\ \text{Ash} = \frac{\text{Ash}}{(\text{TS})} * 100$$

where: TS as defined in equation 3.

Two Stage Sulfuric Acid Hydrolysis for the Determination of Carbohydrates:

Carbohydrate was determined according to NREL protocol No. 002. The total solids content of a portion of the sample dried at 45 ^{0}C was measured at the time of sample preparation. Another portion of the sample was retained for analysis. Approximately 0.3 grams (weighed to the nearest 0.1 milligram) of the 45 ^{0}C dried biomass sample was transferred to a test tube and 3.0 ml of 72 % sulfuric acid was added and stirred. The test tube was placed in a 30 ^{0}C water bath, and incubated for 2 hours with periodic stirring to assure complete mixing and wetting of the sample. This procedure was simultaneously done on a high purity sugar standard mixture containing glucose, xylose, galactose, arabinose, and mannose that had also been dried at 45 ^{0}C. The standard mixture was subjected to the same treatment conditions to estimate and correct for losses due to the destruction of sugars.

After two hours, the samples were removed from the water bath and quantitatively transferred to 250 ml Pyrex™ bottles with teflon lined screw caps. Eighty four ml of distilled water was used to quantitatively transfer the sample, resulting in a 4% acid solution. The sealed bottles were autoclaved for 1 hour at 121 ^{0}C, and cooled before the caps were removed. At this point, a slight variation of the NREL protocol was employed.

Previous studies by a co-worker have found the results of carbohydrate analysis to be more reproducible if the volumes were brought to 100 ml before neutralization rather than measured after neutralization and filtration (Fenske, unpublished results, 1994). Thus, the samples were transferred to a 100 ml volumetric flasks and distilled water added to bring the solution up to volume. Approximately 20 ml of each samples was transferred to a small beaker. Calcium carbonate was added to neutralize the hydrolyzates to a pH of 6 or slightly higher as measured by pH indicator paper. The neutralized hydrolyzates were left standing in a tub filled with ice for 30 to 45 minutes to allow the precipitates to settle. A small portion of the filtrate was prepared for HPLC analysis by passing through a 0.45 μm syringe filter into an autosampler vial.

A series of sugar standards in Milli-Q double distilled water at concentrations of 0.025 mg/ml to 4.0 mg/ml were prepared for the calibration curves for each of the sugars of interest. The calibration sugar standards, the hydrolyzed sugar standard mixture, and the hydrolyzed samples were analyzed by HPLC using a 7.8 x 300 mm, Bio-Rad Aminex HPX-87P column for glucose, xylose, galactose, arabinose, and mannose. The system used was an all-Waters HPLC system, a 401 refractive index detector, and Baseline 810 integration software. The conditions used for the analysis were:

Injection volume:	50 μl.
Mobile phase:	Milli-Q water
Flow rate:	0.6 ml/ min.
Column temperature:	85 ^{0}C
Detector:	refractive index.
Run time:	50 minutes

Calculations:

% Sugar recovered---The amount of each sugar standard recovered after being subjected to the hydrolysis procedure, which will give an estimate of the amount of each individual sugar destroyed as a result of the hydrolysis procedure.

Eq. 6

$$= \frac{\text{(conc. detected by HPLC)}}{\text{(known conc. of sugar before hydrolysis)}} * 100$$

Corrected sugar concentration---the correct sugar concentration for each hydrolyzed samples.

Eq. 7

$$= \frac{\text{(sugar conc. obtained by HPLC)}}{\text{(\% sugar recovered/100)}}$$

% Sugar yield---the percentage of each sugar present in the hydrolyzed samples, on a 105^0 C dry weight basis.

Eq. 8

$$= \frac{\text{(corr. sugar conc.*1 g/1000 mg* volume)}}{\text{(TS)}} * 100$$

where: TS as defined as in equation 3.

To report the carbohydrates in terms of their anhydride form to closer reflect their natural structure in biomass, the following stoichiometric factors were used:

Compound	Reported as	Factor used
glucose	glucan	162/180
xylose	xylan	132/150
galactose	galactan	162/180
arabinose	arabinan	132/150
mannose	mannan	162/180

Acetyl Group Determination:

Sample preparation for acetyl anhydride determination was prepared according to the procedure for carbohydrates determination. After diluting the hydrolysate to 100 ml, the sample was filtered through a 0.22 μm filter film for analysis using HPLC. The separation column used was a 7.8 x 300 mm Bio-Rad HPX-87H. The HPLC system used in the determination of the carbohydrate was the same system used for this analysis. The conditions of analysis were those specified by the NREL procedure No. 015 (1994c).

Sample volume:	50 μl.
Mobile phase:	0.005 M sulfuric acid
Flow rate:	0.6 ml/ min.
Column temperature:	65 ^{0}C
Detector:	refractive index.
Run time:	50 minutes

A set of external calibration standard solutions of acetic acid, with concentrations ranging from 0.025 mg/ml to 1.0 mg/ml was injected prior to the samples. The compound was detected as acetic acid but reported as acetyl anhydride. A correction factor of 43/60 or 0.72 was used to correct for the water of hydrolysis (Karr et al., 1991).

Uronic Acids Determination:

Sample preparation for uronic acids determination was prepared according to the procedure for the determination of carbohydrates. After diluting the hydrolysate to 100 ml, instead of neutralizing with calcium carbonate to pH 6, a fraction of the sample was

neutralized to pH 2.5 with calcium hydroxide. Following neutralization, the sample was placed in a tub of ice to allow the precipitates to settle, and then filtered through a 0.22 µm filter film for analysis using HPLC. The system and analysis conditions were identical to those for acetyl anhydride determination. The difference lies in the external calibration standard solutions. Standard solutions containing glucuronic acid and galacturonic acid in the concentration range of 0.025 mg/ml to 1.0 mg/ml were used. The compounds were detected as glucuronic acid and galacturonic acid.

Determination of Klason Lignin in Biomass:

Lignin was determined using NREL No. 003, which was adapted from Peter Klason (NREL, 1992C). Biomass was dried at 105^0 C, and approximately 1.0 g of the dried sample (weighed to the nearest 0.1 mg) was placed in a test tube. Fifteen ml of 72 % sulfuric acid was added to the test tube. The sample was left standing at room temperature with occasional stirring for 2 hours. The sample was then transferred to a 1000 ml Erlenmeyer flask and diluted to 3 % acid concentration with 560 ml distilled water. The resulting solution was boiled for 4 hours with reflux.

The lignin was vacuum filtered through a medium porosity fritted crucible that had been dried to constant weight and tared. Distilled water was used to wash free all residue clinging to the Erlenmeyer flask and to wash the residue free of acid. The filtrate was saved and the volume recorded for subsequent acid-soluble lignin determination. The crucible containing the lignin was dried at $105 \pm 3\ ^0$C to constant weight, which usually required 8 to 16 hours. The crucible was cooled in a desiccator and weighed as lignin plus

ash. The acid-insoluble ash was determined by incinerating the lignin sample at 575 ± 25^{0}C for 12 to 18 hours. The crucible and its content was cooled in a desiccator and weighed to the nearest 0.1 mg.

Calculation:

$$\textit{Eq. 9} \quad \% \text{ Klason lignin} = \frac{(WLC - WC)}{(\text{initial sample wt.})} * 100$$

where: WLC = weight of lignin plus crucible

WC = weight of crucible.

Determination of Acid Soluble Lignin in Biomass:

To measure acid-soluble lignin, the filtrate from the determination of Klason lignin was analyzed directly using a Shimadzu UV160U UV-Visible Recording spectrophotometer. The absorbance of the filtrate was measured at 205 nm to avoid interference from furfural or hydroxymethylfurfural. A 3% (wt/wt) sulfuric acid solution was used as a reference blank. The sample was diluted 10 fold with distilled water to obtain an absorbance of between 0.2 to 0.7 unit.

Calculation: An absorptivity (extinction coefficient) value of 110 L/g-cm was used to calculate the amount of acid-soluble lignin present. This value is an average of values found for different woods and pulps (Karr and Brink, 1991).

$$\textit{Eq. 10} \quad \% \text{ Acid-soluble lignin} = \frac{[(A/(b*a)*(df)*V]}{[1000cc/1L*W]} * 100$$

Where: A=absorbance at 205 nm

df= dilution factor

b= cell path length of 1 cm

a= absorptivity value of 110L/g-cm

V= filtrate volume (L)

W= initial sample weight in grams

Protein Determination:

Protein determination was adapted from the micro-Kjeldahl method of the AOAC (1990). About 40 to 50 mg (weighed to the nearest 0.1 mg) of corn stover was placed in a 30 ml Kjeldahl flask along with the weighing paper. The moisture content of the sample was also determined. A Kjeldahl digestion pellet containing 1.5 g K_2SO_4 and 0.075 g HgO (VWR) was added along with a few glass boiling beads. To each of the flasks was added 2.4 ml of concentrated H_2SO_4, and the contents heated to boiling. Usually one hour was sufficient for complete digestion, which was indicated by a clear solution. After the flask was cooled and prior to distillation, a small volume of distilled water was added to the flask to dissolve the resultant solids.

The sample was distilled into a 125 ml Erlenmeyer receiving flask containing 2.5 ml of H_3BO_3 and 1-2 drops of methyl red-bromocresol green indicator solution. Eight to 10 ml of 60% NaOH-$Na_2S_2O_3$ solution was added to the sample to neutralize the acid. The distillation was continued until approximately 25 ml of the distillate was collected for titration.

A 0.01 N HCl standardized solution was used to titrate the distilled solution.

Along with the samples, a blank in which everything except the sample was included was run along with the sample to correct for any nitrogen that might have been present in the chemicals.

A correction factor of 6.25 was used in which it was assumed that the protein component of corn stover is 16% (wt/wt) nitrogen.

Calculation:

Eq. 11 % Protein= (C)*(V/W)*(MW of N)*100*CF

where: C= Concentration of HCl (moles/liter)

V= Volume of HCl (ml)

W= Weight of dry sample (g)

CF =Correction factor for nitrogen content in the protein or

100/16 = 6.25

MW= molecular weight of nitrogen (14 g/mole)

Extractives Interference Analysis:

Glucose Supplement:

Two sets of native and 95% ethanol extracted samples were analyzed for glucose according to the method described in the "Two Stage Sulfuric Acid Hydrolysis for the Determination of Carbohydrates" section. In one set, a known amount of pure glucose was added to each sample as a supplement. The theoretical amount of glucose present in each sample, with and without glucose supplement, was calculated using the glucose value

from the native substrate. A comparison was made between the theoretical value and the actual value of glucose measured.

Eq. 12 Theoretical glucose present (mg)=

[(% GLU)*(TS/1-(%extractives/100)]+supplement

where %GLU is the % of glucose in native substrate and TS as defined in equation 3.

Xylose Supplement:

Two sets of native and 95% ethanol extracted samples were analyzed for xylose according to the "Two Stage Sulfuric Acid Hydrolysis for the Determination of Carbohydrates" section. In one set, a known amount of pure xylose was added as a supplement. The theoretical amount of xylose present in each sample, with and without xylose supplement, was calculated using the xylose value from the native substrate. A comparison was made between the theoretical value and the actual value of xylose measured.

Eq. 13 Theoretical xylose present (mg)=

[(% XYL)*(TS/1-(%extractives/100)]+supplement

where %XYL is the % of xylose in native substrate and TS as defined in equation 3.

Klason Lignin Supplement:

Two sets of native and 95% ethanol extracted samples were analyzed for Klason lignin content. In one set of the samples, a known amount of lignin was added as a supplement. The lignin sample for use as the supplement was prepared from native corn

stover without ashing. The acid insoluble ash content in the lignin was determined separately to correct for the amount of total lignin present in the supplemented lignin. The theoretical amount of Klason lignin present in each of the samples, with and without the lignin supplement, was calculated using the lignin value from the native substrate. A comparison was made between the theoretical weight and the actual weight of lignin measured.

Eq. 14 Theoretical lignin present (mg)=

[(% KL)*(TS/1-(%extractives/100)]+supplement

where %KL is the % of Klason lignin in native substrate and TS as defined in equation 3.

Sample Size:

The mean values for the individual components of the native feedstock were based on the following number of independent analyses: Klason lignin, 10; acid soluble lignin, 14; glycans, 14; ash, 18; protein 5; uronic acids, 2; and acetyl groups, 2.

Mean values for the glycans, Klason lignin, acid soluble lignin, and ash of the ethanol extracted corn stover are based on the analyses of four independent ethanol extracted preparations. Protein, uronic acids, and acetyl group values are based on the analyses of two of the ethanol preparations.

Mean values for the individual components in hot water extracted corn stover are based on the analysis of two independent hot water extracted preparations.

Statistical Analysis:

Statistical analysis of the mean values was accomplished using ANOVA on Statgraphics software.

RESULTS AND DISCUSSION

Mass Balance

For this study, an attempt was made to characterize the cell wall composition of corn stover feedstock. Components analyzed included: glucan, xylan, galactan, arabinan, mannan, Klason lignin, acid-soluble lignin, ash, protein, acetyl group, uronic acids, and extractives. Composition of the feedstock was determined following three sample preparations. The three protocols were a) native substrate-no extraction, b) 95% ethanol extracted substrate, and c) hot water extracted sample. High temperature-dilute acid pretreated substrates, whose hemicellulose fractions were largely removed, were also analyzed.

The compositional analysis of native, ethanol extracted, and water extracted corn stover is shown in Table 1. All of the values in Table 1 are expressed in terms of the unextracted native feedstock. The ethanol extracted sample and the water extracted sample were analyzed following the solvent extractions. Therefore, the extraneous substances were removed, making them "extractive-free" samples. The values determined on the extractive-free feedstocks were expressed in terms of the unextracted starting material by accounting for the weight loss of the feedstock during the solvent extractions. The composition of the two extractive-free samples are also given in Table 2. The values in Table 2 are reported on an extractives free basis (i.e. in terms of the weight percent of the extracted sample). The values in Table 1 are the easiest to use when comparing feedstocks for use in biomass-to-ethanol schemes since they provide a summative measure of the actual material that will go into the reactor. That is because the extractives are not

Table 1

Compositional analysis for native, 95% ethanol extracted, and hot water extracted corn stover.
(All results are expressed on the basis of dry, unextracted, native feedstock)

Component % (wt/wt)	Native Substrate*	95% Ethanol	Hot Water
Total Glycans	58.99 [a]	58.18 [a]	52.83 [b]
	(0.16)	(0.46)	(0.23)
Klason Lignin	18.60 [a]	16.56 [b]	15.67 [b]
	(0.22)	(0.09)	(0.31)
Acid Soluble	2.49 [a]	1.71 [b]	1.18 [b]
Lignin	(0.10)	(0.14)	(0.01)
Ash	10.58 [a]	9.23 [a]	5.38 [b]
	(0.26)	(1.00)	(0.29)
Protein	4.19 [a]	4.48 [a]	2.81 [b]
	(0.19)	(0.32)	(0.15)
Uronic Acids	1.97 [a]	2.09 [a]	1.62 [a]
	(0.25)	(0.01)	(0.11)
Acetyl	1.47 [a]	0.25 [b]	0.62 [c]
Group	(0.04)	(0.01)	(0.11)
Extractives	NA	4.88	17.18
		(0.04)	(0.16)
Total:	98.29	97.38	97.29

NA = not applicable
Values in parentheses are standard errors of the mean.
*No extraction
[a, b, c] Mean values in the same horizontal row with different superscript letters were significantly different ($P<0.05$).

removed prior to a feedstock being used in biomass-to-ethanol processes.

Considering the two solvents used in this study, extraction with hot water resulted in a higher percent extractives than extraction with 95% ethanol (17.18% vs. 4.88%, respectively). This difference points out that the extractives content of the feedstock was dependent on the solvent used for the extraction and, hence, extraction conditions must be explicitly defined when an extractives value is presented. In the biomass-to-ethanol studies the actual extractives content of a feedstock is of less interest than the content of the structural components. Consequently, the main reason for removing the extractives of a feedstock is to improve subsequent analyses, particularly the analysis of cellulose, xylan and lignin. The summed components in this study, indicated as "Total" in Table 1, ranged from a low of 97.29% to a high of 98.29%. These summations were essentially the same, irregardless of whether or not the feedstock had been extracted. The indication being that simple extractions do not necessarily improve mass balance numbers when working with corn stover feedstocks.

Table 3 shows the individual glycan content of corn stover. With the exception of glucan, the carbohydrate content of the native substrate displayed no statistical difference from the extracted substrates. Hot water extractions did remove a significant amount of glucan, the result being that the measured glucan content of the water-extracted sample was significantly lower than that of the native substrate. Samples extracted with 95% ethanol showed a decrease in the glucan value also, but not to the extent that it is significantly different from the native substrate. The ethanol extracted value is not significantly different from either of the native or the hot water extracted samples.

Table 2

Compositional analysis of extractive-free corn stover after 95% ethanol and hot water extraction.
(All results are expressed on dry weight basis)

Component (% weight/weight)	Extraction Solvent	
	95% Ethanol	Hot Water
Total Glycans	60.05 (0.13)	63.80 (0.28)
Glucan	35.58	38.38
Xylan	20.25	21.17
Galactan	1.35	1.18
Arabinan	2.87	3.07
Mannan	0.00	0.00
Klason Lignin	17.42 (0.19)	18.92 (0.53)
Acid soluble lignin	1.81 (0.30)	1.40 (0.02)
Ash	9.70 (2.10)	6.50 (0.49)
Protein	4.72 (0.47)	3.39 (0.23)
Uronic Acids	2.20 (0.02)	1.96 (0.18)
Acetyl Group	0.26 (0.02)	0.75 (0.20)
Total	96.16	96.72

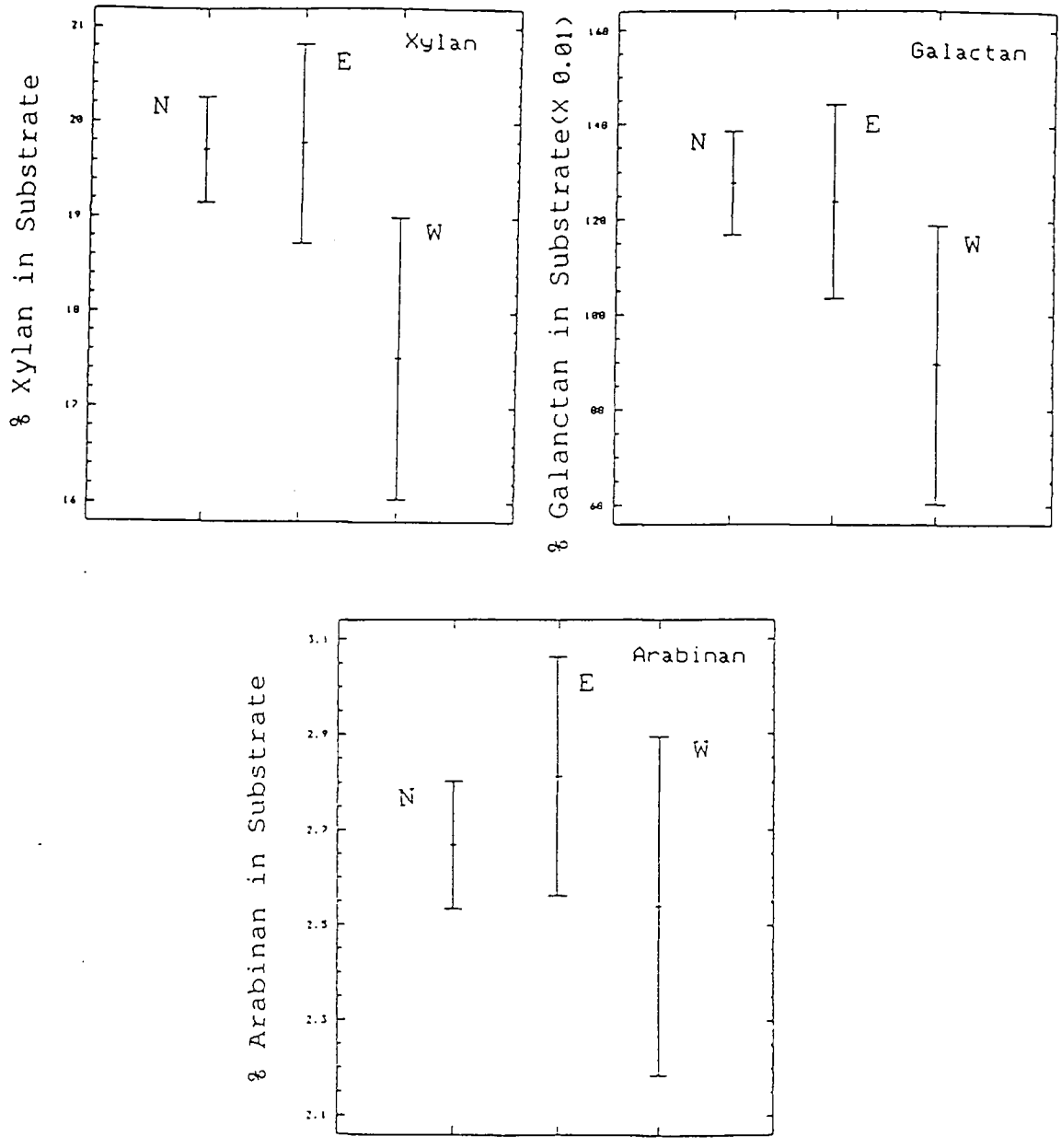
Values in parenthesis are standard deviations of the mean

The values obtained for the three corn stover substrates are in the same range as those reported for other plant materials (Table 4) (Sitton et al., 1979: Tsoumis. 1968: Clausen and Gaddy, 1983; Madan et al., 1983). The component occurring in largest quantity is cellulose, 32-35%, which is analyzed as glucan, followed by hemicellulose or xylan, 18-20%, and Klason lignin, 16-19%.

From tables 1 and 3, it can be seen that total glycan was not affected by ethanol extraction, but was affected by hot water extraction. Statistical analysis of the glucan values for native, ethanol extracted, and water extracted corn stover in Table 1 show there is a significant difference in the glucan content between hot water extracted fraction and the other two samples. Up to 10% of glucan was removed from the substrate via hot water extraction. Browning (1967) had reported that prolonged water extraction can result in conditions similar to that of dilute acid hydrolysis. The pH of the hydrolysate of the water extraction solution was checked with a pH indicator paper and was between pH 5 and pH 6. The pH of the hydrolysate is not low enough to deem it acidic; but nonetheless, more compounds become solubilized during hot water extraction as seen by the decrease in the other glycan values. One explanation may be that in corn stover there is more water soluble than ethanol soluble carbohydrate. This is also indicated by the decrease in the other glycan compositions. Although there was no statistical significant difference in the xylan, galactan, and arabinan content, there was a decrease in all three values from the native to the water extracted substrates (Figure 1). In fact, water extraction resulted in a more extensive extraction of the native substrate than ethanol extraction, as shown by the 3.5 times greater extractives content (Table 1). Except for

Figure 1

95% Confidence interval for the means of xylan, galactan, and arabinan contents (wt/wt) in native, 95% ethanol extracted, and hot water extracted corn stover.



Where N denotes native substrate, E denotes 95% ethanol extracted, W denotes hot water extracted substrate.

Table 3

Glycan composition of native corn stover and solvent-extracted corn stover.
(All results are expressed on the basis of unextracted, dry, raw substrate.)

Component %(wt/wt)	Native Substrate	95% Ethanol	Hot Water
Glucan	35.25[a] (0.45)	34.27[ab] (0.57)	31.79[b] (0.32)
Xylan	19.71[a] (0.40)	19.78[a] (0.66)	17.52[a] (0.04)
Galactan	1.36[a] (0.08)	1.32[a] (0.12)	0.98[a] (0.04)
Arabinan	2.67[a] (0.07)	2.81[a] (0.28)	2.54[a] (0.04)
Mannan	0.00[a] (0.0)	0.00[a] (0.0)	0.00[a] (0.0)
Total	58.99[a]	58.18[a]	52.83[b]

Values in parentheses are standard errors of the mean
[a], [b] Mean values in the same horizontal row with different superscript letters were significantly different ($P<0.05$).

Table 4

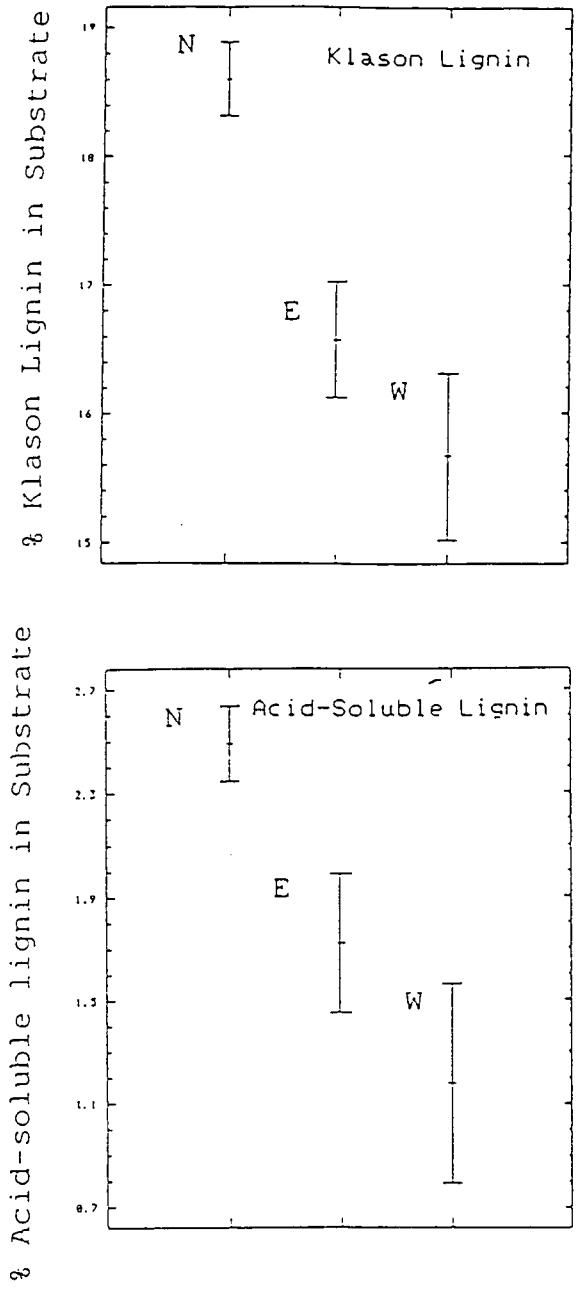
Composition of plant materials from other studies.
(Unless stated otherwise, composition is expressed on dry, unextracted substrate.)

	Researcher			
	Sitton, et al., (1979)	Tsoumis (1968)	Clausen and Gaddy (1983)	*Madan, et al., (1983)
Component % (wt/wt)				
Cellulose	35%	40-45%	30-45%	40-50%
Hemicellulose	15%	15-35%	15-25%	20-35%
Lignin		17-25%	5-15%	15-35%
Extractives				3-10%

*Solvent extracted sample.

Figure 2

95% Confidence interval for the means of Klason lignin and acid-soluble lignin contents (wt/wt) in native, 95% ethanol extracted, and hot water extracted corn stover.



Where N denotes native substrate, E denotes 95% ethanol extracted, W denotes hot water extracted substrate.

uronic acids, up to 50% of ash, protein, and the acetyl group in the native substrate were extracted by water.

Klason lignin and acid soluble lignin values for both solvent extractions were significantly lower from that of native substrate (Figure 2). There are numerous reports stating that residues measuring as Klason lignin are not exclusively lignin, but may include components such as condensed tannins and proteins (Wise and Ratliff, 1941; Browning, 1967; Smelstorius, 1971; Torget et al., 1990). These compounds can condense or precipitate during the strong acid treatments used in the Klason lignin assay. The result of these impurities is an erroneously high value for lignin. The level of Klason lignin in the two pre-extracted substrates was not significantly different, giving the indication that the amount of extractives from both preparations that can be measured as lignin were the same even though the extractives content from water extraction was over 3 times higher than that from ethanol extraction. In addition, direct analysis of the extractives from ethanol extraction indicated that some fractions of the extractives can be analyzed as "apparent" lignin (Table 5). It is noted that the fraction was analyzed as "apparent" lignin and not as the actual lignin because by definition, lignin is insoluble in organic or neutral solvents and therefore should not have been solubilized or isolated along with the extractives (Wayman and Parekh, 1990). Table 5 shows that almost half of the extractives could contribute to the Klason lignin value in native corn stover. When calculated back to the native substrate, the apparent lignin in the extractives is equivalent to approximately 2.4% of the lignin in the native substrate. The difference in Klason lignin values between native substrate and ethanol extracted substrate is about 2% (Table 1). This loss of two percent may be accounted for by the 2.4% of the apparent lignin in the extractives. It is

assumed that the Klason lignin value associated with analysis of extracted substrate more accurately reflects the true lignin content of the feedstock. The higher lignin value in the native substrate is caused by some extractives that can condense/precipitate with the lignin during the acid treatment of the Klason lignin procedure (Wise and Ratliff, 1941; Browning, 1967; Smelstorius, 1971).

The decrease in the ash as well as total glycan contents can also be due to extractives interference. Through ethanol extraction, about 13% of the total ash was lost. From the analysis of extractives, about 34% of the extractives could be analyzed as ash, this is equivalent to 1.6% ash in the native substrate or 15% of the total ash. Similarly, the 0.24% of total glycan in the extractives could make up for some of the total glycan that was lost as a result of ethanol extraction (Table 1). Thus, from the analysis of extractives, it could be assumed that the majority of what is "lost" in the extraction procedure could be accounted for by analyzing the extractives.

The mass balance closure was not greatly affected by pre-extraction of the substrate. This lack of improvement can be explained by the fact that a high percentage of the extractives are measured by the assays that are included in the mass closure. These compounds are measured either as extractives or as individual compounds (such as Klason lignin in Table 5).

From this observation, it ws of interest to see whether the extractives were physically or chemically altered during the isolation procedure. If such a change had occurred then the components observed in the extractives may be different from the endogenous extractives. Thus, the apparent lignin in the extractives may have been an artifact of a chemical or physical change. To show that what was analyzed on the

Table 5

Compositional analysis of extractives obtained from corn stover from a 95% ethanol extraction.
(All results are expressed on dry weight basis)

Component (% weight/weight)	Extractives	% Equivalent in native substrate	% of total component in native substrate
Total Glycans	2.70	0.14	0.24
Glucan	2.08	0.10	0.28
Xylan	0.32	0.02	0.10
Galactan	0.21	0.01	0.74
Arabinan	0.09	0.01	0.37
Mannan	0.00	0.00	0.00
*Klason Lignin	48.29 (0.07)	2.36	12.69
Acid Soluble Lignin	5.71 (0.07)	0.28	11.24
Ash	33.67 (0.78)	1.64	15.50
Protein	5.14 (0.18)	0.25	5.97
Total:	95.51	4.67	NA

* Apparent Klason lignin, may include components of extractives that have condensed in acid during the determination of lignin.
Values in parentheses are standard errors of the mean.
NA= not applicable

extractives was actually from the extractives isolated from the native substrate and not a result of a chemical change, and that what was lost during the extraction could be retrieved in the extractives, an experiment was set up to test the behavior of the extractives after isolation. In this experiment, a sample was pre-extracted and the extractives added back to the pre-extracted sample. Thus, theoretically, all the extractives that were extracted should have remained in the solid mixture. If no chemical reaction took place other than solubilization and desolubilization of the extractives, the solid plus extractives mixture should show chemical composition similar to that of unextracted, native corn stover.

Table 6 shows a comparison of the chemical composition of native corn stover and an ethanol-extracted corn stover to which the extractives have been added back. For the most part, the compositions are the same. There was no net loss in the total mass balance. Klason lignin, acid soluble lignin, protein, ash, and uronic acids showed no effect. Those extraneous compounds that contributed to lignin content in the native corn stover retained their properties and continued to contribute the same effect in lignin determination. Thus, the apparent Klason lignin found in the extractives in Table 5 was actually from the extractives and not a result of chemical or structural reactions that changed the properties of the extractives during the process of extraction and evaporation. In a separate experiment, the extractives were re-extracted to determine if their solubility properties had changed during their extraction and subsequent drying. Approximately 94% of the extracted, dried extractives could be re-extracted with 95% ethanol. Based on this result, we can detect no major changes in the chemical or physical properties of the extractives as a result of the extraction and preparation methods used in this study. It is possible that the

Table 6

Comparison of native corn stover and ethanol extracted corn stover with extractives added back.
(All results are expressed on dry weight basis)

Component (% weight/weight)	Native Substrate*	Extracted with Extractives
Total Glycan	58.99 (0.16)	59.07 (0.17)
Glucan	35.25	35.85 (0.25)
Xylan	19.71	20.82 (0.23)
Galactan	1.36	0.00 (0.00)
Arabinan	2.67	2.40 (0.06)
Mannan	0.00	0.00 (0.00)
Klason Lignin	18.60 (0.22)	19.11 (0.17)
Acid Soluble Lignin	2.49 (0.10)	2.58 (0.06)
Ash	10.58 (0.26)	11.07 (0.26)
Protein	4.19 (0.19)	4.04 (0.20)
Uronic Acids	1.97 (0.25)	2.07 (0.01)
Acetyl Group	1.47 (0.04)	0.76 (0.24)
Total:	98.29	98.70

* No extraction
Values in parentheses are standard errors of the mean.

small loss of galactan reported in Table 6 may still be explained by alterations in the extractives content. However, this is not likely since the cellulose and the xylan contents were not affected.

The chief difference between the two samples was the amount of acetyl group present. More than twice as much acetyl group was found in the native corn stover. Although the two values are different, the difference does not necessarily imply that the acetyl group analysis or the extraction technique were in error or that a reaction occurred that resulted in an alteration of the acetyl group moiety such that it could no longer be detected. Because acetic acid is volatile, some of it might have been lost during the process of evaporation of the ethanol during the preparation of the sample (Karr et al., 1991). Acetyl group determination was achieved by quantifying acetic acid and adjusting the value obtained using a correction factor of 43/60 = 0.72 or (MW compound - MW water)/(MW compound) to account for the water of hydrolysis. Thus, it is more likely that the lower recovery of acetyl group was a result of a loss due to evaporation and not from a change in chemical composition. Despite the loss of some acetyl group, the total mass balance of the two samples are nearly the same.

Hot Water Extraction vs. 95% Ethanol Extraction:

The data of Table 1 indicates that hot water extractions may improve the characterization of carbohydrates in corn stover for some purposes. Extractions with hot water removed relatively higher amounts of glucans than those with ethanol. The extracted glucans would normally be interpreted analytically as cellulose. These glucans

were not actually cellulose because cellulose is not soluble in water. Thus, glucan values measured from hot water extracted samples are likely to be more accurate estimates of the actual cellulose content of the feedstock than values obtained from native or ethanol extracted samples. The xylan content of water extracted sample is expected to be lower than that of the ethanol extracted preparation because of the higher solubility of neutral polysaccharides in water. The results in Table 3 are in agreement with this, more carbohydrate was extracted with hot water than with ethanol. Hot water seemed to be as effective at removing lignin impurities as ethanol - based on the similarities in the Klason lignin values for the two extracted samples.

In addition to solubilization of polysaccharides, hot water extraction also solubilized ash, protein, and the acetyl group, as shown by a decrease in their compositions in Table 1. Extractives that interfered with the Klason lignin assay of native feedstocks were removed from the feedstock by both of these extractions, as shown by the lower lignin contents, from 18.6% to 15.7%, of the extracted samples. For Klason lignin and acid soluble lignin, hot water extraction achieved the same purpose and the same efficiency as 95% ethanol in removing interfering extractives. Statistical data showed no significant difference between Klason lignin and acid-soluble lignin measured on hot water-extracted and 95% ethanol-extracted samples. Thus, if an accurate determination of the lignin and cellulose content of the feedstock is of primary interest, then a simple hot water extraction may be applied instead of the 95% ethanol extraction. This is significant because the ethanol extractions are more time consuming and more expensive. The draw back to the water extraction is that aside from removing extractives that interfered with lignin determination and neutral polysaccharides, it also dissolves a large portion of the

ash, protein, uronic acids, and the acetyl group (Figure 3). Water extracted samples would not be appropriate for the determination of the total quantity of carbohydrate in a native feedstock since a significant fraction of the glycans are extracted with hot water and, thus, would not be measured.

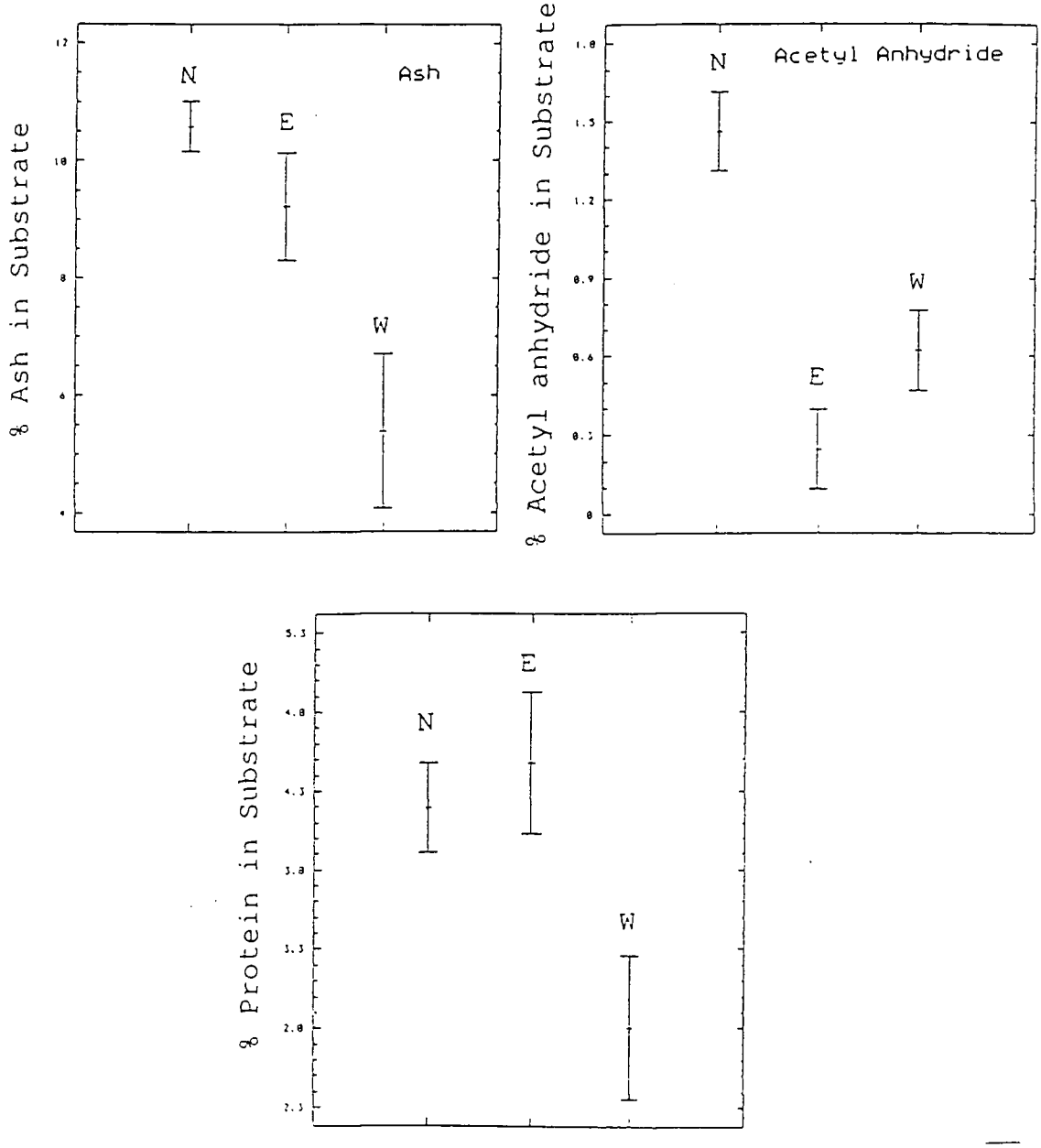
Estimation of the Components

To further illustrate the argument that total glycan determinations do not require pre-extraction, experiments were conducted in which the composition of the native substrate was used to estimate the glycan present in pre-extracted sample. The estimate was tested for its accuracy.

In one experiment, two samples, native and ethanol-extracted substrates, were analyzed for the glucose content. In another set of native and ethanol-extracted samples, a known amount of pure glucose was added as a supplement. The theoretical amount of glucose present in each sample, with or without glucose supplement (calculated using the glucose value from the native substrate), was compared to the amount of glucose actually measured. The results are shown in Table 7. The values under column A represent the theoretical glucose that should have been in the sample vessel. Values under column B indicated the amount of glucose actually measured in the sample vessel. A large ratio of A/B, greater than 100%, would indicate an overestimation of glucose content originally. A ratio of less than 100% would indicate an underestimation. The results in Table 7 show the ratio to be within $100 \pm 2\%$ Thus, it is concluded that the total theoretical values

Figure 3

95% Confidence interval for the means of ash, protein, and acetyl anhydride contents (wt/wt) in native, 95% ethanol extracted, and hot water extracted corn stover.



Where N denotes native substrate, E denotes 95% ethanol extracted, W denotes hot water extracted substrate.

were essentially accurately estimated. This indicates that the native corn stover value can be used to estimate the glucose value in the pre-extracted samples.

In another experiment the same test was applied using the xylose content. The results, shown in Table 8, were similar to those obtained for glucose. The ratios of theoretical values estimated using xylose from native substrate over the actual xylose measured were all close to 100%, suggesting the theoretical values were nearly accurate. Extractives did not interfere with the analysis of xylan in the native substrate to a great extent. The standard error of the mean was 0.74% for the xylan determination. Taking this into consideration, the ratio was not significantly different from 100% at a 95% confidence interval. Thus, the two sugar supplementation experiments further support the argument that the extractives present in native corn stover do not interfere with the determination of the glycan components of the feedstock.

On the other hand, a similar experiment testing the value for the Klason lignin suggested that the presence of extractives causes an overestimation of Klason lignin content. The same experimental scheme was used and the results are shown in Table 9. Lignin sample for use as the supplement was prepared from native corn stover without ashing. The acid insoluble ash content in the lignin was determined separately to correct for the amount of total lignin present in the supplemented lignin. Approximately 23% of the lignin preparation consisted of ash (data not shown). Ratios of the theoretical values over the measured lignin in the extracted corn stover showed the theoretical lignin values had been overestimated. Thus, lignin values obtained by assaying the native corn stover were significantly higher than those obtained by assaying the ethanol extracted samples. It is believed that extractives in the native substrate precipitate and erroneously measure as

Table 7

Estimation and recovery of glucose content using native corn stover value of glucose.

Sample Weight (Dry Weight)	A Theoretical Total Glucose (mg)	B Measured Glucose (mg)	Percent Recovery of Theoretical Glucose
(N) 284.8 mg	398.3[1]	398.3	---
(N+G) 280.0 + 36.4 mg	147.9[2]	144.9	102.1
(N+G) 285.2 + 32.3 mg	145.9[2]	142.6	102.3
(E) 300.1 mg	119.5[3]	122.3	97.7
(E) 301.8 mg	120.2[3]	122.7	98.0
(E+G) 299.2 + 51.0 mg	170.2[2]	171.2	99.4
(E+G) 301.6 + 49.9 mg	170.0[2]	171.8	98.05

[1] = Value based on % glucose in native corn stover per 1 gram sample.
[2] = Values based on % glucose in native corn stover plus supplemented glucose.
[3] = Values based on % glucose in native corn stover.
(N) and (N+G) = Native corn stover and native corn stover plus pure glucose.
(E) and (E+G) = Ethanol-extracted and ethanol extracted substrate plus pure glucose.

Table 8

Estimation and recovery of xylose content using native corn stover value of xylose.

Sample Weight (Dry Weight)	A Theoretical Total Xylose (mg)	B Measured Xylose (mg)	Percent Recovery of Theoretieal Xylose
(N) 297.9 mg	256.3[1]	256.3	---
(N+X) 296.0 + 45.7 mg	121.6[2]	125.0	97.3
(N+X) 296.8 + 39.3 mg	115.4[2]	119.0	97.0
(E) 306.2 mg	78.5[3]	78.9	99.5
(E) 307.4 mg	78.8[3]	79.6	99.0
(E+X) 302.7 + 47.5 mg	125.1[2]	126.7	98.7
(E+X) 302.9 + 41.8 mg	119.4[2]	121.0	98.7

[1] = Value based on % xylose in native corn stover per 1 gram sample.
[2] = Values based on % xylose in native corn stover plus supplemented xylose.
[3] = Values based on % xylose in native eorn stover.
(N) and (N+X) = Native corn stover and native corn stover plus pure xylose.
(E) and (E+X) = Ethanol-extracted and ethanol extraeted substrate plus pure xylose.

Table 9

Estimation and recovery of Klason lignin content using native corn stover value of Klason lignin.

Sample Weight (Dry Weight)	A Theoretical Total Lignin (mg)	B Measured Lignin (mg)	Percent Recovery of Theoretical lignin	Percent Recovery of Added Lignin
(N) 1.0151 g	180.9[1]	180.9	---	---
(N+L) 1.0280 + 0.1251 g	311.1[2]	296.8	104.8	88.60
(N+L) 1.0205 + 0.1040 g	288.6[2]	287.3	100.5	98.74
(E) 1.0329 g	196.4[3]	183.2	107.21	---
(E) 1.0053 g	191.2[3]	175.5	108.9	---
(E+L) 1.0100 + 0.0986 g	290.7[2]	270.8	107.3	94.36
(E+L) 1.1594 + 0.0973 g	307.0[2]	295.1	104.0	93.57

[1] = Value based on % lignin in native corn stover per 1 gram sample.
[2] = Values based on % lignin in native corn stover plus supplemented lignin.
[3] = Values based on % lignin in native corn stover.
(N) and (N+L) = Native corn stover and native corn stover plus lignin.
(E) and (E+L) = Ethanol-extracted and ethanol extracted substrate plus lignin.

lignin. These extraneous components should be removed from the substrate so that the quantification will be more accurate.

In addition, this experiment showed that endogenous extractives did not affect analysis of supplemental Klason lignin. This is illustrated by the percent recovery of the supplemental lignin when it was added to either native or extracted corn stover. The average percent of recovery for both cases is about 94%. Less than 100% of recovery is expected. This is consistent with previous reports of lignin degradation upon reklasonation (Karr and Brink, 1991A).

Pretreated Corn Stover

The main purpose of dilute acid pretreatment of the feedstock is to hydrolyze the hemicellulose and to increase enzyme accessibility to the cellulose fraction. Efficiency of the pretreatment process is determined by the amount of xylan remaining in the solid sample, which reflects the extent of hemicellulose hydrolysis. Low xylan remaining per weight of pretreated substrate indicates a near complete hydrolysis. A large quantity of xylan remaining would brand the pretreatment condition inefficient. The quantity of cellulose fraction remaining is also important.

If, in attempting to hydrolyze a large portion of the hemicellulose, a large portion of the cellulose is also hydrolyzed then the pretreatment is counter-productive. The pretreatment process adds to the cost of the biomass-to-ethanol conversion process. It is crucial that pretreatment conditions be selected such that removal of hemicellulose is maximized but hydrolysis of cellulose is minimized.

In this study, two pretreatment conditions were selected in which one resulted in an extensive hydrolysis and the other in minimal hydrolysis of the hemicellulose fraction. The conditions chosen were: 180 °C, 0.9% (wt/wt) sulfuric acid, and 1.4 minutes ---a harsh condition which hydrolyzes much of the hemicellulose; and 140 °C, 0.6% (wt/wt) sulfuric acid, and 1.0 minute---a mild condition. Results of the carbohydrate and lignin determinations are in Tables 10 and 11.

For the harsh condition, more than 90% per weight of the xylan backbone was hydrolyzed when compared to the original native substrate. The glucan fraction increased by about 12% per unit weight. Thus, for every 1 kilogram of pretreated sample of this condition, almost 500 grams was cellulose.

The data discussed previously with regard to the native feedstock clearly shows that solvent extractions can affect subsequent chemical analyses of a native feedstock. Solvent extractions are generally not done on pretreated feedstocks. However, to our knowledge, no experimental data has been published showing the consequences of this. It is not clear what happens to the extractives during the pretreatment of a feedstock. No reports could be found that investigated the extent that these extractives become insoluble or condense with the lignin under pretreatment conditions. Therefore, the lignin content of pretreated substrates may include extraneous substances.

An ethanol extraction of the pretreated sample was done to determine whether there was any extractives remaining and if so, whether they affected lignin and glycan quantification. The pretreated sample contained about 5.8% (wt/wt) extractives (Table 10). The xylan content was not significantly different whether the sample was extracted or not. The glucan content, however was decreased after the sample was pre-extracted with

95% ethanol. The lower glucan content may be due to the solubilization of glucose molecules hydrolyzed from the amorphous regions of the cellulose during the pretreatment or the solubilization of the glucose moiety from the xylan side chain. It is unlikely that this discrepancy was due to the extractives because ethanol extraction did not show any interference in the native, untreated corn stover.

Klason lignin determination, on the other hand, was affected by the extraction procedure. Some ethanol soluble substance was present in the pretreated sample that later precipitated in the presence of acid during lignin determination. Thus, the pretreatment did not necessarily solubilize the extractives. Because the pretreatment involved the use of acid, some extraneous substances are thought to have precipitated and remained with the pretreated solid. During the ethanol extraction process, these substances were solubilized and removed as extractives, hence, the decrease in the lignin content after extraction.

A similar result was observed in the characterization of the pretreated sample from the mild condition (Table 11). Xylan content of the pretreated sample was unaffected by extraction but a decrease in glucan and lignin content was noted. It is believed that the reasoning used for the harsher condition could also be applied for the mildly pretreated sample. The decrease in the glucan value could be due to the solubilization of the side chain of the xylan backbone or glucose from the amorphous region of cellulose.

The mild pretreatment condition also did not solubilize the extractives. The extractives remaining in the solid precipitated in the presence of acid in the determination of Klason lignin. The ethanol-soluble extractives/lignin was removed in the extraction step and this resulted in the lower Klason lignin value.

Table 10

Cell wall polysaccharides of pretreated corn stover at 180 °C, 0.9 % acid, 1.4 min.
(Results expressed on a basis of dry pretreated sample)

Component (% weight/weight)	Unextracted	Ethanol Extraction
Total Glycans	53.40	49.20
glucan	47.74	44.71
xylan	4.55	4.49
galactan	0.44	0.00
arabinan	0.67	0.00
mannan	0.00	0.00
Klason lignin	25.83 (1.58)	18.89 (0.15)
Acid soluble lignin	1.13 (0.08)	0.83 (0.01)
Extractives	NA	5.78

NA= Not applicable
Values in parentheses are standard errors of the mean

Table 11

Cell wall polysaccharides of prctreatcd corn stover at 140 °C, 0.6 % acid, 1.0 min. (Results expressed on a basis of dry pretreated sample)

Component % (wcight/weight)	Unextracted	Ethanol Extraction
Total Glycans	61.49	58.31
glucan	40.88	38.77
xylan	20.15	19.11
galactan	0.00	0.00
arabinan	0.46	0.43
mannan	0.00	0.00
Klason lignin	21.74 (0.50)	18.29 (0.52)
Acid soluble lignin	1.74 (0.03)	1.46 (0.06)
Extractives	NA	5.03

NA= Not applicable

values of the parentheses arc standard errors of thc mean

From the preliminary results, it is noted that the pretreatment of the feedstock did not remove extraneous substances that interfered with Klason lignin determination. These substances could be removed by extracting the pretreated samples with 95% ethanol. The harsher treatment solubilized more of the extraneous substances. After extraction, the lignin content of the harsher treatment decreased by 25% as opposed to only an 11% decreased on the mildly treated sample. It seems plausible that the lignin fraction of a feedstock is altered to greater extents as the severity of the pretreatment increases (Grohmann et al., 1985). The altered lignin may have increased solubility in 95% ethanol.

Recommendation for Future Work:

Many researchers have reported the interference of extraneous substances in Klason lignin determination. This study confirms those reports. Many suggestions have been made to extract the native feedstocks or other plant materials prior to chemical analysis of the cell wall structure. Although pre-extraction would clearly provide a more thorough and accurate picture of the cell wall structure, for purposes of quantifying total glycan content, protein, ash, and organic acids, extraction is unnecessary. Pre-extraction becomes more important when the cellulose and lignin determinations are involved. Thus, for complete characterization where interest also includes lignin content, pre-extraction is recommended. For that matter, hot water is as effective as 95% ethanol as the solvent of extraction. Not only is it inexpensive, it is nontoxic, and the extraction scheme can be completed in less time.

An alternative and conservative approach for the characterization of herbaceous

feedstocks in biomass-to-ethanol study is to include a solvent extraction step with subsequent analysis of the macrocomponents in both the pre-extracted solid as well as the isolated extractives. This will allow for a more accurate estimate of the cellulose and lignin in the substrate. Furthermore, this approach also allow for an estimation of the total theoretical carbohydrates available for microbial fermentation. Although this approach will require an increase in the number of assays, no new assay is needed because the assays for the determination of the macrocomponents can be applied to the extractives.

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