ABSTRACT

High-Performance Liquid Chromatographic Methods for Quantitative Assessment of Degradation Products and Extractives in Pretreated Lignocellulose

Shou-Feng Chen, Ph.D.

Mentor: C. Kevin Chambliss, Ph.D.

Long-term economic, national security, and environmental concerns have motivated research into renewable fuels from lignocellulosic biomass. Among energy alternatives, biomass-derived ethanol represents one of the more promising commodities for long-term sustainability of transportation fuels. Herbaceous agricultural residues, such as corn stover, represent a major source of lignocellulosic material with considerable potential for use in biomass-to-ethanol schemes.

Currently, the technology for conversion of biomass to ethanol involves dilute acid pretreatment of lignocellulosic materials, followed by enzymatic hydrolysis of cellulose and fermentation of monomeric sugars to produce ethanol. However, a variety of degradation products are produced during dilute acid pretreatment of lignocellulosic biomass, which exert an inhibitory effect on downstream fermentation processes and reduce bio-ethanol conversion. Thus there is an increased demand for reliable analytical methods to advance a better understanding of lignocellulose pretreatment.

Several liquid chromatographic methods are developed for a systematic analysis of various degradation products. High-performance liquid chromatography is the most widely used analytical separation technique, because of its reproducibility, sensitivity, and suitability for separating nonvolatile species, which makes the method ideal for accurate quantitative determinations. A reversed-phase HPLC method with UV detection is developed for simultaneous separation and quantitation of organic acids and neutral degradation products present in the corn stover hydrolysate. On the other hand, inorganic ions and some organic anions, which are present in the water extractive from corn stover, are separated and quantitated by a developed ion chromatographic method with conductivity detection. Sugars and alditols are also determined using high-performance anion chromatography with pulsed amperometric detection.

High-Performance Liquid Chromatographic Methods for Quantitative Assessment of Degradation Products and Extractives in Pretreated Lignocellulose

by

Shou-Feng Chen, B.S.

A Dissertation

Approved by the Department of Chemistry and Biochemistry

_____
David E. Pennington, Ph.D., Interim Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

Approved by the Dissertation Committee

_____
C. Kevin Chambliss, Ph.D., Chairperson

_____
Kenneth W. Busch, Ph.D.

_____
Stephen L. Gipson, Ph.D.

_____
Mary Lynn Trawick, Ph.D.

_____
Ian A. Gravagne, Ph.D.

Accepted by the Graduate School
December 2007

_____
J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Kevin Chambliss for his supervision, advice, encouragement, and patience with me during my graduate studies at Baylor. Thank Dr. Peter van Walsum for his guidance and help in making the research successful.

Special thanks must be extended to Dr. Richard Mowery and Dr. David E. Pennington. Dr. Mowery's knowledge of chemistry and chromatography is what made my research possible. Dr. David Pennington's encouragement is what made my staying for advanced chemistry dream come true. Additional thanks must also go to my committee members Dr. Kenneth Busch, Dr. Stephen Gipson, Dr. Mary Lynn Trawick, and Dr. Ian A. Gravagne for their time and effort to read my dissertation and improve it.

My appreciation also goes to the Department of Chemistry and Biochemistry, as well as Baylor University for allowing me to study here and for the financial support given to me. I also need to thank Sam, Winnie, and all my friends at Baylor.

My parents and family have always given me the utmost, unwavering support that I needed to complete this endeavor. I cannot express how much their love and encouragement has meant to me.

CHAPTER ONE

Renewable Energy from Biomass: Resource and Analytical Technology An Introduction

Alternative Energy from Biomass

Projected Depletion of the World's Petroleum Reserves

Energy consumption has increased steadily over the last century as the world population has grown, and as more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demands of both the United States and other world powers. In view of continuously rising petroleum costs and an impending peak of crude oil resources, as well as the imperative need to lower greenhouse gas emissions, there is escalating pressure worldwide to develop alternative, non-petroleum-based sources of energy.[1-3] Ethanol, produced through the fermentation of sugars, is one of the most important renewable fuels. It not only contributes to a decreased reliance on foreign supplies of petroleum but also reduces the negative environmental impacts that are generated by the worldwide utilization of fossil fuels.[4,5]

The United States interest in fuel ethanol has grown since the oil crises of the 1970s. Current technologies for fuel ethanol production from biological resources in the United States are based on the fermentation of sugar derived from corn starch. It has been used in gasohol or oxygenated fuels since the 1980s, where these gasoline fuels have been mixed with 10% ethanol by volume.[3,4] Production of fuel ethanol from corn starch has steadily increased from 1.5 billion gallons in 2001 to about 3.4 billion gallons in

2004.[6,7] Recently, U.S. automobile manufacturers have announced plans to produce a significant number of flexible-fuel vehicles which can use either gasoline or an ethanol blend-E85 (85% ethanol and 15% gasoline by volume). Using ethanol-blended fuel for automobiles can significantly reduce both petroleum use and greenhouse gas emissions originating from exhaust. As a result, demand for fuel ethanol is expected to increase.[3-5]

Technology resulting in commodity-scale production of ethanol will not likely rely heavily on dry grain mills that employ corn as a starting material. A dramatic increase in ethanol production using current corn–based technology is not practical because corn production for ethanol will compete for the limited agricultural land needed for food and feed production. Also, an increased demand on food crops, such as corn, for production of fuel ethanol could push the price of grain upwards, leading to uneconomical grain-ethanol production.[4-7]

Alternative lignocellulosic materials are potentially capable of meeting the increased demand for ethanol production.[7] Materials such as wood, agricultural residues, grass, and waste paper are commonly referred to as biomass. Biomass is the most abundant renewable energy resource in the world. It is widely abundant as a byproduct of agricultural activities, industrial residues, and domestic wastes and is absent in the human food chain. For these reasons, biomass is perceived to be a relatively inexpensive, renewable feedstock capable of supporting the production of fuel ethanol on a large scale.[3-8]

Environmental Impacts of Bio-ethanol

Production of ethanol from biomass is one way to reduce both the consumption of crude oil and environmental pollution. Blending oxygenates, such as ethanol, in gasoline

is well-recognized to enhance the combustion of fuel. It also substantially reduces the emission of pollutant gases, such as carbon monoxide (CO), sulfur oxides (SO_x) and nitrogen oxides (NO_x), from automobiles.[3,4,9,10] Unlike gasoline, ethanol contains 35% oxygen that helps complete the combustion of fuel and reduces particulate (PM) emissions, which can pose a health hazard to living beings. Furthermore, while the reality of global warming continues to be discussed, the use of fuel ethanol from biomass will significantly reduce net carbon dioxide (CO_2) in the atmosphere because fermentation-derived ethanol is already part of the global carbon cycle, making bio-ethanol an environmentally beneficial energy source. In contrast, carbon dioxide produced from fossil fuel accumulates in the atmosphere, causing greenhouse gas effects which result in hazardous impacts on the environment.[3-5,10] Therefore, fuel ethanol from biomass has been the subject of intensive research in the United States. During the last two decades, technologies for ethanol production from biomass have been developed to the point that large scale production is becoming a reality.[8,9]

Biomass Resources for Ethanol Production

A variety of biomass can be used for near-term bio-ethanol production. Among biomass resources, agricultural residues dominate in terms of tonnage and can serve as renewable feedstocks for ethanol production. According to the United States Department of Agriculture (USDA), corn continues to be one of the major agricultural crops in the United States. The residual remains of the corn plant after the grain has been harvested (i.e., leaf, stalk, cob, etc.) are collectively referred to as corn stover. Corn stover is currently the most abundant agricultural residue available.[11,12] The amount of corn stover that can be sustainably collected on an annual basis is estimated to be 80-100 million dry

tons, a majority of which would be available for ethanol production. Additionally, corn stover has a high carbohydrate content (36.4% glucan, 18% xylan, 3% arabinan, 1% galactan, and 0.6% mannan) and low lignin composition (16.6%).[6,12] Therefore, corn stover is considered to be an excellent feedstock for biomass-to-ethanol conversion.

Biomass Composition

Lignocellulosic materials are heterogeneous complexes mainly composed of cellulose, hemicelluose and lignin. Cellulose, like starch, is a high-molecular-weight linear polymer composed of β-1,4-linked glucose units. However, unlike starch, cellulose favors the ordering of the polymer chains into tightly-packed, highly-crystalline structures that are insoluble in water and resistant to depolymerization. Hemicelluloses are branched polysaccharides, consisting of pentoses (*i.e.*, xylose and arabinose), hexoses (*i.e.*, mannose, glucose, and galactose) and uronic acids. Some of the side chains may also contain acetyl groups of ferulate, depending on the species. Hemicellulose is hydrogen-bonded to cellulose microfibrils, thus forming a network that provides the structural backbone to the plant cell wall. The presence of lignin imparts further strength and provides resistance against diseases.[13-16]

Lignin is an aromatic condensation polymer formed from phenylpropanoid precursors. Lignins are divided into three classes: namely, guaiacyl lignin, syringyl lignin, and *para*-hydroxyphenyl lignin. The terms *para*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) are used to denote the three types of aromatic rings in monomer residues, and the ratio of H/G/S units in lignin is highly dependent upon plant taxonomy. Guaiacyl lignins have a methoxy group attached to aromatic rings at the 3- position, whereas syringyl lignins have methoxy substituents at both the 3- and 5-carbon positions. Lignin

in herbaceous plants also contains *para*-hydroxycinnamic acids (*i.e.*, *para*-coumaric acid, ferulic acid, and sinapic acid).[14,15] Cellulose and hemicellulose are collectively composed of up to 70% carbohydrates (by mass) and thus, represent potential sources of fermentable sugars for ethanol production. The presence of lignin in the cell wall, however, impedes the ability of these polymers to be hydrolytically converted to monomeric sugars by either chemical or biological means.

Biomass Conversion

Extensive research has been performed on conversion of lignocellulosic materials to ethanol in the last two decades. While many process configurations are currently available for ethanol production, most involve three primary steps: pretreatment, enzymatic hydrolysis, and fermentation.[10] The primary purpose of pretreatment is to break down lignin and hemicellulose, thus improving cellulose accessibility to enzyme hydrolysis. Once the enzyme(s) degrade cellulose to glucose monomers, all simple sugars derived from lignocellulosic materials are fermented to ethanol. Unfortunately, most pretreatments not only improve enzymatic hydrolysis, but also produce a variety of alternative degradation products which are potentially inhibitory to downstream enzyme and/or microbial steps in the process. Minimizing the production and effect of inhibitory compounds is widely recognized as one of the two primary roadblocks currently prohibiting optimal efficiency in biomass-to-ethanol conversion. The other is native recalcitrance of cellulose, which is not addressed in experimental work described in this dissertation.

Pretreatment Processes

The pretreatment process is designed to break down biomass by solubilizing hemicellulose, reducing cellulose crystallinity, partially hydrolyzing carbohydrate polymers, and removing lignin. Pretreatment processes for lignocellulosic biomass can be loosely grouped into four categories: physical, biological, hydrothermal, and chemical.[17] Physical pretreatments, which typically demand large amounts of energy, employ chipping, grinding or ball milling to reduce the particle size of lignocellulosic materials, and thus, increase surface area. Biological pretreatments use microorganisms, such as brown-, white-, or soft-rot fungi, to degrade lignocellulosic biomass. The advantages of biological pretreatment include a lower energy requirement, as compared to physical pretreatment, and reduced environmental impacts. However, the rate of hydrolysis in most biological pretreatment processes is very slow.[4,18]

Hydrothermal (or liquid hot water) pretreatments of biomass involve using pressure (350 to 400 psi) to maintain water in the liquid state at elevated temperatures (160 to 200 ºC). This technique can ideally maximize the solubility of sugars from biomass with minimal degradation.[17,19] However, a disadvantage of this process is that a lignin is not effectively decomposed without adding an acid catalyst such as SO_2, thus limiting the efficiency of enzymatic hydrolysis.[20] Chemical pretreatments employ similar conditions to hydrothermal processes, but acids, alkalis, organic solvents, and/or oxidizing agents are typically added to improve sugar recovery. In recent years, dilute acid pretreatment has become one of the most well-studied and near-commercial technologies for biomass-to-ethanol conversion .[4,17-19]

Dilute-Acid Pretreatment

Dilute-acid pretreatment is typically carried out using a mineral acid, such as sulfuric or hydrochloric acid, at temperatures ranging from 120 to 200 ºC and reaction times ranging from seconds to minutes.[4] The advantages of using dilute-acid pretreatments are more effective dissolution of hemicellose and increased enzymatic digestibility of cellulose. Hydrolysis of lignocellulosic materials is typically carried out in two stages to maximize sugar yields. The first stage can be operated under milder conditions, which maximize sugar recovery from the more readily hydrolyzed hemicellulose fraction of biomass. The second stage is optimized at higher temperature to promote recovery and partial hydrolysis of the more recalcitrant cellulose fraction. Liquid hydrolyzates are recovered from each stage and subsequently fermented to alcohol. Residual cellulose and lignin remaining as solids in the hydrolysis reactors serve as boiler fuel for electricity or steam production.[18,19]

Scope of the Dissertation

The majority of experimental work reported in this dissertation has been directed at elucidating fundamental knowledge related to the production of non-carbohydrate degradation products in lignocellulose pretreatment. Recent work describing and modeling the kinetics of lignocellulose hydrolysis has focused almost exclusively on production and release of sugars.[21-24] As a result, relatively little was known about alternative degradation products in hydrolysates at the time this study was initiated. Because non-carbohydrate degradation products are potentially inhibitory to downstream enzymatic and/or microbial processing steps, there is increasing impetus to understand their origin and accumulation trends as a function of pretreatment chemistry. While the

long-term objective of our work is to develop a predictive understanding of biomass pretreatment, initial work was focused on addressing two fundamental questions:

1) What degradation products are formed during pretreatment?
2) How do their concentrations vary as a function of reaction time and reaction temperature?

The development of suitable analytical methodology for monitoring degradation products in pretreatment samples was a requisite first step in meeting stated objectives. The analysis of biomass hydrolysates imposes a formidable analytical challenge since the compounds present in these mixtures vary significantly in terms of molar mass, volatility, ionization state, acid-base properties, and reactivity. Relative concentrations of analytes also vary significantly within and between samples. Gas chromatography (GC) coupled with flame ionization or mass spectrometry detection has been effective in identifying a variety of lignocellulosic degradation products.[25,26] However, these investigations have been primarily qualitative rather than quantitative. Since many compounds of interest are relatively polar, derivatization with a suitable silylating agent is typically required prior to GC analysis. This often results in the presence of multiple peaks for each analyte and complicates quantitative interpretation of chromatographic data. For this reason, techniques employing liquid chromatography have become preferable for quantitative interrogation of hydrolysates. However, most LC analyses of pretreatment samples have suffered from poor chromatographic resolution. Additionally, the scope of most LC analyses has focused on a limited number of analytes, typically belonging to a single compound class (*e.g.*, monomeric sugars, aliphatic acids, furans, etc.)

In Chapter 2, the development of a single method supporting simultaneous analysis of aliphatic acids, aromatic acids, and neutral degradation products (*i.e.*, phenols,

furans, and aldehydes) in pretreatment samples is presented. An analytical extraction procedure was developed, enabling isolation of target compounds from a pretreatment liquor. Additionally, a reversed-phase high-performance liquid chromatography protocol was developed and validated, affording simultaneous separation and quantitative determination of 32 potential analytes with UV detection at 210 nm. The method was subsequently applied to quantify a variety of degradation products in a corn stover hydrolysate. These results have been reported previously in the primary literature (*Journal of Chromatography A* **2006**, *1104*, 54-61). This work represents the first example of simultaneous determination of degradation products having divergent physicochemical properties in a single chromatographic run.

With suitable analytical methodology in hand, the focus of experimental work shifted to provide an assessment of how degradation product concentrations in hydrolysates varied as a function of pretreatment chemistry. A better understanding of the behavior and degree of accumulation of compounds during pretreatment processes could guide the optimization of dilute-acid pretreatments and improve overall process efficiency for biomass-to-ethanol conversion. Lignocellulosic materials are heterogeneous, and the formation of degradation products depends on both the type of biomass utilized as feedstock and the pretreatment conditions. Beginning with Saeman in 1945 [27] and confirmed by many other groups,[28-32] sugar recovery from lignocellulosic materials has been modeled as a pseudo-first-order kinetic process for dilute-acid pretreatments of biomass. The severity factor, Ro (defined by Overend and Chronet as Ro = t*{e^[(T-100)/14.75])}, combines the experimental effects of reaction temperature (T in ºC) and reaction time (t in minutes) to enable comparison of results from different

pretreatments and to facilitate process control.[14,16] At the time our work was initiated, a correlation of the severity relationship with accumulation trends for non-carbohydrate degradation products was absent in literature. Nevertheless, the biomass community operated under the general assumption that the production of inhibitory degradation products was likely correlated with increased reaction severity.

In an effort to more fully understand the relationship between reaction severity and accumulation of non-carbohydrate degradation products in process streams, an experimental design was developed to assess the effect of independently varying reaction time and temperature at constant severity for low, moderate, and high pretreatment severity conditions. Concentrations of degradation products were determined using a reversed-phase high-performance liquid chromatographic method similar to that presented in Chapter 2. Analytical results were interpreted using first-order kinetic models of reaction severity, and this approach unequivocally demonstrated that the classic severity function is not appropriate for predicting accumulation of non-carbohydrate degradation products in lignocellulose pretreatment. These results are presented in Chapter 3.

The balance of experimental work reported in this dissertation was focused on compositional analysis of water-soluble materials present in native corn stover. "Extractives" is the term used to collectively describe the fraction of chemical components that can be recovered from lignocellulosic materials upon extraction with water or ethanol.[33-37] Prior to our study, little was known about the identity or relative concentrations of 'extractive' constituents. However, it had been postulated that components derived from the 'extractive' fraction of biomass were among the most

inhibitory compounds present in bioethanol process streams.[34,35] It had also been demonstrated that aqueous extraction of lignocellulosic materials prior to compositional analysis resulted in reduced compositions for cellulose, hemicellulose, and lignin relative to unextracted samples.[36] Since an overarching theme of this dissertation research was to provide analytical information on potential fermentation inhibitors, it seemed appropriate to include an assessment of aqueous 'extractives' in experimental work.

Chapter 4 describes an analytical study resulting in greater than 90% mass closure for water-soluble materials in 4 of 5 representative corn stover feedstocks. A variety of chromatographic techniques in combination with solid-phase and/or liquid-liquid extraction sample preparations were independently applied to quantify more than 30 previously unidentified constituents of aqueous extracts. Similar to hydrolysates, the composition of extracts consisted of chemicals with widely divergent physicochemical properties. The most significant observation resulting from this study was the discovery that water-soluble sugars represent a significant fraction of the dry weight of corn stover feedstocks. Accordingly, analytical data are interpreted not only in the context of potential microbial inhibition, but also in terms of their potential implications for technical and economic valuations of biomass processing, feedstock storage, and future analyses of feedstock composition. These results were also recently published in the primary literature (*Journal of Agricultural and Food Chemistry* **2007**, *55*, 5912-5918).

Brief Description of Relevant Chromatographic Techniques

High-performance liquid chromatography (HPLC) is a widely used analytical separation technique because of its reproducibility, sensitivity, and suitability for separating nonvolatile species, which makes the method ideal for accurate determination

of compounds derived from plant material. A series of methods utilizing HPLC in combination with three major detection modes was developed for quantitative analysis of target analytes in experimental work. The primary method used to assess lignocellulosic degradation products in pretreatment hydrolysates and aqueous extracts involved reversed-phase chromatography with UV detection. Ion chromatography, which involves the use of ion-exchange chromatography in combination with conductivity detection, was used to determine inorganic ions and select aliphatic acids in aqueous extracts, and carbohydrates and related alditols present in these samples were assessed using high-performance anion-exchange chromatography in combination with pulsed amperometric detection (HPAE-PAD). Each of these chromatographic techniques is discussed in more detail below to provide the reader with requisite background for rationalization of chromatographic behavior reported in Chapters 2-4.

Reversed-Phase High Performance Liquid Chromatography with UV Detection

Reversed-phase chromatography with UV detection is the most widely used liquid chromatography separation strategy. About 75% of all HPLC separations are performed with this approach.[38-43] The term reversed-phase arises from the fact that the separation mode utilizes a non-polar stationary phase with a polar mobile phase, which is the reverse of the situation in normal-phase chromatography.[44,45] In reversed-phase liquid chromatography, separations are based on differences in analyte partitioning between a hydrophobic stationary phase and a polar mobile phase. Analyte retention is dependent upon the gradient condition; in other words, the separation depends on the mobile phase properties of polarity and pH. As a general rule, retention increases with increasing size and/or hydrophobicity of the analytes, allowing polar molecules to elute more readily.

However, very polar compounds, such as low-molecular-weight aliphatic acids that easily ionize in water are poorly retained on the stationary phase and elute almost coincident with the solvent front.[44-47]

The retention of early-eluting acids can be increased by ion suppression, which is particularly useful in the separation of low-molecular-weight acidic compounds. This approach involves suppression of the ionization of these solutes by adding a buffer of appropriate pH to the mobile phase.[48] In this way, the solutes remain either neutral or only partially charged. As a result, retention on the non-polar stationary phase, which is generally governed by hydrophobic interactions, is increased and separation can be accomplished.

UV-visible absorption detectors are the most widely used detectors in liquid chromatography.[49-53] Since many organic compounds absorb to some extent in the UV, these detectors are somewhat universal in application. The detector response, however, depends on how strongly the sample absorbs light at a particular wavelength. The UV-visible absorption detector is operated as a concentration sensitive detector,[52] which provides an output directly related to the concentration of solute in the mobile phase passing through it.

The detector in chromatographic experiments operates on the same principles as a benchtop spectrophotometer.[50] The light source is typically a deuterium lamp, which provides acceptable light intensity from 190 to 400 nm. When measurements at visible wavelengths (400 to 700 nm) are required, a higher-energy tungsten-halide lamp is often used. Light from the lamp passes through a UV-transmitting flow cell connected to the column and strikes on a diode or a phototube that measures the light intensity (I). Usually,

light from the lamp is also directed to a reference diode for measurement of the initial light intensity I_0 (Figure 1.1). The detector electronics then convert the signal from the two diodes into absorbance A, which is transmitted to the data system:

$$A = \log (I_0/I)$$

Analyte concentration (c) in the flow cell is related to absorbance (A), analyte molar absorptivity (ε), and flow-cell path length (L) by the Beer-Lambert Law:

$$A = c\varepsilon L$$

Variable-wavelength detectors also include a means of selecting the wavelength used for detection.[50,53] This wavelength selection is normally achieved with a diffraction grating as illustrated in Figure 1.2. Light from the lamp enters the grating assembly through an entrance slit and is focused on the grating by mirror A. The orientation of the grating can be varied so as to direct monochromatic light of a selected wavelength onto a second mirror B, and from there to the exit slit. For variable wavelength detectors, the grating assembly is positioned between the lamp and the flow cell. Diode-array detectors have the grating assembly positioned after the flow cell, so that light of different wavelengths can be measured simultaneously with an array of sensing diodes as illustrated in Figure 1.3.

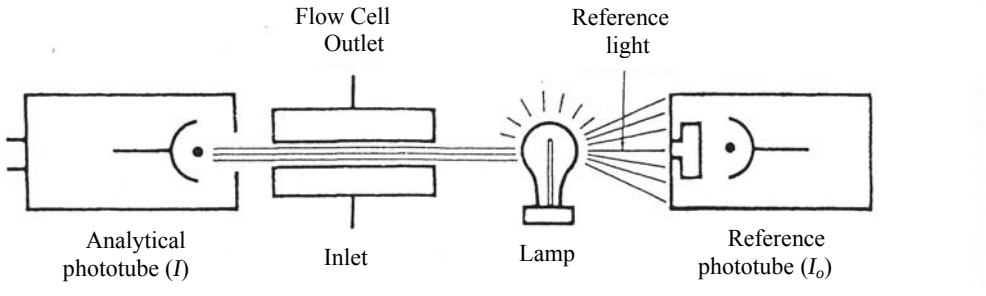


Figure 1.1. Schematic view of a UV detector (Figure adapted from reference 50).

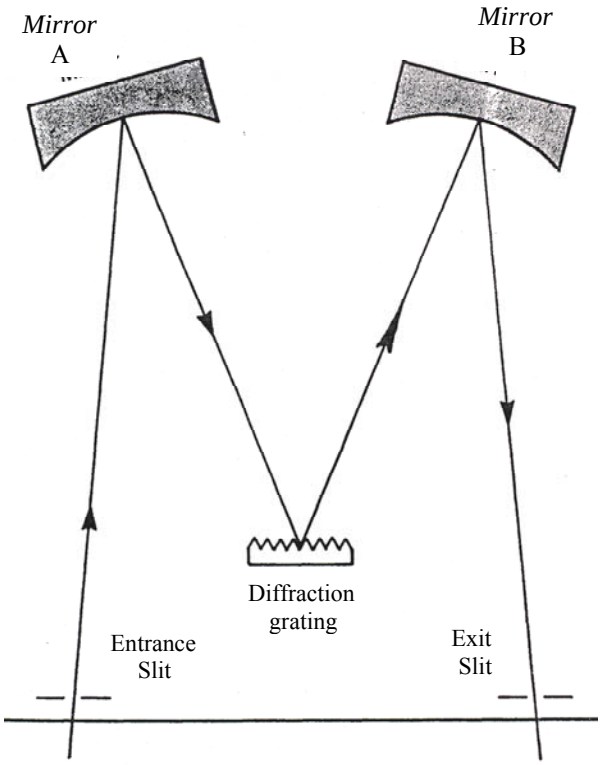


Figure 1.2. Grating assembly for a variable wavelength UV detector (Figure adapted from reference 50).

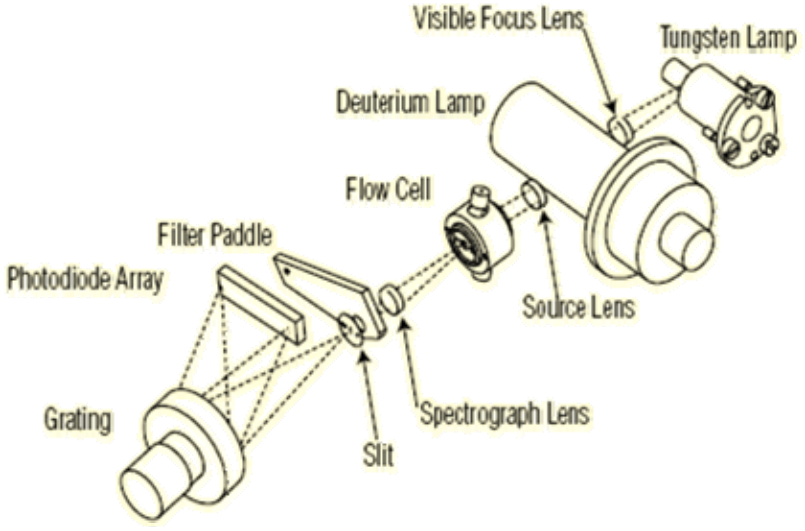


Figure 1.3. Diode-array detector optics (Figure courtesy of Dionex Corporation).

Ion Chromatography with Conductivity Detection

Ion chromatography refers to modern and efficient methods of separating and determining ions based upon ion-exchange resins. Applications of ion chromatography have been historically directed at determination of inorganic anions.[54-60] However, separations of organic acids and alkali/alkaline earth cations are also prevalent in literature.[54-55] The substances commonly analyzed by ion chromatography often lack significant UV absorbance; therefore, a conductivity detector is prominently used in ion chromatography for the analysis of common ions with poor UV absorption properties.

The operating principles of conductivity detection can be illustrated by considering the conductance of eluent prior to and during the elution of a solute ion.[56-57] The conductance change, ΔG, produced when an anionic solute S^- is eluted by an anionic eluent E^- is given by:

$$\Delta G = G_{elution} - G_{background} = [(\lambda_S^- - \lambda_E^-)\, C_S * I_S] / [(10^{-3})K]$$

where C_S is the concentration of the solute, I_S the fraction of the solute present in the ionic form, λ_E^- and λ_S^- the limiting equivalent ion conductance of the competing eluent anion and solute, respectively, and K the cell constant.

Useful sample detectability requires a large difference in the limiting equivalent ionic conductance of the analyte and eluent ions. However, the conductivity detector is a bulk property detector,[57] which senses all ions whether they are from an analyte or from the mobile phase. This presents an obvious problem with respect to detection, namely how to detect low concentrations of ionic analytes in the presence of a high concentration of mobile phase ions. The preferred solution is to neutralize the mobile phase with a suppressor to reduce background conductivity, making it possible to detect the ionic

species of interest. The Anion Self-Regenerating Suppressor (ASRS-ULTRA, available from Dionex Corporation) [58] incorporates an external source of deionized water flowing through the suppressor as a regenerant to achieve mobile phase suppression. The configuration of the suppressor is shown in Figure 1.4.

The suppressor includes two regenerant compartments and one eluent compartment separated by ion-exchange membranes.[58] Electrodes are placed along the length of the regenerant channels. For ion chromatography of anions, the membranes in the suppressor are cation exchange polymers. Consider a separation of anions with sodium hydroxide as the mobile phase eluent. The eluent flows through one side of the ion exchange membrane while a regenerant solution flows in a countercurrent direction on the opposite side of the membrane. When a potential is applied across the electrodes, water is converted to hydrogen and hydroxide ions. Hydrogen ions diffuse across the membrane next to the anode, neutralizing mobile phase hydroxide ions, while sodium ions from the mobile phase diffuse across the other membrane to maintain charge balance at the cathode.

Waste gases, hydrogen from the cathode and oxygen from the anode, are vented with a liquid waste of aqueous sodium hydroxide. Anionic analytes are prevented from penetrating the membrane by the repulsion effect of the anionic functional groups and therefore remain in the eluent stream. Figure 1.5 illustrates the mechanism of suppression.[59]

The result of ion suppression is increased sensitivity at the detector. Since sodium hydroxide from the mobile phase is transferred across the membrane and does not reach

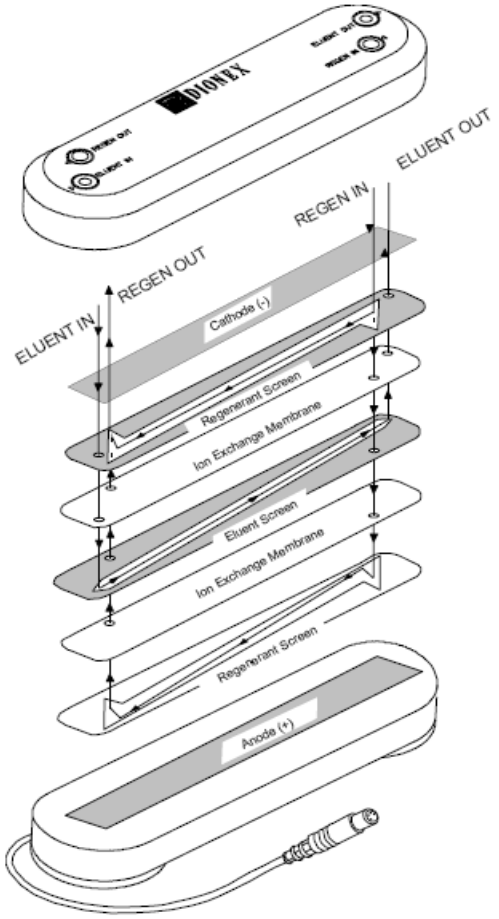


Figure 1.4. Electrode, membrane, and screen configuration in the Anion Self-Regenerating Suppressor, ASRS-ULTRA (Figure courtesy of Dionex Corporation). [58]

the detector, the background conductivity resulting from the mobile phase is near zero. Additionally, it is important to note that the positively charged counterions in the mobile phase are now hydrogen ions, which have an equivalent ion conductance seven times higher than the original sodium counterions. Because the detector responds to the combined conductivity of the negatively charged analyte and positively charged counterion, the observed analyte response is increased relative to that observed in the absence of suppression. Therefore, the suppressor lowers the background conductivity caused by the mobile phase and increases the conductivity of analyte ion pairs.

The retention of sample ions is related to their respective charge and ionic radius. In general, trivalent ions are retained in the stationary phase longer than divalent ions, followed by monovalent ions.[60] Thus, the monovalent nitrate ion elutes prior to divalent sulfate. In addition, for ions of equivalent charge, an ion with a large ionic radius has stronger affinity toward the stationary phase of an anion exchanger (i.e., retention increases with increasing ionic radius). Accordingly, halide ions elute in the following order: fluoride < chloride < bromide < iodide. In some cases, the size of ions often influences retention more strongly than their valency. For example, the divalent sulfate ion elutes prior to monovalent thiocyanate.[59,60]

Selectivity for a range of anions of various affinities can be achieved by gradient elution and the elution order can be changed by adjusting the gradient. Among acidic analytes, selectivity is best for compounds with pKa values below 6. As analyte ionization (dissociation) decreases, so does selectivity. Analytes with pKa values above 7 can be detected under certain conditions, but signal-to-noise ratios are generally poor. Fortunately, all organic acids with carboxylate, sulfonate, or phosphonate functional groups have pKa's below 4.75, so conductivity is a suitable detection method for these species.[54]

The same idea and discussion holds for ion chromatography of cations. The suppressor membranes are anion exchange polymers. These allow anions to pass freely, but exclude cations. Dilute acids such as methanesulfonic acid are used in the mobile phase. In the Dionex Cation Self-Regenerating Suppressor (CSRS),[58] methanesulfonate counterions are replaced by hydroxide generated by the electrolysis of water. This neutralizes the acidic mobile phase and provides the highly conductive hydroxide

counterion to the analyte cations. Inorganic cations detected include the alkali and alkaline earth metals.[60]

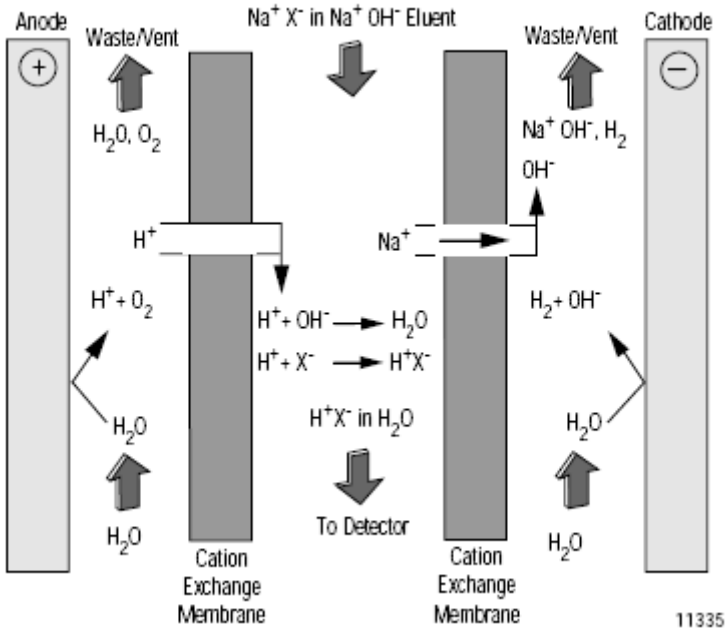


Figure 1.5. Schematic diagram illustrating the mechanism of suppression (Figure courtesy of Dionex Corporation).[59]

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)

High performance liquid chromatography is often preferred for determination of carbohydrates because of their hydrophilicity and low volatility. However, the use of direct UV detection is not feasible for carbohydrates due to the absence of a strong chromophore in the structure of carbohydrates. As a result, an improved chromatographic technique, known as high-performance anion exchange (HPAE), was developed to separate carbohydrates.[61-68] HPAE chromatography takes advantage of the weakly acidic nature of carbohydrates to give highly-selective separations at high pH using a strong anion-exchange stationary phase. The separation is typically coupled with pulsed

amperometric detection to assay carbohydrates that are oxidizable at convenient electrode potentials in aqueous solution.

The term amperometric detection describes a technique in which a constant potential is applied between working and reference electrodes, and the current resulting from oxidation or reduction reactions occurring at the working electrode is measured.[62-65] At high pH, carbohydrates are electrocatalytically oxidized at the surface of a gold electrode by application of a positive potential relative to a Ag/AgCl reference. The current generated is proportional to the carbohydrate concentration, and therefore, carbohydrates can be detected and quantified. However, oxidation or reduction of an analyte at an electrode surface tends to foul the surface, leading to a change in detector response. To prevent signal loss, the electrode surface is cleaned by a series of potential steps that are applied for fixed time periods after detection has been accomplished. [66-68] When detection is performed at regular intervals between cleaning and regeneration, the detection scheme is called pulsed amperometry.

A series of potentials applied for defined time periods is referred to as a waveform. Repeated application of a waveform is the basis of pulsed amperometric detection. The repeating sequence of a triple-potential waveform, which has been used for pulsed amperometric detection of carbohydrates, is illustrated in Figure 1.6.[67]

The potentials of a waveform are designated E1, E2, E3, where E1 is the detection potential. The remaining potentials, E2 and E3, are applied to clean and restore the electrode for subsequent detection. The current due to oxidation of the carbohydrate is measured at the first potential, E1. The second potential, E2, is a more positive potential that oxidizes the gold electrode and cleans the electrode surface. The third potential, E3,

reduces the formed gold oxide on the electrode surface back to gold, thus permitting detection during the next cycle at E1. Optimal values for all waveform parameters can then be determined by systematic variation of one parameter, while holding the other parameters constant.

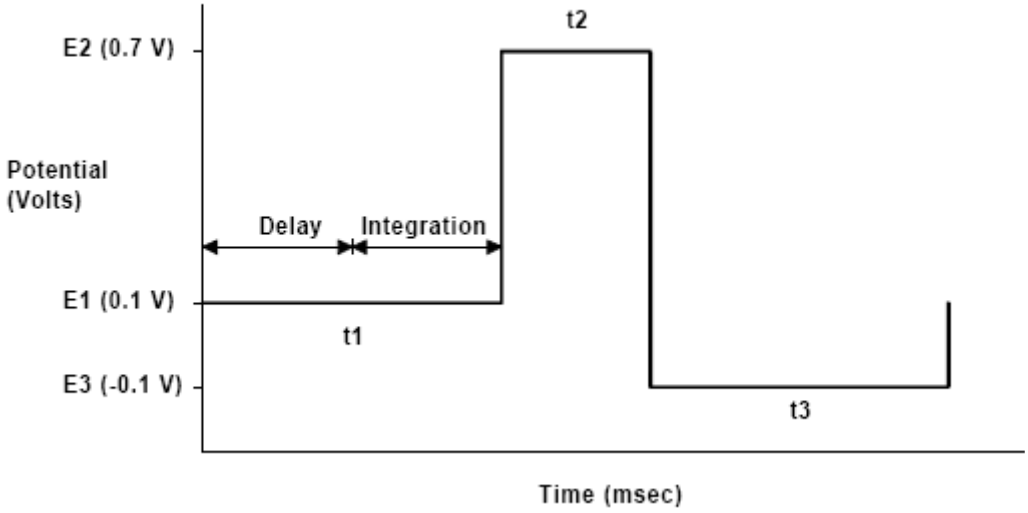


Figure 1.6. Diagram of a triple-potential waveform (Figure courtesy of Dionex Corporation).[67]

Exerting a high positive cleaning potential (E2) of the triple-potential waveform on a gold surface could cause excessive gold oxide formation. The dissolution of gold oxide from the electrode surface results in a slow recession of the gold electrode. Consequently, the detector response decreases, and the reproducibility becomes poor over time. To overcome this drawback, a quadruple-potential waveform for the pulsed amperometric detection has been further developed recently.[67] The repeating sequence of a quadruple-potential waveform is illustrated in Figure 1.7.

The first potential (E1) of the quadruple waveform is the detection potential at which the current from carbohydrate oxidation is integrated. The difference between

triple- and quadruple-potential waveforms is that a quadruple waveform uses negative (E2) rather than positive potential for electrode cleaning. The mechanism of cleaning at a negative potential could be displacement of adsorbed molecules on the electrode surface by hydrogen atoms produced from the reduction of water. Subsequently, a positive potential (E3) is applied for a short time period to maintain a catalytically active electrode, and prevent excessive recession of the gold surface. The fourth potential (E4) is then applied to partially reduce the oxide formed at the positive potential of E3. The formation and then the reduction of gold oxide are thought to be responsible for the creation of catalytic sites on the electrode surface. It is found that omission of this transient step of oxide formation results in a decreased detector response. Compared to the triple-potential waveform, the quadruple-potential waveform greatly improves long-term reproducibility of pulsed amperometric detection.

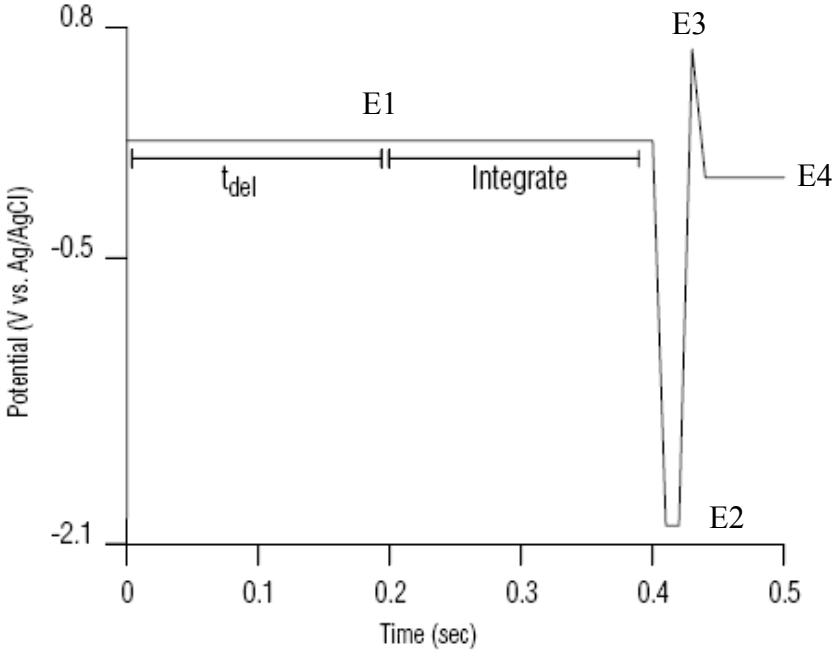


Figure 1.7. Diagram of a quadruple-potential waveform (Figure courtesy of Dionex Corporation).[67]

Carbohydrates and sugar alcohols, the reduced forms of monosaccharides, are weak acids which ionize between pH 11 and 13. Sugars alcohols, such as glycerol, sorbitol and mannitol, have higher pKa values than mono- and disaccharides. Separation of carbohydrates and sugar alcohols can be achieved by using a strong anion-exchange column and choosing an eluent pH near the pKa values of those compounds.[68] Sugar alcohols with higher pKa values elute first, followed by monosaccharides and disaccharides that have lower pKa values. Altering the pH of the eluent, by varying the sodium hydroxide concentration, changes the charge on the compounds. This in turn will change the elution order so that the carbohydrates and sugar alcohols of interest will be resolved from one another.

CHAPTER TWO

High-Performance Liquid Chromatography Method for Simultaneous Determination of Aliphatic Acid, Aromatic Acid and Neutral Degradation Products in Biomass Pretreatment Hydrolysates

Introduction

With the projected depletion of the world's petroleum reserves, there is escalating pressure to develop alternative, non-petroleum-based sources of energy.[68,69] Among energy alternatives, biomass-derived ethanol represents one of the more promising commodities for long-term sustainability of transportation fuels.[70-72] Currently, the most well-studied and near-commercial technology for conversion of biomass to ethanol involves dilute acid-catalyzed pretreatment of lignocellulosic feedstocks, followed by enzymatic hydrolysis of cellulose and fermentation of monomeric sugars to produce ethanol.[73-76] However, the pretreatment product mixture, commonly referred to as hydrolysate, contains not only cellulose and fermentable sugars, but also a wide variety of degradation products such as aliphatic and aromatic acids, phenols, and aromatic aldehydes. Many of these degradation products exert an inhibitory effect on downstream microbial processes,[77-80] thus reducing the overall efficiency for bioconversion of lignocellulosics to ethanol. As a result, there is increasing impetus to develop reliable quantitative analyses for individual degradation products in order to advance a more fundamental understanding of lignocellulose pretreatment as well as subsequent microbial inhibition processes.

Generous effort has been extended towards analysis of degradation products in biomass hydrolysates, with varying degrees of success. Although gas chromatography coupled with flame ionization or mass spectrometry detection has been quite successful in identifying a variety of organic degradation products in lignocellulosic biomass,[81-91] implementation of GC methodologies for quantitative work have suffered from inherent complexitites of derivatizing samples of unknown composition. Liquid-chromatography (LC) methods, employing post-column UV or refractive index detection, have historically suffered from incomplete resolution of analytes. As a result, LC analyses of degradation products in hydrolysate samples have typically employed multiple chromatographic modes and detection strategies, the choice of which depends on analyte class. For example, aliphatic acids have been determined using high performance anion-exchange chromatography with UV[90,91] or conductivity detection,[91-93] ion-exclusion chromatography with UV detection,[94] or electrophoretic methods.[93-95] In contrast, analyses of aromatic acids, furans, phenolic compounds, and aldehydes have typically been accomplished using reversed-phase HPLC with refractive index,[82,96] UV [86, 89, 91-93] or mass spectrometry [92] detection.

Recent advances in column technology have allowed for the separation of relatively polar substances, including a wide spectrum of organic acids with an aqueous mobile phase at low pH, followed by an organic gradient that elutes the more hydrophilic acids. Unlike the standard C-18 column, newer designed reversed-phase columns can be operated with 100% water. Despite these column advances, the main difficulty of using reversed-phase HPLC to separate degradation products in the biomass hydrolysates still arises from the complexity of the matrix. It is impossible to separate a wide spectrum of

degradation products from the raw biomass hydrolysate without fouling the column. Therefore, a relatively simple analytical procedure, which requires an initial precipitation-filtration step, followed by liquid-liquid extraction and subsequent reversed-phase HPLC analysis with UV detection at 210 nm, has been developed in our lab.

In this chapter, we report the first example where aliphatic acid, aromatic acid, furan, aldehyde, and phenolic degradation products are determined simultaneously in a biomass pretreatment hydrolysate using reversed-phase high-performance liquid chromatography with UV detection. This study also represents the first time a validated method for quantitative determination of pretreatment degradation products has been reported in the primary literature. Additionally, it is important to point out that, independent of sample type, the HPLC-UV method validated in this work represents one of very few examples where aliphatic acids, multifunctional-group aromatic acids, and phenolic compounds are simultaneously separated and quantitated in a single chromatographic run. One recent report describes simultaneous determination of 29 organic acid and phenolic compounds in fruit juices using reversed-phase chromatography with an analysis time of approximately 80 minutes.[98] However, the present method enables determination of a similar set of 32 analytes with a 30% decrease in analysis time.

Experimental: Materials and Methods

Chemicals and Reagents

The solvents and reagents acetonitrile (HPLC far UV grade, Acros, Fair Lawn, NJ), methyl *tertiary*-butyl ether = MTBE (EM Science, Gibbstown, NJ), methanol

(Sigma-Aldrich, St. Louis, MO), phosphoric acid (J. T. Baker, Philipsburg, NJ), and ammonium bicarbonate (EM Science, Gibbstown, NJ) were reagent grade or better and used as received. The internal standard, *para-tert*-butylphenoxyacetic acid (Alfa Aesar, Ward Hill, MA) and 32 reference standards (Sigma-Aldrich St. Louis, MO): formic acid, malic acid, lactic acid, acetic acid, maleic acid, succinic acid, methylmalonic acid, fumaric acid, propanoic acid, levulinic acid, itaconic acid, gallic acid, 5-hydroxymethylfurfural (5-HMF), 2-furoic acid, furfural, adipic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, phenol, 4-hydroxybenzaldehyde, vanillic acid, syringic acid, vanillin, benzoic acid, syringaldehyde, 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3-hydroxy-4-methoxycinnamic acid, 4-hydroxycoumarin, *ortho*-toluic acid and *para*-toluic acid were purchased in the highest available purity and used as received. Corn Stover was obtained from the National Renewable Energy Laboratory (Golden, CO) and used as received. Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system.

Preparation of Standards

Thirty-two reference compounds reflecting a wide range of potential analytes were selected based on previous reports of hydrolysate composition.[77,81-97] Reference standards and calibrators were prepared from the group of purchased reference standards using water as the diluent. All solutions were prepared in sufficient quantity to provide replicate analyses for each individual study and stored at 4 °C. The internal standard solution of *para-tert*-butylphenoxyacetic acid was prepared at a concentration of 2.5 mM in methanol.

High-Performance Liquid Chromatography Analysis

All HPLC analyses were carried out using a Dionex® DX-600 series liquid chromatograph (Dionex Corp., Sunnyvale, CA). The HPLC system consisted of an AS50 autoinjector, DG2410 degassing module, GS50 gradient pump, LC30 chromatography oven and UVD170U ultraviolet detector. Chromatographic separation was achieved using a 150 mm x 4.6 mm YMC™ Carotenoid S-3 column (Waters Corporation, Milford, MA). This is a C30 reversed-phase column, withstanding mobile phase compositions up to 100% water. An RP 18 Opti-Guard® column (Alltech Associates, Deerfield, IL) was employed to protect the analytical column. Gradient separations were carried out using aqueous 0.05% (v/v) phosphoric acid (pH 2.2-2.3) and water-acetonitrile (10:90) as the A and B solvents, respectively. The nonlinear gradient elution profile employed to achieve chromatographic separation is given in Table 2.1. Additional parameters employed in HPLC analyses were as follows: injection volume, 25 µl; column temperature, 30 ºC; flow rate, 1 ml/min; detection wavelength, 210 nm.

Quantitation of target analytes was accomplished using a multipoint internal standard calibration curve. Calibration solutions were prepared by successive dilutions of a stock solution consisting of the neat chemicals dissolved in water. A constant amount (26 µg) of *para-tert*-butylphenoxyacetic acid was added as an internal standard, and each calibration solution was carried through the entire sample preparation procedure prior to HPLC analysis. Response factors at 210 nm were determined for each analyte by dividing the peak area of the analyte by the peak area of the internal standard, and calibration curves were constructed by plotting a linear regression ($r^2 \geq 0.99$) of the average response factor

(n = 5) versus analyte concentration for all calibration standards analyzed. Calibration curves were then used to directly determine analyte concentrations in hydrolysate samples.

Identification of degradation products in hydrolysates was accomplished by combining assessment of retention time data, UV absorbance ratios at four wavelengths (i.e., A_{254}/A_{210}, A_{275}/A_{210}, A_{300}/A_{210}) and spiking tests. Tentative identification of analytes required that multiple absorbance ratios ($A_{\lambda 1}/A_{\lambda 2}$) at a given retention time were consistent for both reference and hydrolysate samples. To further confirm analyte identity in hydrolysates, each sample was spiked with a suitable amount of a reference mixture to exactly double the concentration of perceived analytes, and the samples were reanalyzed. The criteria employed for positive identification required: 1) that the retention time of a given analyte in hydrolysate samples fell within ± 2% of the average retention time observed for the compound in replicate analyses of a reference standard, 2) that absorbance ratios observed at the retention time of a given analyte agreed within ± 15% to the average absorbance ratios observed for that compound in replicate analyses of a reference standard, and 3) that an expected doubling of analyte concentration was observed in a subsequent analysis of the spiked hydrolysate. It is also important to point out that most aliphatic acids do not absorb appreciably above 210 nm, and identification of these analytes was based solely on retention time and spiking tests. For this reason, the identity of these analytes in hydrolysate samples may be considered tentative, pending more conclusive spectroscopic analysis (e.g., mass spectrometry).

Table 2.1. HPLC Gradient Elution Profile[a]

	Mobile phase composition (%)	
Time (min)	0.05% H_3PO_4	90% Acetonitrile
0	100	0
2	100	0
15	90	10
24	90	10
43	65	35
89	0	100
114	0	100
120	100	0

[a] Additional chromatographic parameters are defined in the Experimental Section.

Hydrolysate Sample Preparations

The pretreatment process was carried out in two Techne SBL-2D high-temperature fluidized sand baths with TC-8D temperature controllers that maintained the temperature in the bath to ± 1 ºC. Reactor vessels for generating corn stover hydrolysates were constructed from 316 stainless steel tubing. All reactor vessels were equipped for pressurization and steam heating. Two temperature-controlled sand baths were employed for sample generation. One was maintained at the desired reaction temperature, and the other was set 40 ºC above the desired reaction temperature and used for preheating the reaction vessel.

The corn stover hydrolysate analyzed in this work was generated using a previously reported procedure.[78] Corn stover was pretreated in 100 mL of 1% (v/v) sulfuric acid at a solids concentration of 100 g/L. The mixture was reacted in a 150 mL 316 stainless steel pressure vessel. Temperature control was achieved by pre-heating the reactor for 3 minutes in a sand bath at 200 ºC. The reactor was immediately transferred to a second sand

bath at 160 ºC for 8 minutes. Quenching was accomplished by immersing the reactor in an ice bath. Particulates were removed by filtration through 0.45-μm membrane filters, and samples were stored at 4 °C until processed for HPLC analysis.

Sample Preparation and Extraction Procedures

All reference samples, hydrolysate samples and calibration standards were prepared and extracted using the following procedure. Samples were initially treated with solid ammonium bicarbonate (pH 7-8) and stored at 4 °C for 30 minutes. Samples were subsequently filtered using a 0.2-μm syringe filter. The filtrate was adjusted to pH 1-2 with concentrated sulfuric acid. Five milliliter aliquots of each sample were subsequently transferred to a 50 mL centrifuge tube, and 50 μL of the methanolic internal standard mixture (26 μg) was added prior to extraction. Samples were contacted two times with 45 mL portions of MTBE on a rotating wheel at 25.0 ± 0.1 °C for 15 minutes. Following each extraction, samples were centrifuged at 4500 rpm for 3 minutes to ensure complete phase disengagement. The volume of the combined MTBE extracts was reduced to 1-2 mL under a stream of N_2 at 55 °C, using a Zymark® Turbovap LC™ concentration workstation (Zymark Corp., Hopkinton, MA). At this point, 1.5 mL of water was added to the MTBE mixture, and the remaining MTBE was evaporated under a stream of N_2 at 55 °C. The resulting aqueous mixture was quantitatively transferred to a volumetric flask and diluted to 5 mL with water. Aliquots of each sample (1.5 mL) were then transferred to 2 mL autosampler vials prior to HPLC analysis.

Extraction Efficiency

The efficiency of analyte extraction using MTBE was determined using a modified literature procedure.[95] Two groups of controls were prepared in purified water and extracted using the sample preparation procedure described above. The first group was spiked with a precisely known concentration of each analyte and internal standard prior to extraction, while the second group was spiked with the internal standard only. Following extraction, the same concentration of each analyte added to samples in group one was added to samples from the second control group. Both groups were analyzed by HPLC, and the ratio of response factors obtained for samples from control groups one and two were used to calculate values of percent recovery for each analyte:

$$\%\ \text{recovery} = \frac{A_{X1}/A_{IS1}}{A_{X2}/A_{IS2}} \times 100\%$$

where A_{X1}, A_{IS1}, A_{X2} and A_{IS2} represent the peak areas for the analyte (X) and internal standard (IS) in groups one and two, respectively. Reported values of percent recovery for the 32 reference compounds represent the average of three replicate determinations plus or minus one standard deviation (n = 3).

Results and Discussion

What Degradation Products are Formed?

Pretreatments at elevated temperatures and acidic conditions result in not only the production of fermentable sugars but also a wide range of degradation products, such as aliphatic acids, furan derivatives, and a variety of aromatic and phenolic compounds. The degradation products are considered to be potential fermentation inhibitors, which is a limiting factor in the conversion of lignocellulosic materials to ethanol.[24] These inhibitors

can be divided into three groups based on their origin: (1) compounds released during pretreatment, such as acetic acid; (2) sugar degradation products, such as furfural and 5-hydroxymethylfurfural; (3) lignin degradation products, resulting in a range of phenolic and aromatic compounds.[23,24] Primary degradation pathways are schematically presented in Figure 2.1.[14,15]

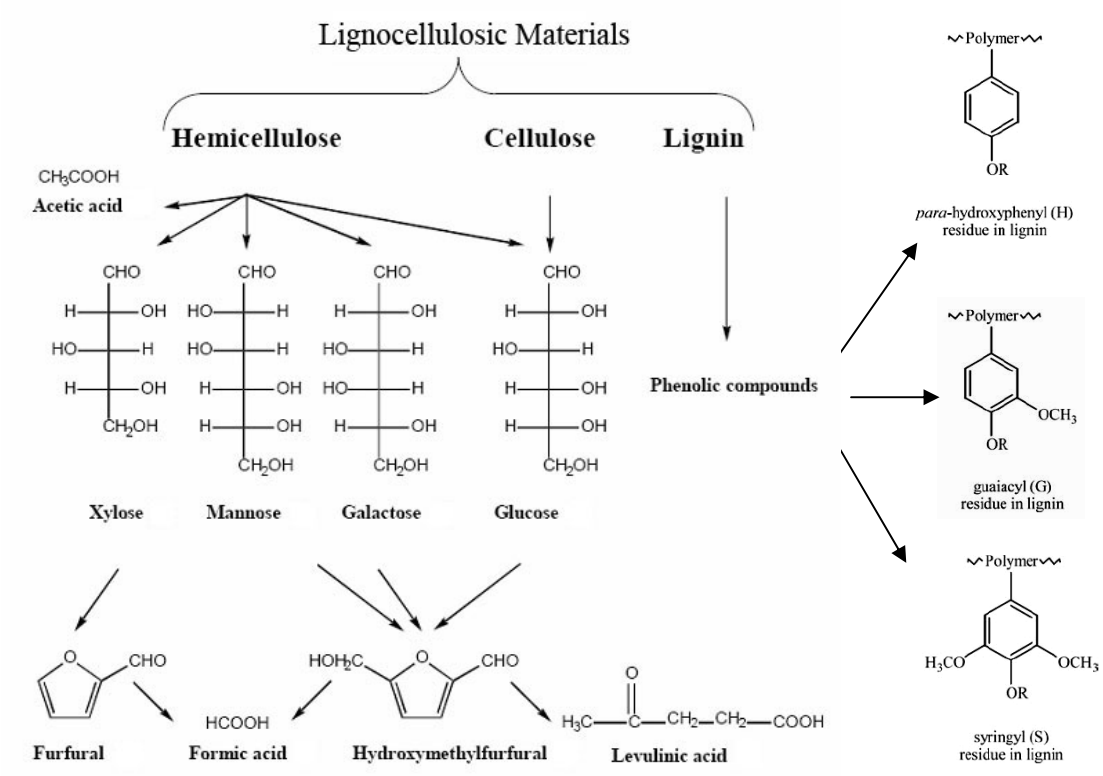


Figure 2.1. Possible degradation pathways that are operative during pretreatment of lignocellosic materials (Figure adapted from references 14 & 15).

When hemicellulose is degraded, xylose, mannose, acetic acid, galactose, and glucose are liberated. Cellulose is hydrolyzed to glucose. At high temperature and pressure, xylose is further degraded to furfural. Similarly, 5-hydroxymethylfurfural (5-HMF) is formed from hexose degradation in acidic solution. Acetic acid is ubiquitous in hemicellulose hydrolysates of lignocellulosics biomass, where hemicellulose and to

some extent lignin are acetylated.[15] Formic acid can be formed when furfural and 5-HMF are broken down. Levulinic acid is also a secondary product from degradation of 5-HMF. Phenolic and aromatic compounds are generated from partial breakdown of lignin and have also been reported to be formed during carbohydrate degradations.[14-16]

Vanillic acid and vanillin, formed from the degradation of the guaiacylpropane units of lignin, have been detected in hydrolysates from willow, spruce, poplar, red oak, pine, and corn stover.[23] Syringaldehyde and syringic acid, formed from the degradation of syringyl propane units, have been reported in hydrolysates of hard wood and corn stover.[24] 4-Hydroxybenzoic acid, which is esterified with alcoholic hydroxyl groups of lignin, is liberated during hydrolysis.[16]

Selection of Analytes

Selection of analytes was based on their ubiquity in previous analyses of hydrolysate composition and commercial availability. A review of degradation product formation and the effect of these products on subsequent microbial fermentations recently appeared in the literature.[77] In this paper, the authors identify 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, syringaldehyde, and syringic acid among the more commonly identified phenolic compounds derived from lignin. Various phenylpropane derivatives, such as cinnamic acids, are also mentioned as general products of acid hydrolysis. Predominant sugar decomposition products identified are furfural, 5-hydroxymethylfurfural, and 2-furoic acid. Aliphatic acids typically found in hydrolysates include acetic acid (originating from acetylated functionalities on both lignin and hemicellulose), formic acid (derived from decomposition of both sugars and lignin), and levulinic acid (produced upon further decomposition of 5-hydroxymethylfurfural [100]).

Lactic acid is reported as a common hydroxycarboxylic acid. An earlier review cites the additional importance of alternative C4-C9 aliphatic acids and aromatic acids.[101] The analytes selected for method development activities clearly represent the majority of ubiquitous degradation products of potential interest to the biomass community. Notable omissions include coumaric acid and Hibbert's ketones. The reported HPLC method affords quantitative determination of additional analytes with little modification.

Method Development and Validation

The methodology described here provides a robust quantitative procedure for simultaneous determination of a wide variety of degradation products in biomass pretreatment hydrolysates. The method involves an initial precipitation-filtration step, followed by liquid-liquid extraction with MTBE and subsequent HPLC analysis with UV detection at 210 nm. The choice of 210 nm as the detection wavelength enabled reliable monitoring of not only aromatic compounds, which are typically monitored at longer wavelengths (*e.g.*, 254 nm or 280 nm), but also carboxylic acids in the sample that do not contain a C=C double bond and thus do not appreciably absorb at longer wavelengths. As demonstrated in Figure 2.2, a non-linear gradient elution profile, utilizing 0.05% (v/v) aqueous phosphoric acid (pH 2.3) and 10:90 water-acetonitrile, respectively, as the A and B solvents (Table 2.1) and a C30 stationary phase, results in near-baseline resolution of 32 analytes in just over 40 minutes. Initial method development activities employed a C18 stationary phase. However, the C30 column gave better peak symmetry and improved resolution of analytes with very little change in retention behavior.

Resolution of analytes in this separation was found to be highly dependent upon mobile phase pH. For example, when the initial pH of the A solvent was increased to 2.5,

a dramatic reduction in the resolution of aliphatic acids was observed concomitant with changes in chromatographic selectivity. Further increases in the pH of the A solvent resulted in a gradual collapse of early eluting analytes into the solvent front. At pH 2.3, all of the organic acids are protonated. Thus, analyte retention is primarily governed by hydrophobic interactions between the analytes and the C30 stationary phase. Under these conditions of ion suppression, appreciable retention of low-molecular weight organic acids is achieved and a general increase in retention time is expected with increasing number of carbon atoms in the backbone of target analytes. (*i.e.*, with increasing molecular weight). However, many pretreatment degradation products possess multiple oxygenated functionalities that noticeably affect their retention behavior (*e.g.*, the family of benzoic acid derivatives).

Although an initial goal of method development activities was direct analysis of biomass pretreatment samples, preliminary analyses of hydrolysates revealed that some level of analytical sample preparation would be required. A relatively simple cleanup procedure was devised, involving an initial precipitation-filtration step followed by liquid-liquid extraction. The initial adjustment of sample pH to 7-8 resulted in the formation of a brown precipitate in hydrolysate samples. While the composition of this precipitate remains unknown, omitting this step in the analysis procedure resulted in a substantial decline in column performance after only a few injections. Additionally, extraction of samples with MTBE served to isolate potential analytes away from alternative pretreatment products (*e.g.*, salts, sugar monomers and water-soluble oligomers) and greatly simplified resulting chromatograms.

Experiments were performed to determine the extraction efficiency of MTBE for removal of target analytes from acidic solution (pH 1-2). Methylene chloride was also investigated as an extraction solvent in preliminary work. However, while the recovery of

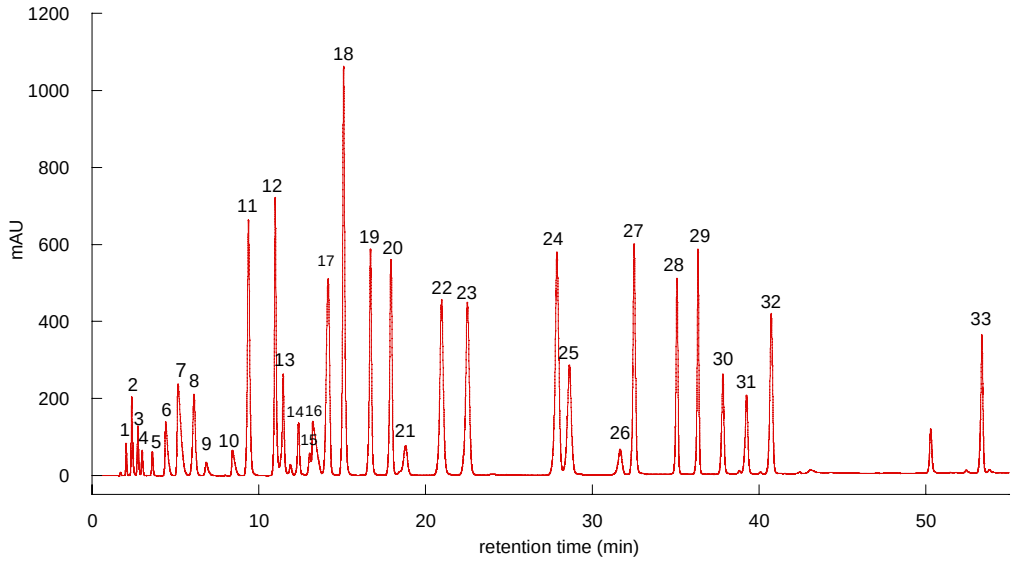


Figure 2.2. Sample chromatogram of an aqueous reference standard. Detection at 210 nm. Peak identifications are as follows: (1) formic acid; (2) malic acid; (3) lactic acid; (4) acetic acid; (5) maleic acid; (6) succinic acid; (7) methylmalonic acid; (8) fumaric acid; (9) propanoic acid; (10) levulinic acid; (11) itaconic acid; (12) gallic acid; (13) 5-hydroxymethylfurfural; (14) 2-furoic acid; (15) furfural; (16) adipic acid; (17) 3,4-dihydroxybenzoic acid; (18) 3,5-dihydroxybenzoic acid; (19) 3,4-dihydroxybenzaldehyde; (20) 4-hydroxybenzoic acid; (21) phenol; (22) 4-hydroxybenzaldehyde; (23) vanillic acid; (24) syringic acid; (25) vanillin; (26) benzoic acid; (27) syringaldehyde; (28) 4-hydroxy-3-methoxycinnamic acid; (29) 3-hydroxy-4-methoxycinnamic acid; (30) 4-hydroxycoumarin; (31) *ortho*-toluic acid; (32) *para*-toluic acid, and (33) *para-tert*-butylphenoxyacetic acid.

neutral analytes (*i.e.*, aldehydes, furans, and mono-functional group phenols) into methylene chloride appeared higher than the recovery of these analytes using MTBE, the recovery of organic acids was much lower, especially for the most water-soluble aliphatic acids. Accordingly, MTBE was employed in subsequent method development and application studies. Average recoveries at two concentrations (0.5 and 5 mM for aliphatic

analytes and 0.01 and 0.1 mM for aromatic analytes) ranged from 20 to 99% and are reported in Table 2.2. At both low and high concentrations, the recovery of the majority of analytes exceeds 60%. Notable exceptions include the low-molecular-weight aliphatic acids (*i.e.*, formic, malic, lactic, acetic, and propanoic acids), furfural and phenol. Average recoveries for these aliphatic acids ranged from 28 to 60%, consistent with the increased aqueous solubility of these compounds as compared to other analytes included in the study. In contrast, the origin of the low recoveries observed for furfual and phenol (ca. 20 and 50%, respectively) is not obvious. Nevertheless, these aliphatic acids, furfural and phenol are among the more dominant degradation products quantified in a hydrolysate (see below), and low recoveries of these analytes were deemed acceptable for application of the method to biomass pretreatment samples.

Due to considerable variation in extraction recoveries for these analytes, it was presumed that the best approach to quantitation would require that all calibrators be carried through the sample cleanup procedure prior to HPLC analysis and that the UV response for each analyte be normalized using an internal standard. This approach enables reliable determination of analyte concentrations in the original sample with no dependence on the efficiency of analyte extraction beyond obvious sensitivity limitations. Analysis of hydrolysate samples according to this method results in a near continuum of peaks out to an analysis time of 50 minutes (Figure 2.3). This significantly complicated the selection of an internal standard possessing similar extraction behavior to the analytes of interest yet not co-eluting with alternative peaks in the chromatogram. After multiple candidate trials, it was determined that *para-tert*-butylphenoxyacetic acid was a suitable choice.

A series of high-purity reference standards dissolved in water was employed to determine the analytical merits of the HPLC method. Following sample preparation and subsequent HPLC analysis, response factors were determined by dividing the peak area of

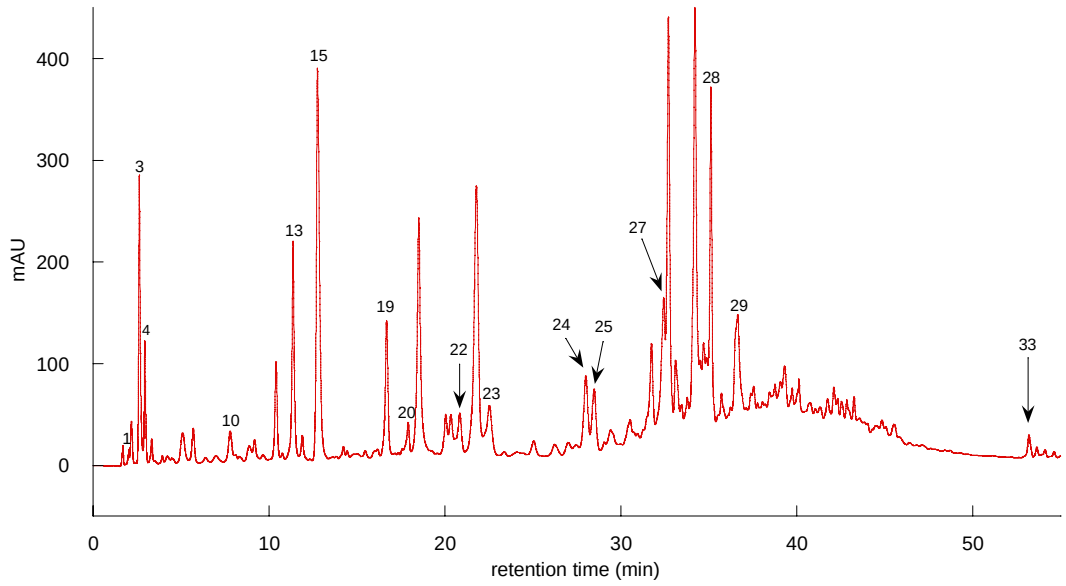


Figure 2.3. Chromatogram of a corn stover hydrolysate prepared by treatment of milled corn stover with aqueous 1% (v/v) sulfuric acid. Detection at 210 nm. (1) formic acid; (3) lactic acid; (4) acetic acid; (10) levulinic acid; (11) itaconic acid; (13) 5-hydroxymethylfurfural; (15) furfural; (19) 3,4-dihydroxybenzaldehyde; (20) 4-hydroxybenzoic acid; (22) 4-hydroxybenzaldehyde; (23) vanillic acid; (24) syringic acid; (25) vanillin; (27) syringaldehyde; (28) 4-hydroxy-3-methoxycinnamic acid; (29) 3-hydroxy-4-methoxycinnamic acid and (33) *para-tert*-butylphenoxyacetic acid.

the analyte by the peak area of the internal standard. Calibration curves were constructed by plotting the average response factor (n = 5) versus analyte concentration for all reference standards analyzed. The retention time, linear dynamic range (LDR), correlation coefficient (r^2) and limit of detection (LOD) determined for each analyte are reported in Table 2.2. The range of investigated concentrations varied considerably due to large differences in analyte extinction coefficients. However, the reported LDRs typically span

three orders of magnitude with correlation coefficients for a linear regression exceeding 0.99. The y-intercept values for the 32 analytes ranged from −0.0331 to 0.1247 milliabsorbance units (mAU) with an average intercept of 0.03 ± 0.04 mAU. The limit of detection (LOD) for aliphatic and aromatic analytes ranged from 1.1 to 17 μM and 7 to 267 nM, respectively. It was discovered after the fact that a significant amount of maleic acid and fumaric acid were present as impurities in the purchased malic acid standard. As a result, our knowledge of the true concentrations of these analytes in calibration solutions was suspect. For this reason, the LDR and LOD merits determined for these analytes are not reported in Table 2.2. However, it is important to point out that a linear detector response with correlation coefficients exceeding 0.99 was observed for each analyte, and it is not unreasonable to expect that the method would enable their reliable determination with higher purity reference standards.

Method intra-day (within day) and inter-day (between days) accuracy and precision were also evaluated at two concentrations (high and low) for each analyte over a five day period (Table 2.3). All reference standards and calibrators were prepared on day 1 and stored at 4 °C between analyses. Calibration curves were constructed on day 1, as described above, and employed for the duration of the study. Method accuracy was measured as the average relative error between experimentally determined concentrations for five replicate analyses and prepared target concentrations. Method precision was measured as the average relative standard deviation (RSD) for experimentally determined concentrations. For both intra-day (day 1) and inter-day (days 3 and 5) assays, the RSDs and relative errors between experimentally determined and prepared target concentrations were typically less than 10%, demonstrating excellent accuracy and precision for the method. Notable exceptions

Table 2.2. Retention Time, LDR, LOD and % Recovery of Analytes

	analyte	retention time (min)	LDR (mM)	r^2	LOD (μM)	Recovery (%)	
						low conc.	high conc.
1	formic acid	2.05	0.005-5.00	0.9971	4.5	40 ± 5	27 ± 6
2	malic acid	2.39	—	0.9996	—	33 ± 2	31 ± 2
3	lactic acid	2.75	0.025-5.00	0.9988	6.1	55 ± 1	54 ± 5
4	acetic acid	3.02	0.005-5.00	0.9979	2.9	47 ± 2	31 ± 7
5	maleic acid	3.46	—	0.9983	—	90 ± 3	74 ± 5
6	succinic acid	4.62	0.005-5.00	0.9997	2.9	78 ± 8	80 ± 5
7	methylmalonic acid	5.18	0.005-5.00	0.9995	1.5	82 ± 8	86 ± 3
8	fumaric acid	5.86	—	0.9996	—	92 ± 1	88 ± 3
9	propionic acid	7.01	0.025-5.00	0.9933	9.1	51 ± 5	32 ± 6
10	levulinic acid	8.67	0.025-5.00	0.9986	17	66 ± 5	77 ± 2
11	itaconic acid	9.44	0.0002-0.2	0.9995	0.043	83 ± 8	86 ± 3
12	gallic acid	11.12	0.005-5.00	0.9994	1.1	45 ± 8	88 ± 2
13	5-hydroxymethylfurfural	11.63	0.0004-0.4	0.9997	0.088	71 ± 4	78 ± 5
14	2-furoic acid	12.23	0.00008-0.08	0.9990	0.043	72 ± 7	78 ± 1

Table 2.2 (cont.). Retention Time, LDR, LOD and % Recovery of Analytes

	analyte	retention time (min)	LDR (mM)	r^2	LOD (μM)	Recovery (%)	
						low conc.	high conc.
15	furfural	13.25	0.0004-0.4	0.9836	0.267	20 ± 5	20 ± 3
16	adipic acid	13.81	0.005-5.00	0.9996	1.1	84 ± 7	87 ± 1
17	3,4-dihydroxybenzoic acid	14.38	0.00008-0.08	0.9994	0.014	86 ± 9	89 ± 2
18	3,5-dihydroxybenzoic acid	15.26	0.00008-0.08	0.9994	0.007	85 ± 7	88 ± 2
19	3,4-dihydroxybenzaldehyde	16.89	0.00008-0.08	0.9998	0.012	82 ± 3	89 ± 2
20	4-hydroxybenzoic acid	18.12	0.00008-0.08	0.9997	0.015	88 ± 9	90 ± 3
21	phenol	19.03	0.00008-0.08	0.9916	0.068	34 ± 9	41 ± 6
22	4-hydroxybenzaldehyde	21.31	0.0002-0.2	0.9995	0.094	77 ± 6	88 ± 2
23	vanillic acid	22.91	0.00008-0.08	0.9998	0.027	86 ± 8	89 ± 2
24	syringic acid	28.34	0.00008-0.08	0.9997	0.019	86 ± 8	89 ± 2
25	vanillin	29.03	0.0004-0.08	0.9994	0.12	66 ± 5	84 ± 2
26	benzoic acid	31.92	0.00008-0.08	0.9997	0.051	62 ± 8	75 ± 2
27	syringaldehyde	32.83	0.00008-0.08	0.9994	0.021	70 ± 10	88 ± 2
28	4-hydroxy-3-methoxycinnamic acid	35.35	0.00008-0.08	0.9996	0.024	87 ± 9	89 ± 2

Table 2.2 (cont.). Retention Time, LDR, LOD and % Recovery of Analytes

	analyte	retention time (min)	LDR (mM)	r^2	LOD (μM)	Recovery (%) low conc.	Recovery (%) high conc.
29	3-hydroxy-4-methoxycinnamic acid	36.61	0.00008-0.08	0.9991	0.016	79 ± 8	89 ± 3
30	4-hydroxycoumarin	38.09	0.00005-0.05	0.9982	0.013	76 ± 9	90 ± 5
31	*ortho*-toluic acid	39.49	0.00008-0.08	0.9995	0.044	64 ± 9	77 ± 2
32	*para*-toluic acid	41.01	0.00008-0.08	0.9992	0.002	70 ± 6	82 ± 2
	Internal Standard:						
33	*para*-*tert*-butylphenoxyacetic acid	53.71					

[a] Reference standards constituted in 18 MΩ water were employed in the determination of these parameters. See text for chromatographic details.
[b] Investigated linear dynamic range (LDR).
[c] Limit of detection (LOD), calculated as three times the standard deviation in the background signal. UV detection at 210 nm.
[d] Recoveries for liquid-liquid extraction into MTBE, calculated as the average (n = 3) plus or minus one standard deviation in the least significant digit. See text for details.
[e] Initial aqueous concentrations of degradation products were 0.5 mM and 0.01 mM, respectively, for aliphatic and aromatic analytes.
[f] Initial aqueous concentrations of degradation products were 5 mM and 0.1 mM, respectively, for aliphatic and aromatic analytes.

Table 2.3. Intra- and Inter-day Accuracy and Precision.

		Day 1			Day 3			Day 5		
Analyte	Target conc. (mM)	Mean (mM)	RSD	% E	Mean (mM)	RSD	%E	Mean (mM)	RSD	%E
1	4.0	4.5 ± 0.3	6%	13%	4.7 ± 0.2	5%	17%	4.6 ± 0.2	6%	14%
	0.40	0.52 ± 0.04	8%	29%	0.45 ± 0.09	19%	17%	0.47 ± 0.08	17%	17%
3	4.0	4.17 ± 0.07	2%	4%	4.28 ± 0.08	2%	7%	4.1 ± 0.1	2%	6%
	0.40	0.41 ± 0.01	2%	3%	0.42 ± 0.03	8%	5%	0.42 ± 0.01	1%	5%
4	4.0	4.1 ± 0.2	4%	1%	4.3 ± 0.2	5%	6%	4.3 ± 0.3	7%	7%
	0.40	0.41 ± 0.03	8%	3%	0.42 ± 0.03	8%	5%	0.42 ± 0.02	6%	4%
6	4.0	4.08 ± 0.07	2%	2%	4.03 ± 0.03	1%	1%	3.96 ± 0.03	1%	−1%
	0.40	0.41 ± 0.01	2%	1%	0.39 ± 0.02	5%	−3%	0.44 ± 0.02	1%	−5%
7	4.0	4.05 ± 0.07	2%	1%	4.03 ± 0.05	1%	1%	3.98 ± 0.05	1%	0%
	0.40	0.41 ± 0.01	3%	3%	0.40 ± 0.02	5%	0%	0.38 ± 0.01	1%	0%
9	4.0	4.0 ± 0.1	3%	−1%	4.0 ± 0.2	6%	−3%	4.0 ± 0.3	7%	−3%
	0.40	0.44 ± 0.02	5%	11%	0.43 ± 0.04	8%	8%	0.43 ± 0.03	7%	8%
10	4.0	4.05 ± 0.05	1%	1%	4.02 ± 0.04	1%	0%	4.07 ± 0.05	1%	2%
	0.40	0.44 ± 0.05	10%	9%	0.41 ± 0.03	7%	2%	0.41 ± 0.03	7%	3%
11	0.32	0.323 ± 0.002	1%	1%	0.323 ± 0.003	1%	1%	0.318 ± 0.004	1%	−1%
	0.032	0.0321 ± 0.0007	2%	0%	0.032 ± 0.001	4%	1%	0.0320 ± 0.0002	1%	0%

Table 2.3 (cont.). Intra- and Inter-day Accuracy and Precision.

		Day 1			Day 3			Day 5		
Analyte	Target conc. (mM)	Mean (mM)	RSD	% E	Mean (mM)	RSD	%E	Mean (mM)	RSD	%E
12	0.04	0.0366 ± 0.0008	2%	−8%	0.0379 ± 0.0009	2%	−5%	0.037 ± 0.003	9%	−8%
	0.004	0.0030 ± 0.0004	14%	−26%	0.0039 ± 0.0004	11%	−3%	0.0033 ± 0.0005	14%	−17%
13	0.32	0.329 ± 0.004	1%	3%	0.331 ± 0.009	3%	3%	0.326 ± 0.003	1%	2%
	0.032	0.032 ± 0.001	3%	1%	0.033 ± 0.002	5%	2%	0.0326 ± 0.0003	1%	2%
14	0.08	0.080 ± 0.001	1%	−1%	0.081 ± 0.002	3%	−1%	0.082 ± 0.002	2%	2%
	0.008	0.0084 ± 0.0003	3%	5%	0.0081 ± 0.0004	5%	2%	0.008 ± 0.0003	4%	0%
15	0.32	0.31 ± 0.01	5%	−4%	0.32 ± 0.01	3%	1%	0.32 ± 0.02	4%	1%
	0.032	0.029 ± 0.003	9%	−9%	0.037 ± 0.003	8%	16%	0.036 ± 0.002	5%	11%
16	4.0	4.08 ± 0.07	2%	2%	4.03 ± 0.07	2%	1%	3.95 ± 0.03	1%	−1%
	0.40	0.39 ± 0.01	1%	−2%	0.40 ± 0.02	4%	1%	0.386 ± 0.003	1%	−3%
17	0.08	0.081 ± 0.001	1%	1%	0.080 ± 0.001	1%	0%	0.0796 ± 0.0004	1%	0%
	0.008	0.0078 ± 0.0001	2%	−2%	0.0080 ± 0.0002	3%	0%	0.0077 ± 0.0001	1%	−3%
18	0.08	0.0822 ± 0.0008	1%	3%	0.081 ± 0.001	1%	1%	0.080 ± 0.001	0%	0%
	0.008	0.0079 ± 0.0002	2%	−2%	0.0079 ± 0.0002	3%	−1%	0.0077 ± 0.0001	0%	−4%

Table 2.3 (cont.). Intra- and Inter-day Accuracy and Precision.

		Day 1			Day 3			Day 5		
Analyte	Target conc. (mM)	Mean (mM)	RSD	% E	Mean (mM)	RSD	%E	Mean (mM)	RSD	%E
19	0.08	0.0813 ± 0.0005	1%	2%	0.081 ± 0.001	2%	2%	0.080 ± 0.001	1%	0%
	0.008	0.0077 ± 0.0002	3%	−3%	0.0080 ± 0.0005	6%	0%	0.0079 ± 0.0006	8%	−1%
20	0.08	0.081 ± 0.001	1%	1%	0.081 ± 0.001	1%	1%	0.080 ± 0.001	1%	−1%
	0.008	0.0080 ± 0.0002	2%	0%	0.0081 ± 0.0003	3%	1%	0.0080 ± 0.0001	1%	0%
21	0.08	0.080 ± 0.003	4%	0%	0.069 ± 0.006	8%	−14%	0.063 ± 0.007	11%	−21%
	0.008	0.0081 ± 0.0008	10%	1%	0.007 ± 0.002	27%	−9%	0.005 ± 0.003	60%	−38%
22	0.32	0.322 ± 0.002	1%	1%	0.322 ± 0.005	2%	1%	0.325 ± 0.005	2%	1%
	0.032	0.0333 ± 0.0004	1%	4%	0.034 ± 0.001	4%	5%	0.0334 ± 0.0006	2%	4%
23	0.08	0.0815 ± 0.0009	1%	2%	0.080 ± 0.001	1%	0%	0.0802 ± 0.0006	1%	0%
	0.008	0.0081 ± 0.0001	2%	1%	0.0081 ± 0.0002	3%	2%	0.0080 ± 0.0001	1%	0%
24	0.08	0.0809 ± 0.0007	1%	1%	0.0809 ± 0.0009	1%	1%	0.0810 ± 0.0006	1%	1%
	0.008	0.0079 ± 0.0002	2%	−1%	0.0083 ± 0.0003	3%	4%	0.0080 ± 0.0001	1%	1%
25	0.08	0.0810 ± 0.0006	1%	1%	0.078 ± 0.002	2%	−2%	0.079 ± 0.003	4%	−2%
	0.008	0.0081 ± 0.0001	2%	1%	0.0082 ± 0.0004	5%	3%	0.0081 ± 0.0003	3%	1%

Table 2.3 (cont.). Intra- and Inter-day Accuracy and Precision.

		Day 1			Day 3			Day 5		
Analyte	Target conc. (mM)	Mean (mM)	RSD	% E	Mean (mM)	RSD	%E	Mean (mM)	RSD	%E
26	0.08	0.080 ± 0.002	2%	−1%	0.081 ± 0.002	3%	2%	0.083± 0.003	3%	3%
	0.008	0.0090 ± 0.0005	5%	13%	0.0081 ± 0.0006	7%	1%	0.0081 ± 0.0002	3%	1%
27	0.08	0.0811 ± 0.0004	0%	1%	0.081 ± 0.001	1%	1%	0.080 ± 0.001	1%	0%
	0.008	0.0078 ± 0.0001	1%	−1%	0.0080 ± 0.0003	3%	0%	0.0080 ± 0.0001	1%	−1%
28	0.08	0.0806 ± 0.0009	1%	1%	0.079 ± 0.001	1%	−1%	0.079 ± 0.001	2%	−2%
	0.008	0.0078 ± 0.0002	2%	0%	0.0082 ± 0.0002	3%	2%	0.0079 ± 0.0001	1%	−1%
29	0.08	0.0812 ± 0.0005	1%	2%	0.080 ± 0.001	1%	0%	0.079 ± 0.001	1%	−1%
	0.008	0.0077 ± 0.0002	2%	0%	0.0081 ± 0.0002	2%	2%	0.0079 ± 0.0001	1%	−1%
30	0.04	0.0389± 0.0009	2%	−3%	0.0379 ± 0.0005	1%	−5%	0.036 ± 0.001	1%	−11%
	0.004	0.0036 ± 0.0001	4%	−10%	0.0038 ± 0.0002	5%	−5%	0.0035 ± 0.0002	5%	−12%
31	0.08	0.080 ± 0.001	2%	0%	0.0807 ± 0.0009	1%	1%	0.0807 ± 0.0007	1%	1%
	0.008	0.0082 ± 0.0002	2%	2%	0.0082 ± 0.0002	3%	2%	0.0081 ± 0.0001	1%	1%
32	0.08	0.0799 ± 0.0009	1%	0%	0.0801 ± 0.0006	1%	0%	0.0810 ± 0.0008	1%	1%
	0.008	0.0081 ± 0.0002	3%	1%	0.0083 ± 0.0003	4%	4%	0.0081 ± 0.0002	2%	1%

include formic acid (analyte 1) and phenol (analyte 21). No general trend in performance metrics was observed for formic acid over the five-day period (RSDs ranged from 6%-19% and relative error ranged from 8%-20%). Thus, compromised accuracy and precision observed for this analyte are presumably due to the fact that it is not well retained and elutes very close to the solvent front. In contrast, a steady decrease in accuracy and precision was observed for phenol over the five-day period, especially at the lower concentration, suggesting that this analyte is not stable under these conditions over the time frame of the experiment. However, the performance metrics for phenol were excellent on day 1 of the study (*i.e.*, RSD and relative error ≤ 10% for both examined concentrations). A secondary observation from this investigation was that performance metrics were generally improved for aromatic analytes as compared to aliphatic acids. This presumably reflects increased sensitivity of the UV detector for aromatic compounds. Finally, it is important to point out that triplicate sample injections were assessed on day 1 of the study, and it was determined that the error associated with the chromatographic step in this analysis is essentially negligible compared to that associated with the cleanup procedure.

Method Application

In order to demonstrate the utility of developed analytical methodology for analysis of biomass hydrolysates, the HPLC method was applied to a corn stover pretreatment sample. Based on previous analyses of lignocellulosic feedstocks,[81-97] it was anticipated that the choice of corn stover would result in aliphatic carboxylic acids, furans and the complete spectrum of ketone, aldehyde, and acidic degradation products derived from all three lignin monomers being present in the sample. Indeed, a wide variety of compounds were represented, as evidenced by the near continuum of peaks in Figure 2.3.

Table 2.4. Quantified Degradation Products in a Corn Stover Hydrolysate.[a]

	Analyte	*Cx* (mM)[b]	RSD[c]	recovery[d]
1	formic acid	2.8 ± 0.1	4%	104%
3	lactic acid	41.0 ± 0.2	1%	100%
4	acetic acid	25.6 ± 0.2	1%	101%
10	levulinic acid	1.5 ± 0.2	13%	103%
13	5-hydroxymethylfurfural	0.701 ± 0.004	1%	104%
15	furfural	18.7 ± 0.1	1%	101%
19	3,4-dihydroxybenzaldehyde	0.066 ± 0.001	2%	97%
20	4-hydroxybenzoic acid	0.021 ± 0.001	5%	96%
22	4-hydroxybenzaldehyde	0.076 ± 0.003	4%	101%
23	vanillic acid	0.034 ± 0.001	3%	104%
24	syringic acid	0.034 ± 0.001	3%	106%
25	vanillin	0.060 ± 0.001	2%	104%
27	syringaldehyde	0.079 ± 0.001	1%	99%
28	4-hydroxy-3-methoxycinnamic acid	0.153 ± 0.003	2%	102%
29	3-hydroxy-4-methoxycinnamic acid	0.034 ± 0.001	3%	110%

[a] See text for details. The criteria employed for analyte identification are described in Materials and Methods (High-Performance Liquid Chromatography Analysis).
[b] Average concentration of analyte determined for a corn stover hydrolysate plus or minus one standard deviation ($n = 5$) in the least significant digit.
[c] Precision measured as the relative standard deviation (R. S. D.) for five replicate measurements.
[d.] Average analyte recovery for a spiked hydrolysate ($[C_S/2C_X] \times 100\%$; where C_S represents the analytical concentration determined for the spiked sample; $n = 3$)

The concentrations of identified degradation products in this sample are given in Table 2.4 and are in general agreement with previous analyses of lignocellulosic hydrolysates.[90-96] The most abundant degradation products appear to be aliphatic acids (especially lactic and acetic acids), furfural, 4-hydroxy-3-methoxycinnamic acid, 5-hydroxymethylfurfural, and 4-hydroxy-3-methoxycinnamic acid with additional analytes present at trace levels. Note that the precision observed for five replicate analyses

of this hydrolysate (RSD in Table 2.4) was not statistically different than that observed for analysis of reference standards constituted in water, despite a significantly more complex background. More significantly, calculated recoveries for a spiked sample (Table 2.4) also suggest that excellent accuracy can be expected for quantitative determination of these analytes in pretreatment samples. Finally, it is important to point out that repeated analysis of this hydrolysate over a five-day period revealed no statistically relevant changes in analyte concentrations suggesting general stability of pretreatment samples over this timeframe when stored at 4 °C.

Conclusions

A relatively simple analytical procedure for the determination of potentially inhibitory degradation products derived from lignocellulosic biomass was developed and validated using 'clean' reference samples. Intra-day and inter-day accuracy and precision assessments combined with application of the procedure to a corn stover hydrolysate confirm that the method is reliable, robust and suitable for analysis of biomass pretreatment samples. The use of liquid chromatography in combination with UV detection should render the method available to a wide variety of users and attract the attention of other researchers investigating biomass pretreatment and microbial inhibition processes. Moreover, recent work describing and modeling kinetics and mass transfer aspects of lignocellulose hydrolysis have focused almost exclusively on production and release of monomer and oligomer sugar products.[102-109] Improved and simplified analytical procedures such as the method reported here will enable more comprehensive analysis of other chemical conversions taking place during the thermochemical pretreatment processes. One potential drawback of the methodology reported here is that

UV detection substantially limits the number of sample components that can be uniquely identified in hydrolysate samples. Continuing work in our laboratory is focused on alleviating this caveat by using liquid chromatography in combination with diode array detection and tandem mass spectrometry.

CHAPTER THREE

Pseudo Reaction Kinetics of Organic Degradation Products in Dilute-Acid-Catalyzed Corn Stover Pretreatment Hydrolysates

Introduction

Limited crude oil supplies and rising oil prices, along with increasing concern about the environmental impact of their use, has increased emphasis on the use of biomass resources for production of fuels and other chemicals currently derived from petroleum.[110,111] For bio-ethanol production, it is important to release fermentable sugars from lignocellulosic biomass.[112,113] Dilute acid pretreatment followed by enzymatic hydrolysis is the typical process used to convert lignocellulosic materials to ethanol.[116-119] However, the hydrolysis temperature, reaction time and acid concentration influence not only the generation of sugars but also the accumulation of a variety of potentially inhibitory degradation products. During acid pretreatment, each individual degradation product has a different sensitivity to acid. Consequently, the degree of accumulation of each degradation product is expected to vary considerably with pretreatment conditions.[120-122] Biomass pretreatments are commonly compared using a mathematical function that combines reaction temperature and time into a single equation.

The severity factor has been much used for reporting on biomass pretreatment, especially in the context of sugar recovery.[123-138] It was first proposed in its commonly used form (Equation 1) by Overend and Chornet in 1987 as the "reaction ordinate," Ro (in minutes):[133]

$$Severity = Ro = t \times e^{\left(\frac{Tr - Tb}{\omega}\right)} \quad (1)$$

in which t is time in minutes, Tr is the absolute reaction temperature, Tb is a base temperature (usually 373 K) and ω (in kelvin, K) is a fitting parameter, which is typically assigned a value of 14.75.

The purpose of the severity function is "to trade duration of treatment and the temperature of treatment such that equivalent final effects…are obtained." [133] In this regard, the severity function expresses a kinetic dependence on temperature that is similar to the Arrhenius dependence of rate on temperature where the rate constant k is related to the absolute temperature T and activation energy E_a. Indeed, the 'P' factor proposed by Brasch and Free,[137] from which the severity function is derived, is a commonly applied approximation to the Arrhenius relation in which reaction rates approximately double for every 10 ºC increase in temperature. This relation can be expressed as:

$$\frac{rate_{Tr}}{rate_{Tb}} = 2^{\left(\frac{Tr - Tb}{10}\right)} \quad (2)$$

in which Tr and Tb are as in equation 1. The similarity in form between Equation 1 and Equation 2 is evident, and Equation 1 expresses a reaction rate that increases by a factor of 1.971 for every 10 ºC increase in temperature when ω = 14.75. Equations 1 and 2 also infer an apparent activation energy that is a function of temperature. According to Chum, Johnson, Black and Overend, [134] the factor ω is related to the activation energy via:

$$\omega = \frac{T_f^2 R}{E_a} \quad (3)$$

in which T_f is a temperature chosen to be in the middle of the experimental conditions (floor temperature, K in kelvin), R is the universal gas constant and E_a is the apparent activation energy.

The severity function was first proposed for aqueous pretreatment (without addition of acid) and implies that overall kinetics follow a first order concentration dependence. To incorporate the effect of varying acid concentration, Chum et al.[138] proposed the combined severity factor (CS), which also assumes a first order rate contribution from the acid catalyst:

$$\text{Combined severity (CS)} = \log(\text{Ro}) - \text{pH} \quad (4)$$

Chum and coworkers found that the combined severity function gave a better fit to acid-catalysed organosolv data than did the pH-independent severity function. In this same study it was reported that for removal of xylan and glucan from aspen wood, a value of $\omega = 11 \pm 1$ in the calculation of CS gave a better fit to experimental results than the commonly applied assumption that $\omega = 14.75$. For removal of lignin, a value of $\omega = 10 \pm 1$ was found to give optimal fit to their data.

There have been several reports seeking to correlate pretreatment severity (Equation 1) or combined severity (CS) (Equation 4) to fermentability of pretreated hydrolysates. Tengborg et al.[139] found that sulfuric acid pretreatment of sprucewood gave optimal sugars near CS 3.0 but that fermentability declined at this combined severity. Larsson et al.[140] conducted an extensive study of dilute acid hydrolysis of sprucewood at 76 different conditions, over a combined severity range of 1.4 to 5.4. Their study looked at concentrations of glucose, mannose, xylose, furfural, 5-hydroxymethylfurfural, acetic acid, formic acid and levulinic acid as well as the

fermentability of the hydrolysates by *Saccharomyces cerevisiae.* Their results showed optimal sugar accumulations between combined severities (CS) of 2.0 and 3.4, maximum concentrations of furans in the vicinity of CS 3.2 to 3.6 and increasing acid concentrations with increasing CS. Fermentability, as measured by ethanol yield and productivity, decreased with increasing CS, with the greatest decreases occurring at approximately CS 3.

Bouchard et al.[125] presented an analysis that characterized the general chemical properties of pretreatment products without identifying individual compounds. Results were presented characterizing qualities such as molecular weight distribution, abundance of O-acetyl groups, or the relative distribution of chemical bond types as determined by FTIR. Decomposition kinetics of xylose, galactose, mannose, glucose, furfural, and 5-hydroxymethylfurfural have been investigated over varying severities toward the end of enhancing methane fermentation.[141-144] Degrees of deacetylation of lignocellulose have also been shown to correlate well to the severity factor.[128]

The objective of experimental work reported in this chapter was to increase understanding of the influence of reaction severity contributions to the accumulation of a wider variety of potential fermentation inhibitors during dilute sulfuric acid pretreatment. Chapter 2 of this dissertation describes the development of an HPLC procedure enabling simultaneous determination of 32 potentially inhibitory compounds in a dilute-acid hydrolysate. In the present study the previously reported protocol was modified to enable screening of 41 target compounds. The accumulation of nineteen ubiquitous degradation products was charted as a function of pretreatment time and temperature, and the resulting data were analyzed using various forms of the severity function. This work

represents the first application of newly developed analytical methodology to the assessment of pretreatment reactions and clearly demonstrates that the method is sufficiently robust and reliable to yield useful information on fundamental aspects of chemical pretreatment.

Experimental: Materials and Methods

Chemicals and Reagents

The solvents and reagents included: acetonitrile = MeCN (HPLC far UV grade, Acros, Fair Lawn, NJ), methyl *tertiary*-butyl ether = MTBE, ammonium bicarbonate (EM Science, Gibbstown, NJ), methanol, sulfuric acid, phosphoric acid (J. T. Baker, Philipsburg, NJ). All chemicals were reagent grade or better and used as received. The internal standard, *para*-*tert*-butylphenoxyacetic acid (Alfa Aesar, Ward Hill, MA) and 41 reference standards (Sigma-Aldrich St. Louis, MO): formic acid, lactic acid, acetic acid, maleic acid, succinic acid, methylmalonic acid, fumaric acid, propanoic acid, levulinic acid, itaconic acid, gallic acid, 5-hydroxymethylfurfural (5-HMF), 2-furoic acid, furfural, adipic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, phenol, 4-hydroxybenzaldehyde, vanillic acid, syringic acid, vanillin, benzoic acid, syringaldehyde, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, 4-hydroxycoumarin, *ortho*-toluic acid, malonic acid, glutaric acid, 2-hydroxy-2-methylbutyric acid, homovanillic acid, caffeic acid, 4-hydroxyacetophenone, *para*-coumaric acid, sinapic acid, and salicylic acid, 2,5-dihydroxybenzoic acid and *para*-toluic acid were purchased in the highest available purity and used as received. Corn stover was supplied by Mark Ruth at the National

Renewable Energy Laboratory in Golden, CO. Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system.

Preparation of Standards

Forty-one reference compounds reflecting a wide range of potential analytes were selected based on previous reports of hydrolysate composition.[77,81-97,145] Reference standards and calibrators were prepared from the group of purchased reference standards using water as the diluent. All solutions were prepared in sufficient quantity to provide replicate analyses for each individual study and stored at 4 °C. The internal standard solution of *para*-*tert*-butylphenoxyacetic acid was prepared at a concentration of 5 mM in methanol. Note that the concentration of internal standard utilized in this work is higher than that employed in Chapter 2. This procedural change was made to minimize the contribution of background absorbance to the integrated peak area of the internal standard.

High-Performance Liquid Chromatography Analysis

The HPLC protocol utilized in this work enabled pretreatment hydrolysates to be screened for 41 target compounds in a single chromatographic run and was only slightly modified from that reported in Chapter 2. Ten additional compounds (malonic acid, glutaric acid, 2-hydroxy-2-methylbutyric acid, homovanillic acid, caffeic acid, 4-hydroxyacetophenone, *para*-coumaric acid, sinapic acid, salicylic acid, and 2,5-dihydroxybenzoic acid) were added to the analyte list, and the HPLC gradient was adjusted to optimize resolution of all target analytes (Table 3.1). Otherwise, sample preparation and analysis followed protocols identical to those reported in Chapter 2.

Representative chromatograms depicting typical HPLC traces for a calibration solution and a hydrolysate are shown in Figure 3.1.

Identification of degradation products in hydrolysate samples was accomplished by comparing UV absorbance and retention time data with reference standards. Quantitation of target analytes was achieved using multipoint calibration curves. Detailed criteria for compound identification and quantitation are described in Chapter 2.

Table 3.1. HPLC Gradient Elution Profile[a]

Time (min)	Mobile phase composition (%)	
	0.05% H_3PO_4	90% Acetonitrile
0	100	0
2	100	0
16.5	90	10
26.5	90	10
39	70	30
43	70	30
64	38	62
65	0	100
100	0	100
120	100	0

[a] Additional chromatographic parameters are defined in the Experimental Section. See text for details.

Experimental Design and Hydrolysate Sample Preparation

Hydrolysis was carried out at three levels of reaction severity, as defined by Overend and Chornet (Equation 1).[133] Twelve experimental conditions were selected with reaction time varied between 2 and 64 minutes and temperature varied between 160 ºC and 200 ºC. Table 3.2 specifies the twelve experimental conditions tested and the

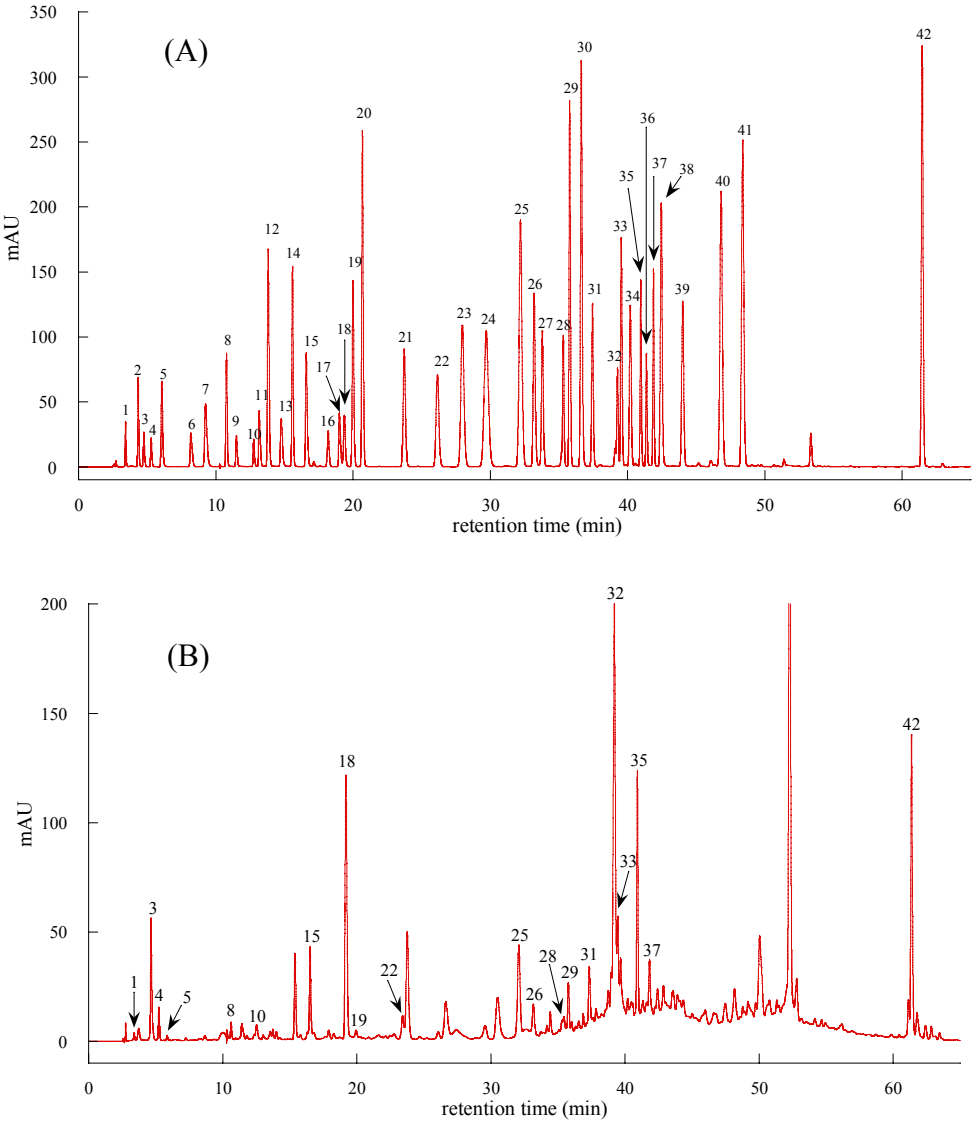


Figure 3.1. (A) Chromatogram generated by HPLC analysis of 41 prepared standards, and (B) Chromatogram generated by HPLC analysis of a hydrolysate sample. Peak identifications are as follows: (1) formic acid; (2) malonic acid; (3) lactic acid; (4) acetic acid; (5) maleic acid; (6) succinic acid; (7) methylmalonic acid; (8) fumaric acid; (9) propionic acid; (10) levulinic acid; (11) glutaric acid;(12) itaconic acid; (13) 2-hydroxy-2-methylbutyric acid; (14) gallic acid; (15) 5-HMF; (16) 2-furoic acid; (17) adipic acid; (18) furfural; (19) 3,4-dihydroxybenzoic acid; (20) 3,5-dihydroxybenzoic acid; (21) 3,4-dihydroxybenzaldehyde; (22) 4-hydroxybenzoic acid; (23) 2,5-dihydroxybenzoic acid; (24) phenol; (25) 4-hydroxybenzaldehyde; (26) vanillic acid; (27) homovanillic acid; (28) caffeic acid; (29) syringic acid; (30) 4-hydroxyacetophenone; (31) vanillin; (32) *para*-coumaric acid; (33) syringaldehyde; (34) benzoic acid; (35) ferulic acid; (36) sinapic acid; (37) 3-hydroxy-4-methoxycinnamic acid;(38) salacylic acid; (39) 4-hydroxycoumarin; (40) *ortho*-toluic acid; (41) *para*-toluic acid, and (42) *para-tert*-butylphenoxyacetic acid.

resulting values of log(Ro). The center point, occurring at a reaction time of 8 minutes and temperature of 180 ºC, was used to calculate activation energy via Equation 3. The corn stover hydrolysate analyzed in this work was generated as described in Chapter 2.[78] However, reactions were carried out in the presence of 0.7% (w/v) sulfuric acid with initial corn stover solids at 10 g/L. These pretreatment conditions are more comparable with envisioned commercial processes than the conditions employed in Chapter 2.

Table 3.2. Experimental Design and Total Concentration of Degradation Products.

Experimental Condition	Temperature (ºC)	Reaction Time (min)	Log (Ro)[a]	Concentration (mM)[b]
1	180	2	2.66	49
2	170	4	2.66	28
3	160	8	2.67	21
4	200	2	3.25	143
5	190	4	3.25	108
6	180	8	3.26	82
7	170	16	3.27	57
8	160	32	3.27	45
9	200	8	3.85	182
10	190	16	3.85	145
11	180	32	3.86	109
12	170	64	3.86	85

[a] Ro: Severity factor (See Equation 1)
[b] Total concentrations of 19 quantified degradation products

Results and Discussion

Effect of Reaction Severity on Degradation Product Accumulation

In an effort to more fully understand the relationship between reaction severity and accumulation of lignocellulosic degradation products in process streams, pretreatment of corn stover was carried out at twelve different conditions of time and

temperature (Table 3.2), and the resulting hydrolysates were screened for 41 target compounds (Figure 3.1A) using HPLC. Nineteen compounds (Figure 3.1B) meeting the identification criteria outlined in Chapter 2 were consistently detected in samples spanning the full range of tested severity conditions. The total concentration of all quantified analytes is listed for each condition in Table 3.2. Accumulated concentrations of individual degradation products are summarized in Figures 3.2 and tabulated in Appendix A.

These data clearly indicate that the effect of temperature on accumulation of pretreatment byproducts is inadequately accounted for in the classic severity function (Equation 1). For example, severity conditions 1-3, 4-8, and 9-12 correspond to variable temperature reactions carried out at constant log(Ro) = 2.66, 3.26 and 3.86, respectively. Since reaction severity in each grouping is constant over all conditions, it would normally be expected that product concentrations would also be essentially constant. This is obviously a faulty expectation, as a general increase in concentration with increasing temperature at constant log(Ro) was observed for most products, independent of their respective level of accumulation. Moreover, in some cases, higher temperature reactions carried out at the low or intermediate severity condition resulted in greater accumulation of a given degradation product than that observed for lower temperature reactions carried out at higher severity (*i.e.*, longer times). These same trends were also preserved when the total concentration of measured degradation products was considered (Table 3.2). The most significant implication of these data is that the severity function, as it is commonly applied, appears to be a poor tool for predicting accumulation trends of biomass degradation products.

Empirical Modifications of the Severity Function

In order to develop a more predictive relationship between pretreatment conditions and degradation product concentrations, the value of ω (the denominator in the exponent of the severity function) needed to be modified. It is important to note that a similar empirical approach for improving the predictive capability of the severity equation was reported by Chum et al.[138] in which they found that ω = 11 offered an

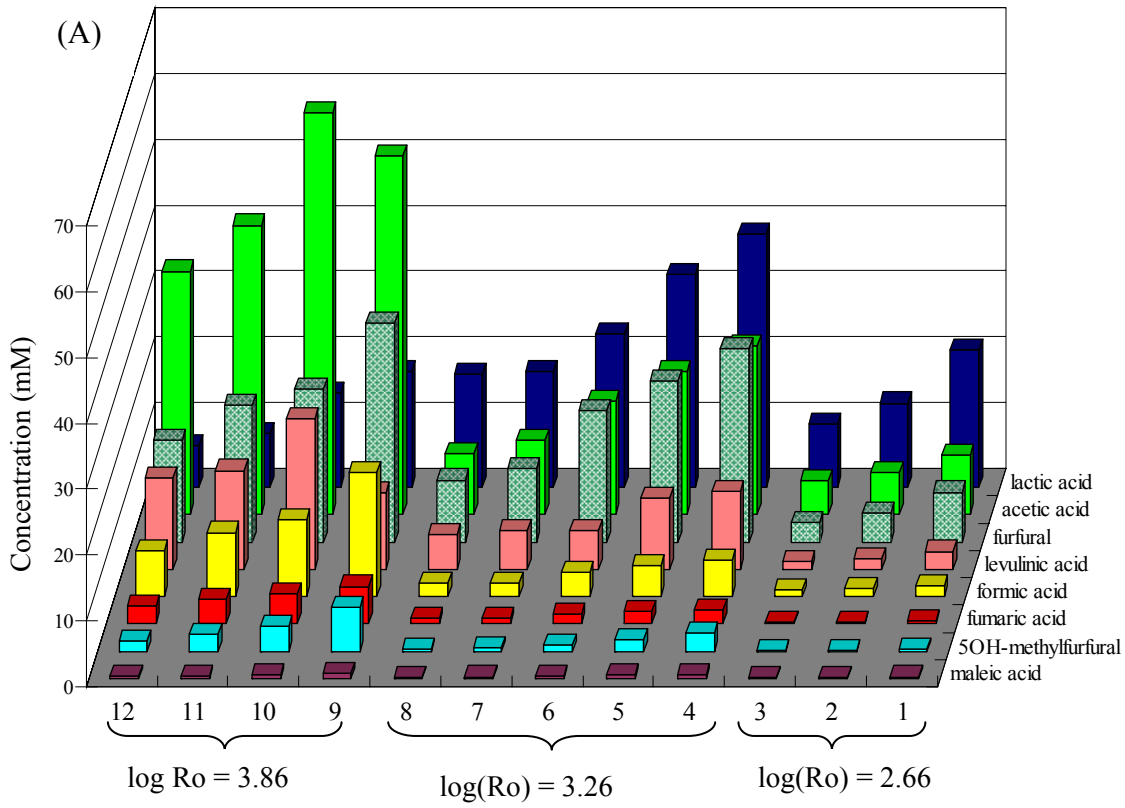


Figure 3.2. (A) Accumulation of degradation products vs temperature at constant severities of log(Ro) = 2.66, 3.26 and 3.86 for higher concentrations of eight identified compounds.

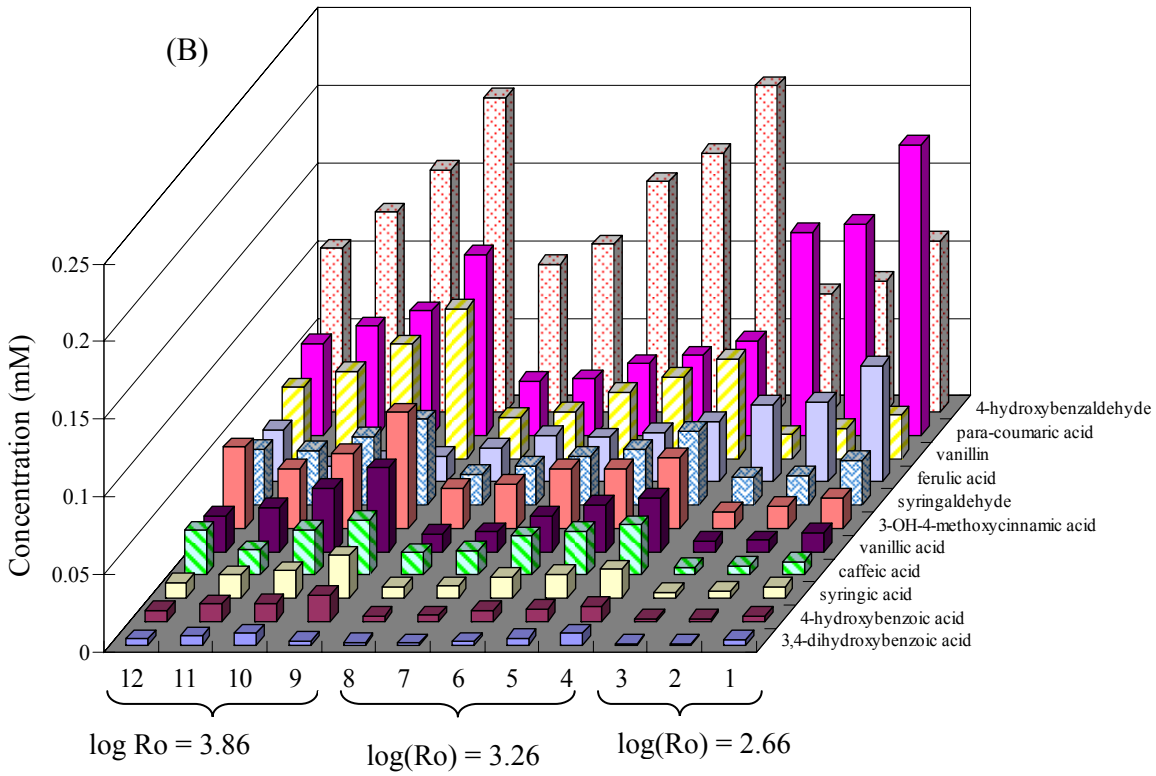


Figure 3.2. (B) Accumulation of degradation products vs temperature at constant severities of log(Ro) = 2.66, 3.26 and 3.86 for lower concentrations of eleven identified compounds.

improved fit of experimental data characterizing dissolution of xylan and glucan while ω = 10 was optimal for dissolution of lignin. Figure 3.3 presents accumulation data for formic acid versus different formulations of the severity function. It can be seen in Figure 3.3A that for ω = 14.75, which is the value commonly used for analysis of biomass pretreatment data, the severity function offers virtually no discrimination between different reaction conditions. However, improved correlations were obtained by decreasing the value of ω. As demonstrated by the correlation coefficients (r^2) shown in Figures 3.3B and 3.3C, an exponent denominator on the order of 9.5 provided the best linear fit of experimental data for formic acid. The observation of a linear correlation for formic acid is likely related to its presence in hydrolysates as a stable end product under

the experimental conditions applied. Consequently, it is expected to accumulate throughout the pretreatment reaction.

Similar treatments of concentration data for alternative degradation products monitored in this work enabled optimization of the exponent denominator ω for all identified compounds. Table 3.3 lists the ω values found to be most effective at providing a correlated response of concentration to reaction severity and the regression coefficient (r^2) for their identified correlation. Plots of r^2 versus ω, justifying the selection of optimized fitting parameters are included in Appendix B for all identified compounds.

An overall outcome of the work summarized in Table 3.3 is that predictive correlations were identified for 13 of 19 analytes. Graphical representations of these optimized correlations are given in Appendix C along with the specific mathematical relationship used to fit experimental data for each compound. Additional degradation products for which linear correlations with reaction severity were initially identified included acetic acid, fumaric acid, and 5-hydroxymethylfurfural. However, as demonstrated in Figure 3.4 and 3.5, analytical concentrations for fumaric acid and 5-HMF at low severities were better explained by a power function than by a linear relationship.

Similarly, observed correlations for the remaining analytes were also found to be non-linear, possibly indicating that these compounds are intermediates in one or more specific degradation pathways. Representative non-linear correlation data are shown for vanillin and *para*-coumaric acid in Figures 3.6 and 3.7, respectively. Note that even the

Table 3.3. ω Value and r^2 for Each Identified Analyte.

Analyte		ω (K)	fit	r^2
1	formic acid	9.5	linear	0.99
3	lactic acid	12	polynomial	0.64
4	acetic acid	11.5	linear	0.95
5	maleic acid	2	logarithmic	0.93
8	fumaric acid	10.5	linear	0.98
	fumaric acid	10.5	power	0.99
10	levulinic acid	11	logarithmic	0.80
15	5-hydroxymethylfurfural	8	linear	0.97
	5-hydroxymethyfurfural	8	power	0.99
18	furfural	5	logarithmic	0.93
19	3,4-dihydroxybenzoic acid	4.5	logarithmic	0.54
22	4-hydroxybenzoic acid	7	power	0.97
25	4-hydrobenzaldehyde	1.5	logarithmic	0.91
26	vanillic acid	6	power	0.97
28	caffeic acid	6.5	logarithmic	0.80
29	syringic acid	4.5	power	0.96
31	vanillin	6	power	0.99
32	*para*-coumaric acid	16	power	0.78
33	syringaldehyde	6	logarithmic	0.86
35	ferulic acid	17	power	0.72
37	3-hydroxy-4-methoxycinnamic acid	8	polynomial	0.97

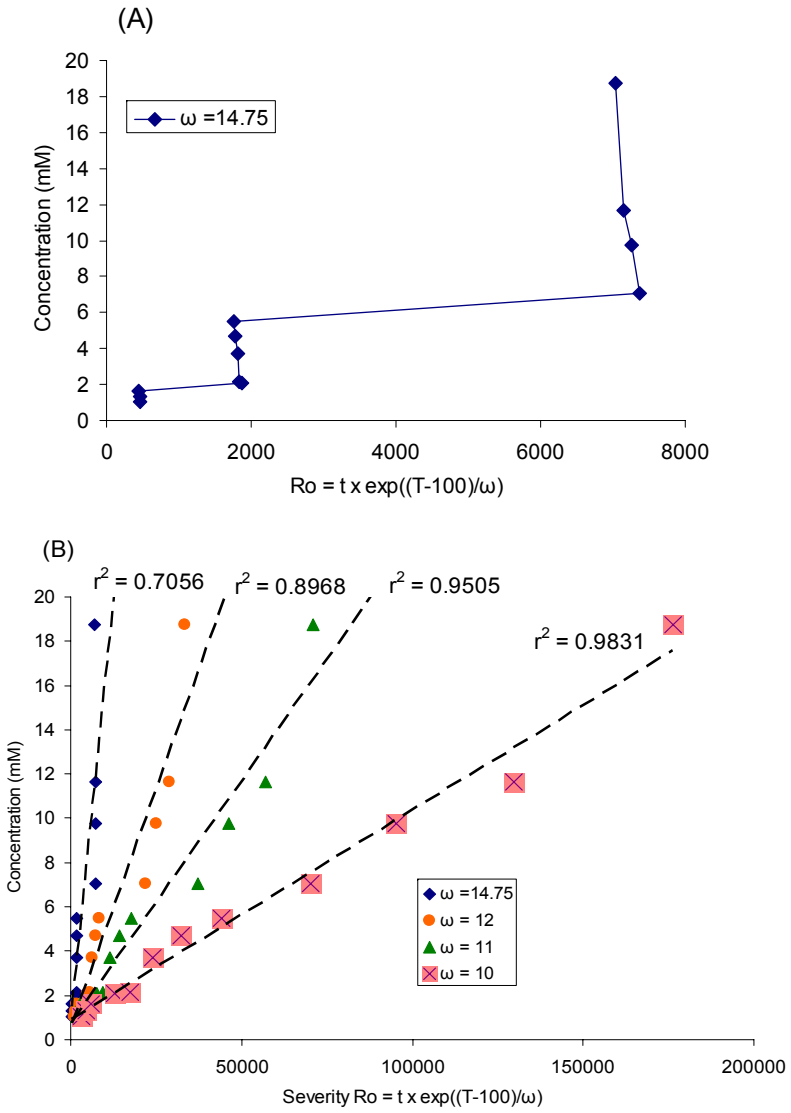


Figure 3.3. Concentrations of formic acid vs reaction severity. (A) ω = 14.75. (B) and (C) A linear fit with different values of ω (14.75, 12, 11, 10, 9.5, 9, and 8) the denominator in exponent term. Plotted points represent averages of triplicate measurements.

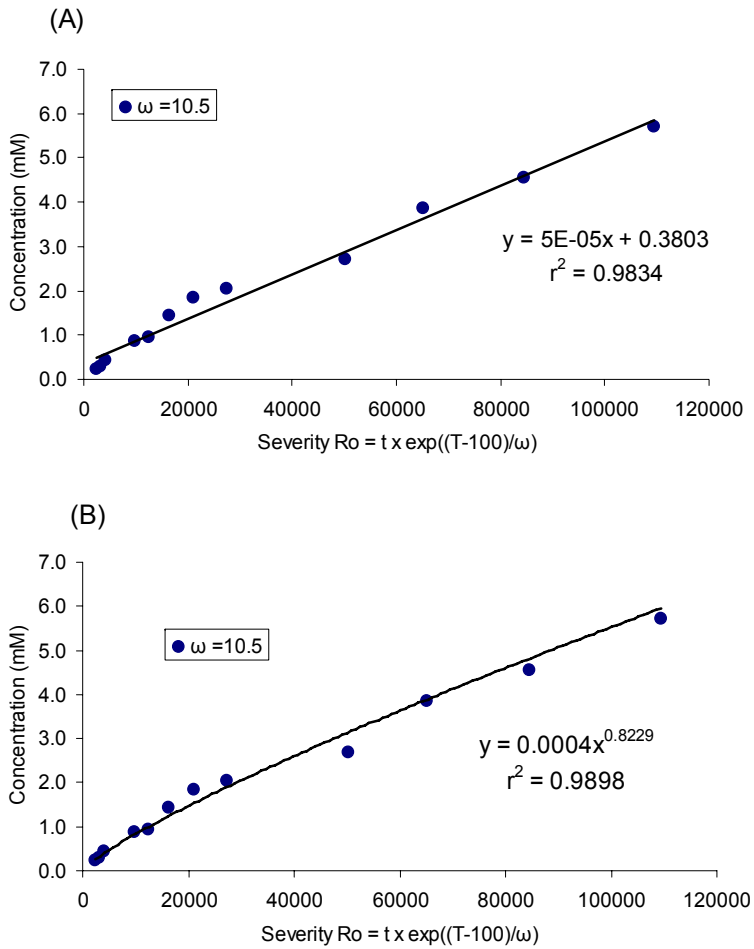


Figure 3.4. Fumaric acid with (A) a linear fit and (B) non-linear fit.

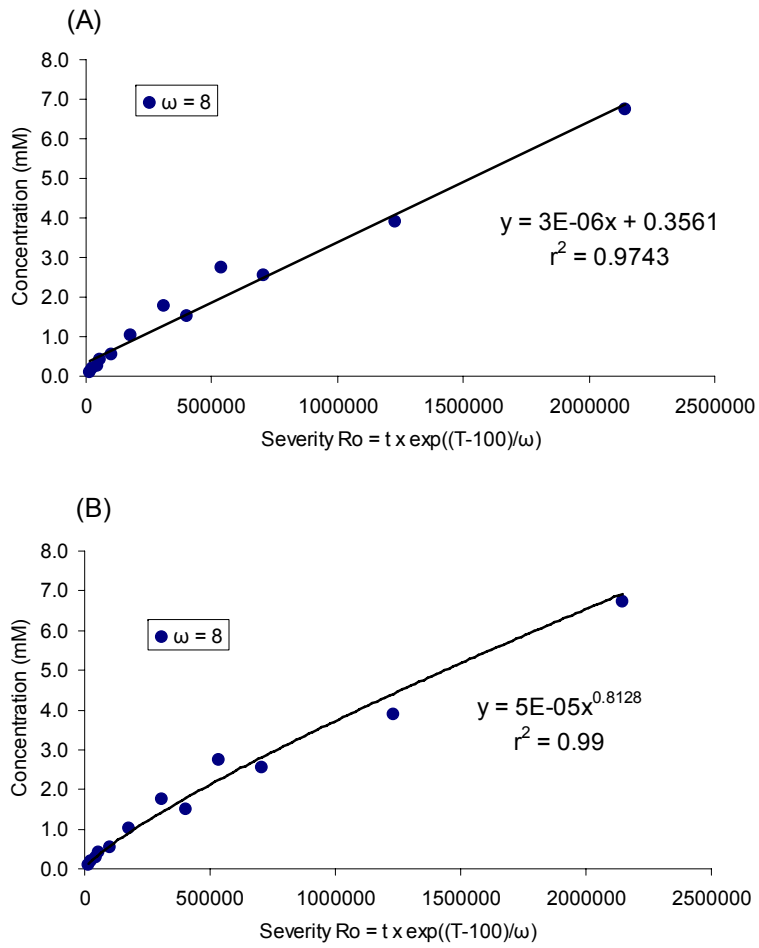


Figure 3.5. 5-HMF with (A) a linear fit and (B) non-linear fit.

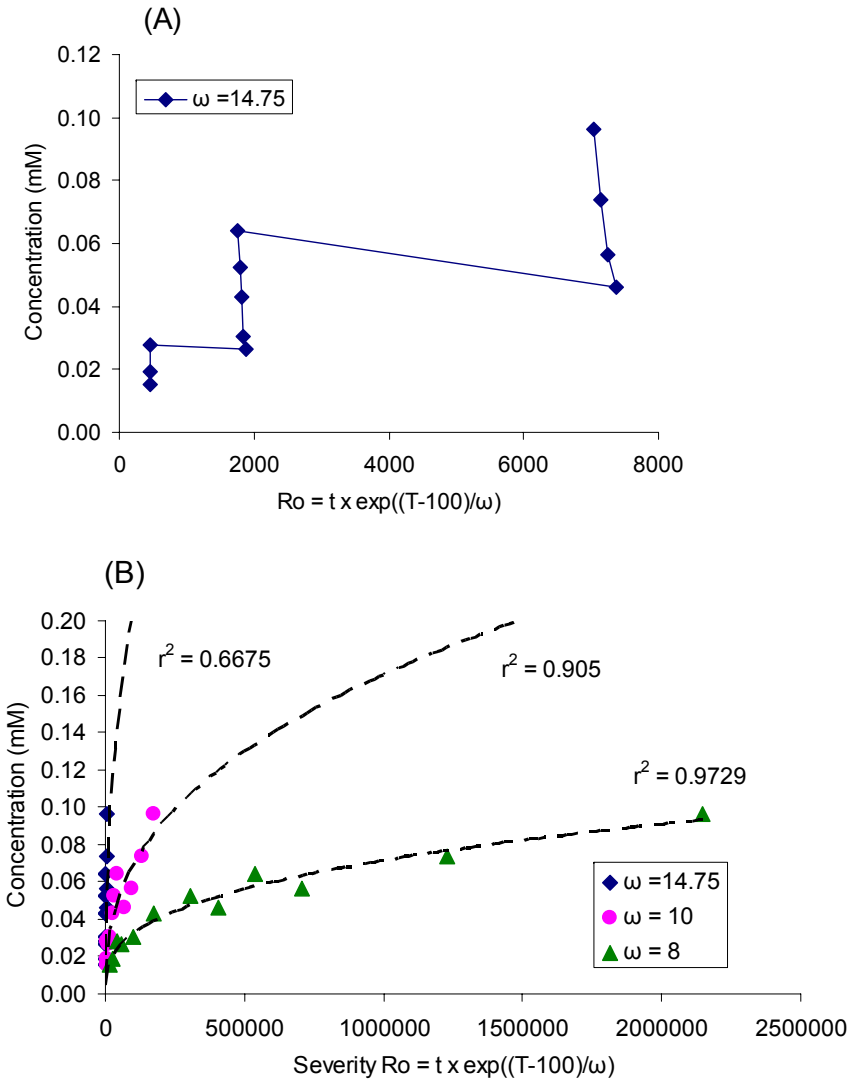


Figure 3.6. Concentration of vanillin vs reaction severity. (A) ω = 14.75, (B) and (C) A non- linear fit with different values of ω (14.75, 10, 8, 7, 6, and 5), the denominator in exponent term. Plotted points represent averages of triplicate measurements.

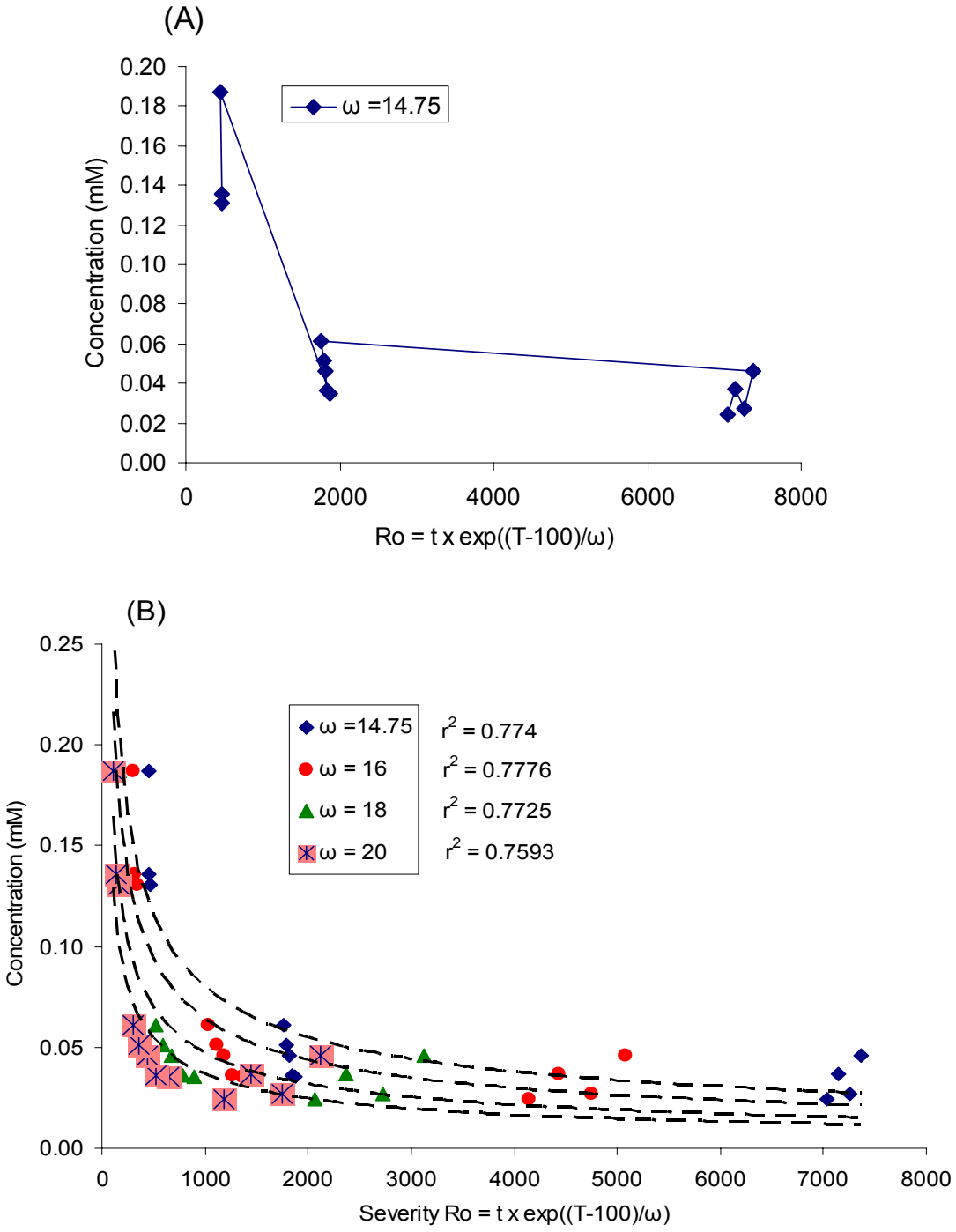


Figure 3.7. Concentration of *para*-coumaric acid vs reaction severity. (A) ω = 14.75. (B) A non-linear fit with different values of ω (14.75, 10 and 8), the denominator in exponent term. Plotted points represent averages of triplicate measurements.

optimized correlation for *para*-coumaric acid (ω = 16) provides relatively poor discrimination between reaction conditions at constant severity. The relatively poor predictive ability of this fit to experimental data is also reflected in the correlation coefficient (r^2 = 0.78). Poor fitting ($r^2 \leq 0.8$) was also observed for lactic acid, levulinic acid, 3,4-dihydroxybenzoic acid, caffeic acid, and ferulic acid. Thus, it is unlikely that optimal correlations identified for these compounds in Table 3.3 will be beneficial in predicting accumulation trends in future work.

A final observation from Table 3.3 is that all optimized values of ω resulting in acceptable fitting of experimental data ($r^2 \geq 0.85$) were less than 14.75. This result implies that the traditional severity function (Equation 1 with ω = 14.75) underestimates the temperature contribution to degradation product accumulation. Indeed, analyses of covariance between concentration and temperature and between concentration and reaction time indicated that temperature was the dominant factor influencing accumulation of compounds for which optimized ω values fell below 14.75. Thus, it is likely that the reactions resulting in the accumulation of most compounds measured in this study have higher activation energies than predicted by the standard severity function. This can be seen by applying Equation 3 to ω values identified in Table 3.3.

Reaction Kinetics

Overend and Chornet [133] are clear that the severity function should not be used to infer reaction mechanisms in biomass pretreatment, as clearly these correlations are strictly empirical. That said, it remains true that several of the compounds measured demonstrate accumulation trends that appear to be first order with respect to the reaction ordinate. Of the compounds quantified in this study, formic acid, acetic acid, fumaric acid

and 5-HMF demonstrated initial accumulation trends over the reaction times measured that were sufficiently linear to enable calculation of initial reaction rates. The linearity of these data was assessed assuming a non-zero concentration at time zero (*i.e.*, the time at which reaction timing began at the desired reaction temperature). Some reaction would have occurred during the preheating period (see Experimental Section for details). Therefore, the concentration at time zero was determined by an average regression from the five temperature experiments. Figure 3.8 illustrates representative kinetic data for formic acid. This and similar plots for acetic acid, fumaric acid, and 5-HMF were used to determine initial rates (via the slopes) at each reaction temperature. (It is acknowledged that more data points and shorter reaction durations would strengthen the certainty of kinetic analyses.)

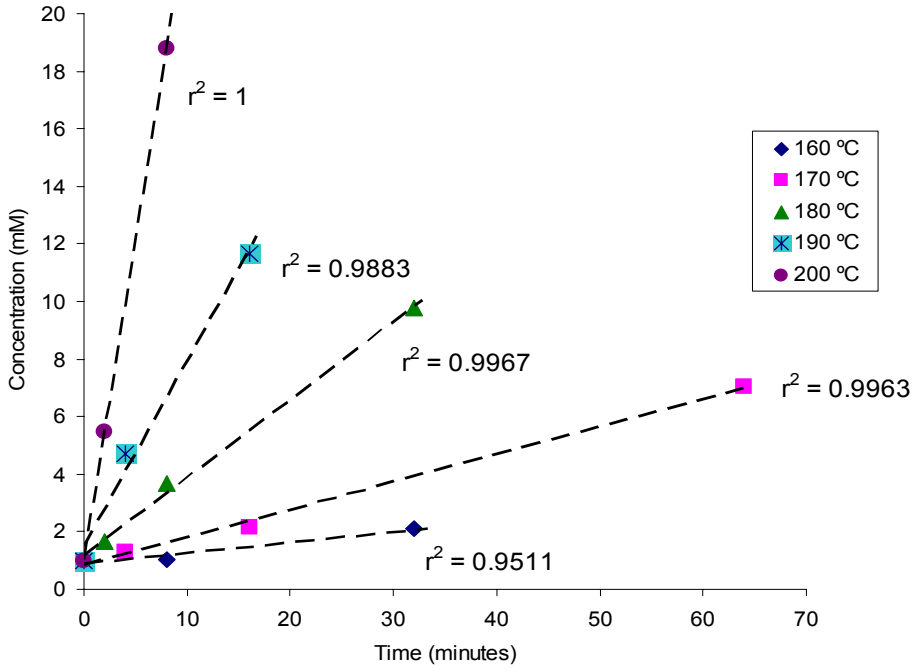


Figure 3.8. Accumulation of formic acid vs time at different temperatures. Point at time zero was estimated, not measured.

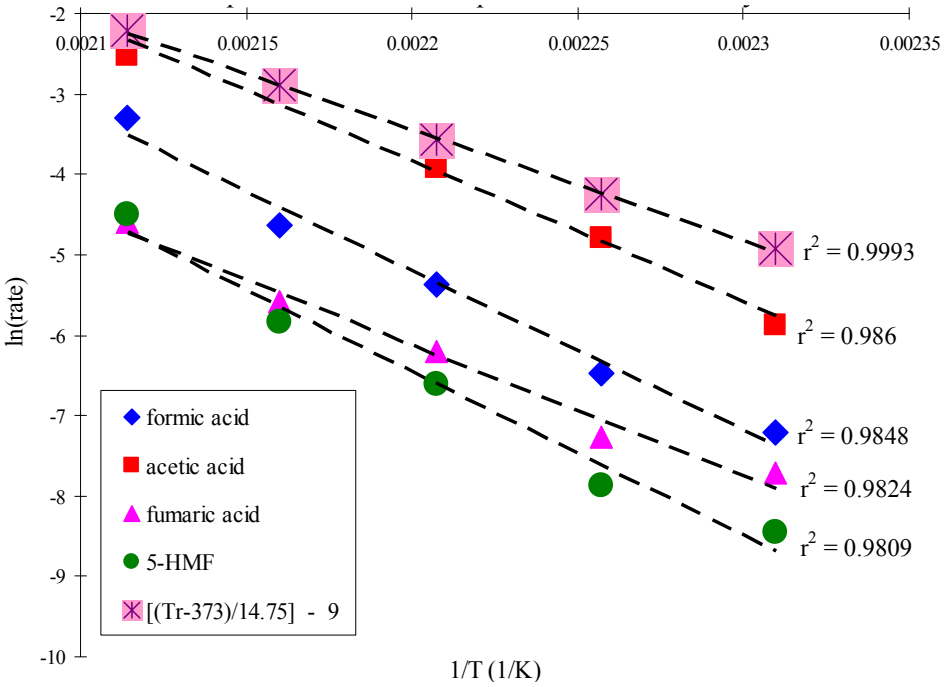


Figure 3.9. Arrhenius plots for formic acid, acetic acid, fumaric acid and 5-HMF.

Arrhenius plots (Figure 3.9) were subsequently constructed to determine the effective activation energy E_a demonstrated by formic acid, acetic acid, lactic acid, and 5-HMF. Activation energies were calculated via the relationship slope = $-E_a/R$ (where R = 8.314 kJ mol^{-1} K^{-1}) and are listed in Table 3.4. Also shown in Table 3.4 are the corresponding activation energies associated with the classical severity function (Ro) over this same temperature range as well as values predicted by Equation 3 using optimized values of ω identified in Table 3.3 for each compound. It can be seen that for all four compounds, the experimental activation energy is higher than that assumed by the severity function and in rough agreement with the values calculated via Equation 3. These data collectively suggest that formic acid, acetic acid, lactic acid, and 5-HMF display behavior consistent with higher activation energies than are inherent in the standard severity function (Equation 1). This hypothesis may also be applicable to

alternative compounds measured in this study that exhibited improved correlation with reaction severity when ω was adjusted below 14.75.

Table 3.4. Activation Energies Determined via Arrhenius Plots

Analyte	Slope (160 – 200 °C) [b]	E_a (kJ / mol) [c]	E_a via eq'n 3(kJ / mol) [d]
Formic acid	-19727	164	180
Acetic acid	-17601	146	148
Fumaric acid	-16254	135	163
5-HMF	-20326	168	213
Ro [a]	-13880	115	116

[a] Ro (min): Severity factor (See Equation 1). ω (K) = 14.5, T*b* = 373 K
[b] Slope obtained from Arrhenius plots. See Figure 3.9.
[c] E_a = -(Slope/R), R = universal gas constant.
[d] E_a is calculated according to Equation 3. ω (K) = 14.5, T_f (floor temperature) = 453 K, R = universal gas constant.

Conclusions

The severity function as it is commonly described functioned poorly as a means of discriminating between different reaction conditions making use of variable combinations of temperature and reaction time. It was found that most of the products measured demonstrated kinetics that indicated a greater influence of temperature on their rate of accumulation than is predicted by the widely used severity function. It was found that manipulation of the temperature contribution to the severity function could in some cases result in a monotonic response of product accumulation to reaction severity, and that this required manipulation was different for different compounds. Thus, there appears to be no one severity function that can describe a universal effect on accumulation trends for these various products. Kinetic calculations carried out on four compounds that demonstrated linear initial accumulation rates indicated that in these four

cases, the calculated activation energy E_a was higher than that assumed by the standard severity function.

CHAPTER FOUR

Compositional Analysis of Water-Soluble Materials in Corn Stover

Introduction

Corn stover has been identified as a promising feedstock for biomass-to-ethanol conversions.[11,146-148] The term, corn stover, refers to the above-ground portion of a corn plant (i.e., leaves, cobs, husks, and stalks) that remains after corn has been harvested for grain. It has been estimated that greater than 60 million tons of corn stover can be sustainably collected and used to produce over 3 billion gallons of ethanol transportation fuel on an annual basis.[11] Additional attributes of corn stover for biomass-to-ethanol processing include its proximity to existing grain-to-ethanol production facilities[146,147] and its amenability to conventional harvesting practices.[148]

Any technical or economic valuation of a feedstock is inherently dependent upon detailed knowledge of its chemical composition. Accepted analytical procedures for compositional analysis of biomass[149] enable near-quantitative mass closure on a dry-weight basis. However, total water- and/or ethanol-soluble materials are typically quantified gravimetrically and identified only as extractives.[150-153] Previous work has demonstrated that extractives can affect macrocomponent compositional determinations affiliated with analysis of herbaceous biomass (*e.g.*, Klason lignin, total glucan, ash, protein).[153] Most notably, it was demonstrated that hot water extraction prior to analysis resulted in a significant reduction in the measurable glucan content of corn stover, fescue and switchgrass. Additionally, it has been postulated that constituents of the extractive

fraction are potent fermentation inhibitors in biomass-to-ethanol conversion.[34,35] These results provide strong impetus to further investigate the composition of water-soluble materials in herbaceous feedstocks.

The objective of the present study was to characterize unknown water-soluble constituents of corn stover. Five representative samples with diverse chemical composition were extracted and investigated in a side-by-side comparison. Multiple analytical protocols were developed over the course of the study, collectively affording identification and quantitative assessment of over 30 chemical constituents that were common to each sample. Compositional data for carbohydrates, alditols, organic acids, inorganic ions, and a tentatively-identified oligomeric fraction of aqueous extracts are reported as a percentage of total water-soluble materials in corn stover, and results are interpreted in terms of their potential impact on biomass processing, feedstock storage, and future analyses of feedstock composition.

Experimental: Materials and Methods

Corn Stover Feedstocks and Chemical Reagents

Feedstocks were supplied by the National Renewable Energy Laboratory (NREL), Golden, CO. Five samples (NREL sample ID: Kramer 33A14, 2302-079, 2870-061, 2302-115 and 2893-026) were selected from a larger collection of corn stover feedstocks at NREL.[147,151] A brief history of each stover is provided in Table 4.1. The only criterion employed in selecting feedstocks for the present study was diverse chemical composition. All chemicals and reference standards were reagent grade or better, obtained from

commercial vendors, and used as received. Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system.

Table 4.1. Historical Data for Analyzed Corn Stover Feedstocks.

NREL ID	Seed Company	Location Grown	Harvest Year
Kramer 33A14	Pioneer Hi-Bred International	Wray, CO	2002
2302-079	Monsanto	Oskaloosa, IA	1997
2870-061	Syngenta Seed Company	Fond du Lac, WI	1997
2320-115	Monsanto	Perry, IA	1997
2893-026	Pioneer Hi-Bred International	Fond du Lac, WI	1997

Sample Preparation

Each stover sample was 'milled' for 90 seconds using a commercial coffee grinder. Milled stover was subsequently screened using a 40-mesh sieve, and material passing through the sieve was collected and utilized in compositional determinations. It was assumed that chemical fractionation does not occur during the sieving step.

Water Extraction

In a typical extraction, 8 g of sieved stover was added to a Soxhlet thimble and extracted for 10 hours as described in NREL Laboratory Analytical Procedure (LAP) Determination of Extractives in Biomass.[150] Heating was adjusted to achieve a siphon rate of 4-5 cycles per hour. Aqueous extracts generated for compositional determinations were quantitatively transferred to volumetric flasks and diluted to 200 mL prior to analysis. For determination of percent extractives, aqueous extracts (prepared from independent water extractions) were evaporated under N_2 at 40 °C using a Zymark Turbovap LC concentration workstation (Zymark Corp., Hopkinton, MA), and residues

were dried to constant weight in a vacuum oven at 40 °C. The moisture content of each feedstock was independently determined using the "convection oven method" described in NREL LAP Determination of Total Solids in Biomass.[154] Approximately 1 g of each sieved stover was placed in a pre-weighed aluminum weighing dish and dried to constant weight at 105 °C. Percent total solids, defined as:

$$\% \text{ Total Solids} = \frac{\text{Weight}_{\text{dry pan plus sample}} - \text{Weight}_{\text{dry pan}}}{\text{Weight}_{\text{sample}}} \times 100$$

was calculated for each tested feedstock and used to determine the oven dry weight (ODW) for each extracted sample:

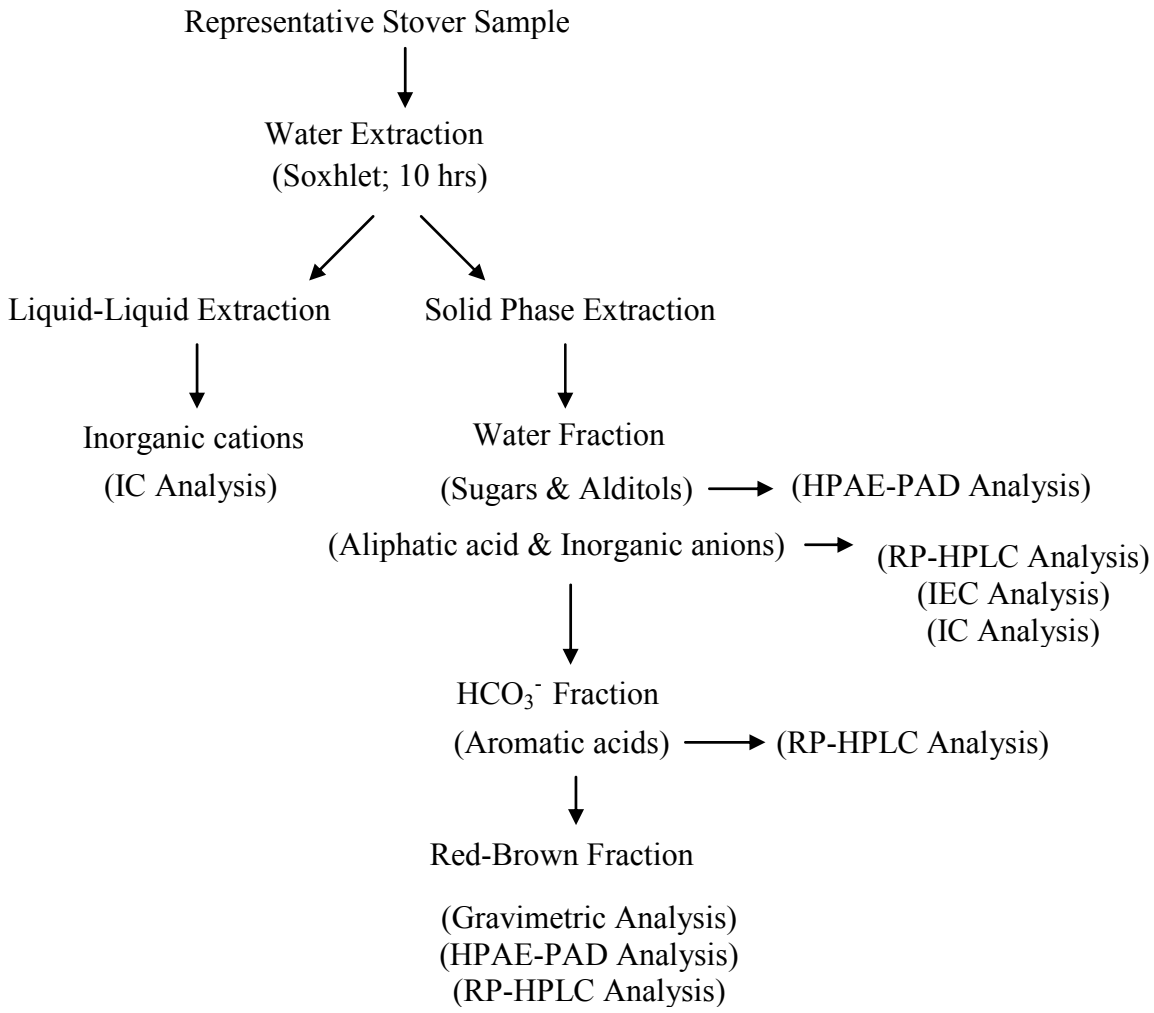
$$\text{ODW} = \frac{(\text{Weight}_{\text{thimble plus sample}} - \text{Weight}_{\text{thimble}}) \times \% \text{ Total solids}}{100}$$

Percent extractives was subsequently calculated using the equation below:

$$\% \text{ Extractives} = \frac{\text{Weight}_{\text{dry flask plus extractives}} - \text{Weight}_{\text{dry flask}}}{\text{ODW}_{\text{sample}}} \times 100$$

Fractionation and Compositional Analysis of Aqueous Extracts

The analytical approach employed for identification and quantitation of water-soluble materials in corn stover involved fractional clean-up of aqueous extracts followed by one or more chromatographic analyses. Details of clean-up procedures and affiliated chromatographic methods developed during this study are described below with specific reference to the class of compounds assessed using each experimental protocol. A summary of the employed analytical approach is given in Scheme 1.



Scheme 1. Systematic approach for fractionation and compositional analysis of aqueous extracts.

Water Fraction: Sugars and Related Alditols

A 2-mL aliquot of aqueous extract was loaded onto a Supelclean ENVI-Chrom P solid phase extraction (SPE) cartridge that had been preconditioned with 15 mL methanol followed by 15 mL water. The cartridge was rinsed with slightly less than 23 mL water, and the combined eluate was diluted to 25 mL in a volumetric flask. After a subsequent 5-fold dilution, the sample was analyzed for monomeric sugars and related alditols via high performance anion-exchange chromatography with pulsed amperometric detection

at a disposable gold disk electrode (HPAE-PAD). A second aliquot from the diluted eluate was also screened for sucrose, glucose, and fructose (by Lekh Sharma) using a colorimetric enzyme assay available commercially from R-Biopharm. Spectroscopic monitoring affiliated with the enzyme assay was carried out on a Perkin-Elmer Model Lambda 35 UV-visible spectrophotometer.

Assessment of oligosaccharides was conducted using a procedure similar to that described in NREL LAP Determination of Sugars, Byproducts, and Degradation products in Liquid Fraction Process Samples.[155] Briefly, a 5 mL aliquot was taken from the 25 mL sample described above and hydrolyzed with 4% sulfuric acid at 121 °C for one hour. A series of sugar recovery standards were treated in the same manner to correct for potential degradation. Once the samples cooled to room temperature, the pH was adjusted to 5-6 with calcium hydroxide, and the resulting mixtures were loaded onto preconditioned Supelclean ENVI-Chrom P SPE cartridges. Each cartridge was rinsed with water and eluates were diluted to 25 mL. These samples were transferred directly to autosampler vials and analyzed for sugar content (*i.e.*, the sum total of monosaccharides present in hydrolysate resulting from treatment of aqueous extracts with sulfuric acid at elevated temperature and pressure) using the HPAE-PAD method. The amount of water-soluble oligomeric sugar present in analyzed stover samples was assessed by subtracting the summative monosaccharide content (excluding fructose) measured in native aqueous extracts from the total sugar content measured in the corresponding hydrolysate.

All HPAE-PAD analyses were carried out on a Dionex DX-600 series liquid chromatograph equipped with a DG2410 degassing module, GP50 gradient pump, AS3500 autoinjector (10 μL sample loop), LC30 chromatography oven, and ED40

electrochemical detector. The target sugars and related alditols were detected using quadruple-potential waveform pulsed amperometry using conditions specified in Table 4.2. Chromatographic separation (Figure 4.1) was achieved at 27 °C using a 50 mm × 4 mm CarboPac PA100 guard column and two 250 mm × 4 mm CarboPac PA100 analytical columns connected in series and isocratic elution (mobile phase = aqueous 10 mM NaOH at 0.8 mL/min).

Table 4.2. Detection Waveform for Carbohydrates and Alditols

Potential (V)		Time (sec)	Integration
E1	+0.10	0.00	
	+0.10	0.20	Begin
	+0.10	0.40	End
E2	-2.0	0.41	
	-2.0	0.42	
E3	+0.60	0.43	
E4	-0.10	0.44	
	-0.10	0.50	

Water Fraction: Aliphatic Acids and Inorganic Anions

An independent 2-mL aliquot of aqueous extract was loaded onto a second preconditioned Supelclean ENVI-Chrom P SPE cartridge (see above). The cartridge was rinsed with slightly less than 3 mL water and the combined eluate was diluted to 5 mL in a volumetric flask. Polar organic acids and inorganic anions in this sample were analyzed via ion chromatography (IC) with suppressed conductivity detection (ASRS-ULTRA 4 mm Suppressor). Chromatographic separation (Figure 4.2A) was carried out

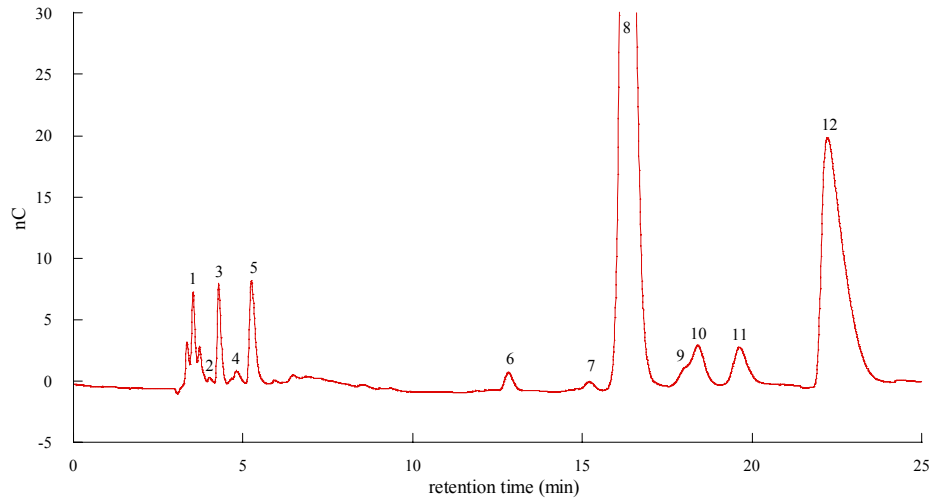


Figure 4.1. Representaive chromatogram for sugars and related alditols. 1, glycerol; 2, mannitol; 3, arabitol; 4, sorbitol; 5, xylitol; 6, arabinose; 7, galactose; 8, glucose; 9, mannose; 10, xylose; 11, sucrose; 12, fructose.

at 30 °C using a 50 mm × 4 mm IonPac AS11-HC guard column and 250 mm × 4 mm IonPac AS11-HC analytical column connected in series and gradient elution (1-200 mM aqueous KOH; flow rate, 1.2 mL/min): 1 mM KOH to 30 mM in 25 minutes, step to 50 mM at 25.1 minute, step to 100 mM at 35.1 minute, and step back to 1 mM at 40.1 minute. In each chromatographic run the suppressor current was held constant at 150 mA from 1 to 35 minutes and stepped to 340 mA after 35 minutes. Complementary screening of aliphatic acids in this sample was also conducted via independent ion-exclusion (IEC) and reversed-phase (RP-HPLC) separations with UV detection at 210 nm. Ion-exclusion separations (Figure 4.2B) were carried out at 55 °C using a 300 mm × 7.6 mm IC-Pak column (Waters Corp., Milford, MA) and isocratic elution (0.01 N aqueous H_3PO_4). Reversed-phase separations (Figure 4.2C) were carried out using the procedure described below for analysis of aromatic acids. All three chromatographic analyses were carried out on a Dionex DX-600 series liquid chromatograph equipped with a DG2410 degassing

module, GP50 gradient pump, AS50 autoinjector (10 μL sample loop), LC30 chromatography oven, UVD170U multi-wavelength ultraviolet detector, and ED40 electrochemical detector.

Water Extract: Inorganic Cations

An independent 1-mL aliquot of aqueous extract was combined with an equal volume of 100 mM aqueous methanesulfonic acid and extracted with 1 mL *n*-butanol. The clear, colorless aqueous phase was analyzed directly for cations via ion chromatography with suppressed conductivity detection (CSRS-ULTRA 4 mm Suppressor at a 125 mA). Chromatographic separation (Figure 4.3) was achieved at 40 °C using a 50 mm × 5 mm IonPac CS16 guard column and 250 mm × 5 mm IonPac CS16 analytical column connected in series and isocratic elution (mobile phase = 48 mM aqueous methanesulfonic acid at 1 mL/min).

Bicarbonate (HCO_3^-) Fraction: Aromatic Acids

The SPE cartridge used to isolate sugars and related alditols from the aqueous extract was subsequently rinsed with 10 mL of an aqueous 2% sodium bicarbonate solution. The eluate was collected and acidified to pH 1.8 with phosphoric acid. This sample was extracted with methyl-*tertiary*-butyl ether and screened for a variety of aromatic acids (and alternative lignocellulosic degradation products) using a previously reported reversed-phase liquid chromatography (RPLC) procedure in Chapter 2. Chromatographic separation (Figure 4.4) employed an RP 18 Opti-Guard column (Alltech, Deerfield, IL) and 250 mm × 4.6 mm YMC Carotenoid S-3 analytical column

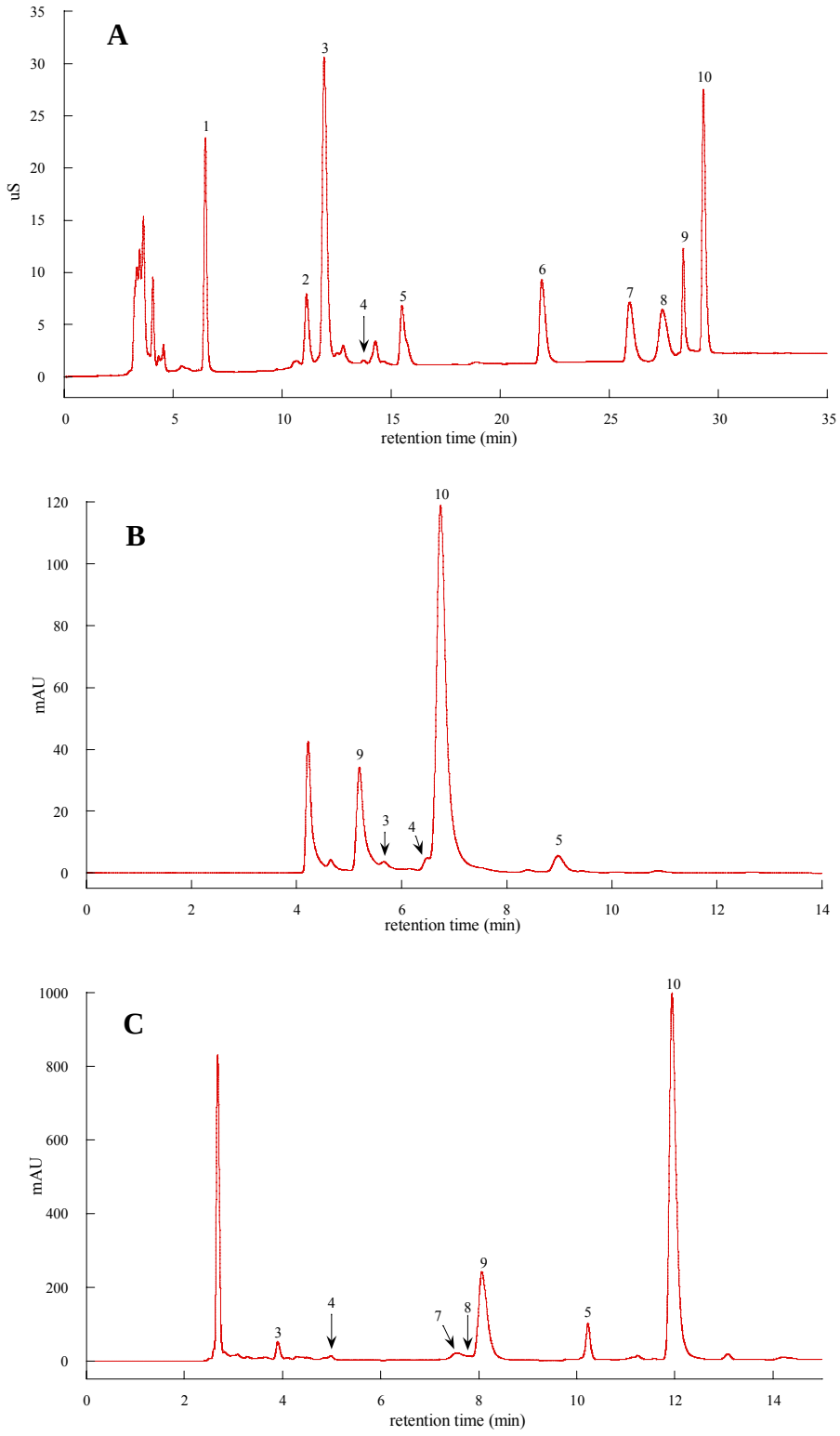


Figure 4.2. Representative chromatograms resulting from analysis of (A) IC, (B) IEC, and (C) RP-HPLC 1, chloride; 2, nitrate; 3, malic acid; 4, maleic acid; 5, fumaric acid, 6, phosphate; 7, citric acid; 8, isocitric acid; 9, *cis*-aconitic acid; and 10, *trans*-aconitic acid.

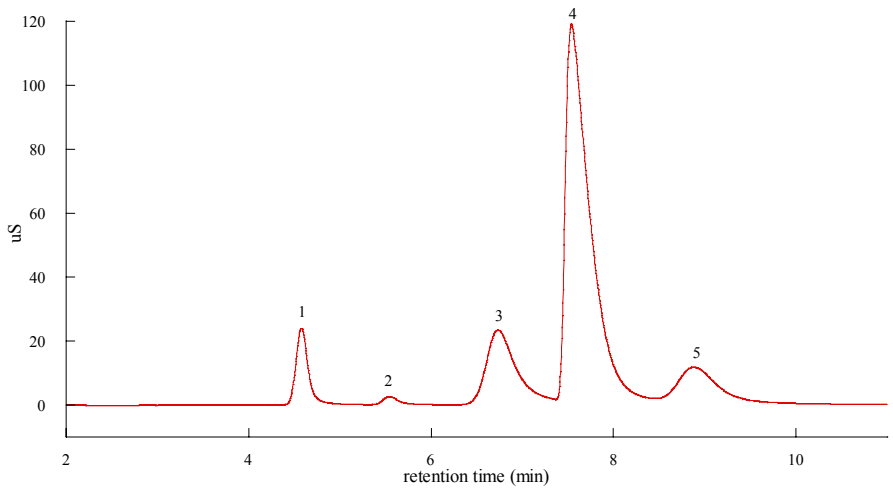


Figure 4.3. Representative chromatogram for inorganic cations. 1, sodium; 2, ammonium; 3, magnesium; 4, potassium; and 5, calcium.

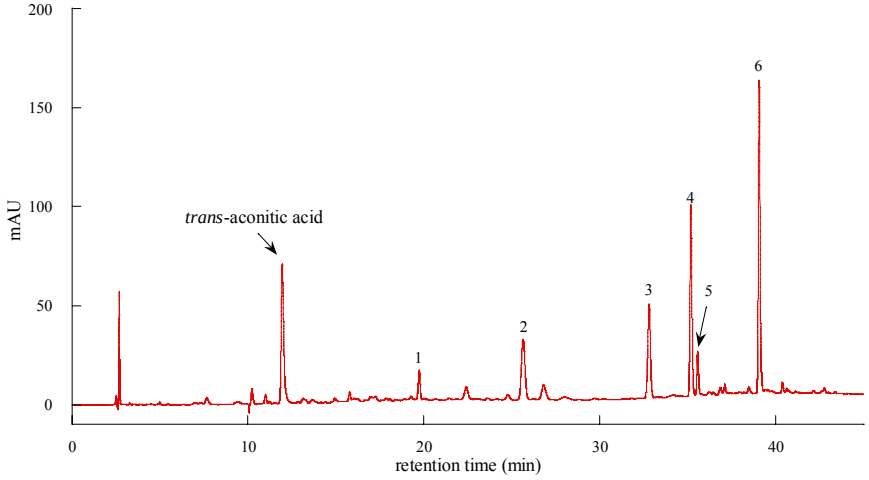


Figure 4.4. Representative chromatogram for aromatic acids. 1, 3,4-dihydroxybenzoic acid; 2, 4-hydroxybenzoic acid; 3, vanillic acid; 4, caffeic acid; 5, syringic acid; 6, *para*-coumaric acid.

(Waters Corp., Milford, MA) connected in series and a non-linear gradient, consisting of 0.05% (v/v) aqueous H_3PO_4 and acetonitrile.

Red-Brown Fraction

Following successive rinses with water and 2% aqueous sodium bicarbonate, a narrow band of material that was reddish-brown in color remained at the head of the SPE cartridge used to isolate carbohydrate and aromatic-acid fractions from the aqueous extract. The band was eluted with 5 mL 1:1 water-acetonitrile, the solvent was evaporated under N_2 at 40 °C, and the quantity of remaining non-volatile material was assessed gravimetrically. Qualitative analyses directed at characterization of material(s) in the 'red-brown' fraction were also performed. The residue remaining after solvent evaporation was initially reconstituted in 5 mL water, extracted with MTBE and analyzed 4% sulfuric acid at elevated temperature (as described above) and analyzed via HPAE-PAD. A third 5 mL sample was treated with 4% sulfuric acid and subsequently extracted with 95:5 *n*-butanol:hydrochloric acid using a previously reported procedure.[156] Following phase separation, the colored *n*-butanol phase was back-extracted with 2% aqueous sodium bicarbonate, and the resulting aqueous extract was analyzed via RPLC.

Identification and Quantitation of Analytes

The criteria employed for analyte identification varied with compound class. Monomeric sugars, alditols, and inorganic ions were assigned based on retention time and an expected doubling of analyte concentration (± 20%) in subsequent analyses of spiked samples. Although retention time is not a unique qualifier of identity, this approach was deemed acceptable due to the unique nature of the analytical response expected for

individual compounds when pulsed amperometry or conductivity is employed for analyte detection. In contrast, assignment of aliphatic acids required confirmation of a retention time match with a reference standard in three complementary separations (*i.e.*, anion exchange, ion exclusion, and reversed phase). Identification of aromatic acids was based on a comparison of retention time and UV absorbance data with reference standards as described previously in Chapter 2. As evidenced by the chromatograms in Figures 4.1-4.4, this approach resulted in assignment of nearly all major peaks in sample fractions derived from water extracts. Table 4.3 summarizes the various components that were identified along with the analytical tool(s) used to support assignment of their identity.

Quantitation methods also varied with compound class. Sugars and related alditols were determined via HPAE-PAD using multipoint, external standard calibration curves. External standard calibration curves were also employed in the determination of aliphatic acids and inorganic ions via ion chromatography. In contrast, aromatic acids were determined using an internal standard calibration approach. Constituents in the strongly-retained 'red-brown' fraction were quantified gravimetrically. Mean values reported in Tables 4.4-4.8 were based on triplicate extractions of corn stover, and excepting aromatic acids, assume quantitative recovery of analytes in all sample preparation steps.

Results and Discussion

Mass Balance for Water-Soluble Materials

Water-soluble materials accounted for as much as 27% of the dry weight of corn stover feedstocks utilized in this study, clearly justifying the importance of understanding

Table 4.3. Summary of Major Components Found in Corn Stover Extracts

Identified Components	HPAE-PAD[a]	RP-HPLC[b]	AX[c]	IEC[d]	CX[e]	UV[f]
Sugar/Alcohols						
glycerol	√					
mannitol	√					
arabitol	√					
sorbitol	√					
xylitol	√					
arabinose	√					
galactose	√					
glucose	√					
mannose	√					
xylose	√					
sucrose	√					
fructose	√					
Organic Acids						
maleic acid		√	√	√		
malic acid		√	√	√		
fumaric acid		√	√	√		
cis-aconitic acid		√	√	√		
trans-aconitic acid		√	√	√		√
citric acid		√	√			
isocitric acid		√	√			
3,4-dihydroxybenzoic acid		√				√
4-hydroxybenzoic acid		√				√
vanillic acid		√				√
caffeic acid		√				√
syringic acid		√				√
para-coumaric acid		√				√
Inorganic Anions			√			
chloride			√			
nitrate			√			
phosphate						
Inorganic Cations						
sodium					√	
ammonium					√	
calcium					√	
potassium					√	
magnesium					√	

[a] HPAE-PAD: High-performance anion-exchange chromatography with pulsed amperometric detection. [b] RPLC: Reversed-Phased Liquid Chromatography. [c] AX: Anion-exchange chromatography. [d] IEC: Ion Exclusion Chromatography. [e] CX: Cation-exchange chromatography. [f] UV: Ultraviolet absorption spectroscopy.

the composition of extractives. However, the mass percent of extractives varied by almost a factor of two between samples (Table 4.4). While a detailed explanation of compositional variation among stover samples is beyond the scope of this work, previous studies have demonstrated differing chemical composition in various stovers derived from maize hybrids[157] and between different anatomical fractions (i.e., leaves, cobs, husks, and stalks) of corn plants[147] and corn stover.[158] Crop maturity[147] and storage method[158] have also been shown to influence composition. Stover feedstocks utilized in this study were composite samples, collected from multiple locations in different years. Thus, some variation in chemical composition is to be expected. All values reported in Table 4.4 are consistent with previous analyses of extractives in herbaceous biomass.[153]

Table 4.4. Mass Percent of Water-Soluble Materials in Oven-Dried Corn Stover [a]

Sample ID	Mean (n=3), (% dry weight)	RSD (%)
Kramer 33A14	14.2	0.90
2302-079	20.3	0.15
2870-061	16.4	0.56
2302-115	26.7	0.61
2893-026	14.3	0.94

[a] Values based on 10-hour Soxhlet extractions. See text for details. RSD = relative standard deviation.

Compositional analysis of aqueous extracts resulted in greater than 90% mass closure for extractives in four of five corn stover feedstocks (Table 4.5 & Figure 4.5). As described in more detail below, monosaccharides were the largest contributors to overall mass balance for extractives. Additional compounds identified in water extracts and assessed in this work include various alditols, organic acids, and inorganic ions. A visible band of material that was reddish-brown in color and could not be eluted with

water, aqueous sodium bicarbonate, or acetonitrile was consistently retained near the top of solid phase extraction cartridges used to clean-up aqueous extracts prior to analysis. It was later discovered that the colored band could be eluted with a 1:1 water-acetonitrile mixture, and gravimetric analysis of the residue remaining after solvent evaporation demonstrated that this fraction of the aqueous extract (*i.e.*, the red-brown fraction) also contributed significantly to the mass balance for water-soluble materials (10-18%). The mass percentages of individual constituents in each compound class identified in Figure 4.5 are reported in Tables 4.6-4.8, and notable features of these data are discussed below.

Table 4.5. Composition of Extractives in Corn Stover

	Sample ID[a,c]				
Component	Kramer	2302-079	2870-061	2302-115	2893-026
monosaccharides + sucrose	42(1)	42(2)	48(2)	57(2)	30(<0.1)
red-brown fraction	17(2)	13(1)	16.0(5)	10(1)	18(1)
inorganic cations[b]	13.62	11.01	8.98	6.31	10.55
organic acids	15.34(4)	21.20(4)	7.43(2)	9.14(1)	9.75(5)
alditols	5.2(3)	3.4(1)	6.4(4)	4.3(3)	7.3(5)
inorganic anions[b]	4.07	2.14	4.40	3.03	4.81
Total	97(2)	93(2)	91(2)	90(2)	80(1)

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit.
[b] The standard deviation of reported means was < 0.001 in all cases.
[c] See individual tables for more detail.

Carbohydrates

Monosaccharides, primarily glucose and fructose, represented 30-46% of the dry weight of water-soluble materials in tested feedstocks (Table 4.6). Note that the glucose

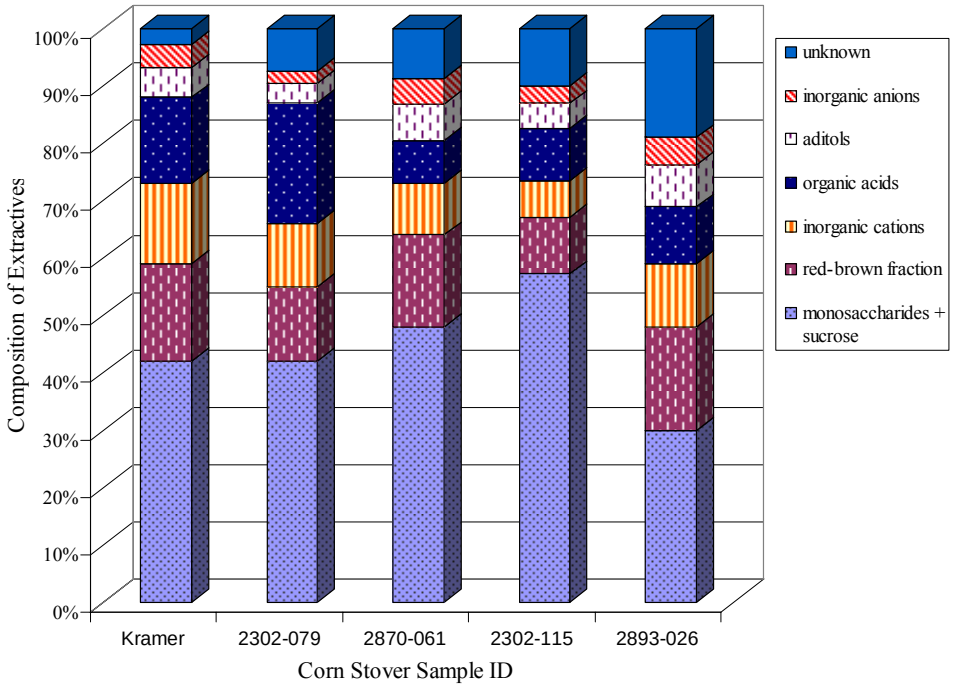


Figure 4.5. Composition of extractives in corn stover (expressed as a percentage of oven-dried water-soluble material recovered from the native feedstock).

quantities reported here are more than sufficient to explain the apparent reduction in glucan content reported previously for analysis of structural carbohydrates in water-extracted corn stover.[153] Additionally, it is noteworthy that approximately equal amounts of glucose and fructose were detected in all samples, suggesting that the presence of these sugars may be derived from a common sucrose origin. This observation led to divergent hypotheses that one or more sample preparation/analysis steps may promote hydrolysis of extracted sucrose or that latent enzyme activity may be present in harvested feedstocks.

A simple experiment was subsequently conducted to evaluate the potential of procedural factors to affect sucrose hydrolysis. An aqueous solution of sucrose at pH 5.4 (the typical pH of corn stover extracts) was refluxed for 10 hours in a Soxhlet apparatus. Once the solution cooled to room temperature, an aliquot was analyzed for sucrose,

glucose, and fructose via HPAE-PAD and an independent colorimetric enzyme assay. Results for both analyses were consistent and indicated essentially negligible quantities of glucose and fructose (less than 5% of sucrose originally added to the aqueous solution). These results suggest that the extraction and analysis protocols utilized in this study do not contribute significantly to sucrose hydrolysis.

The confirmed presence of water-soluble sugar in corn stover has potentially significant implications for technical and economic valuations of bioconversion processes as well as feedstock storage practices. The data in Tables 4.4 and 4.6 collectively demonstrate that fermentable sugars represent as much as 12% of the dry weight of corn stover feedstocks. Other sources of water-soluble sugar include oligomeric sugar and sugars derived from the red-brown fraction of aqueous extracts (see below). However, the recommended analytical procedure for determination of total carbohydrates in biomass requires that extractives be removed from feedstocks prior to analysis.[159] As a result, the contribution of water-soluble sugars to total carbohydrates is not typically considered in technical and economic models of biomass-to-ethanol conversion.

In practice, implications of this oversight could have both positive and negative consequences. For example, the presence of water-soluble glucose in feedstocks would be expected to result in increased ethanol yields relative to those predicted by current models. In contrast, water-soluble fructose would likely have a negative impact on ethanol yield in processing schemes involving dilute acid. Under these conditions, fructose is rapidly degraded to 5-hydroxymethylfurfural,[160] which is a known fermentation inhibitor.[161] Lastly, it is important to point out that the confirmed presence of water-soluble sugars in corn stover raises an interesting question related to biomass

storage practices, namely whether outdoor storage of feedstocks in rainy climates has an impact on feedstock composition.

Table 4.6. Mass Percent of Sugars and Related Alditols in Water Extracts of Corn Stover.[a]

	Sample ID				
Analyte	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
glucose	18(1)	20(1)	24(2)	21(1)	15.0(4)
fructose	22(2)	19(2)	22(2)	24(2)	11.6(7)
sucrose	0.9(1)	1.53(3)	0.36(6)	11(1)	ND[b]
xylose	0.53(5)	0.36(2)	0.69(8)	0.115(8)	2.2(2)
arabinose	0.27(2)	0.150(3)	0.49(4)	0.057(5)	0.8(2)
galactose	0.21(2)	0.097(4)	0.34(2)	0.13(2)	0.36(5)
mannose	0.43(5)	0.54(2)	0.27(4)	0.23(2)	0.3(1)
Total Sugars	42	42	48	57	30
glycerol	3.4(3)	1.7(1)	3.6(4)	3.2(3)	3.1(5)
xylitol	0.43(3)	0.74(2)	1.26(7)	0.472(8)	1.7(2)
arabitol	0.56(4)	0.63(2)	1.2(1)	0.42(4)	2.0(2)
sorbitol	0.69(4)	0.296(8)	0.18(2)	0.15(2)	0.26(3)
mannitol	0.11(1)	0.097(3)	0.13(1)	0.08(1)	0.22(4)
Total Alditols	5.2	3.4	6.4	4.3	7.3

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit.
[b] ND = not detected.

Alditols and Aliphatic Acids

Various alditols and aliphatic acids identified in aqueous extracts (Tables 4.6 and 4.7, respectively) have been cited among the top 30 value-added chemicals that can be derived from biomass.[162] Glycerol, sorbitol, xylitol, arabitol, malic acid, and fumaric acid are identified in the first tier of chemical building blocks (*i.e.*, the top 12), while citric and aconitic acid fall into the second tier. To our knowledge, the present study represents the first demonstration these chemicals are among the primary constituents of

purely aqueous extracts of corn stover. While commercial syntheses of many of these compounds are relatively straightforward, the same is not true of aconitic acid (1-propene-1,2,3-tricarboxylic acid). The primary commercial source of this compound has been its recovery as a by-product of sugar-cane processing since the 1950's.[163]

The presence of water-soluble aconitic acid may indicate a novel opportunity to lower overall processing cost for biomass-to-ethanol conversion via its recovery from aqueous process streams. In living systems, aconitic acid is a ubiquitous intermediate of the Krebs cycle (this statement also applies to most other acids in Table 4.7), and *trans*-aconitic acid is synthesized in maize (*Zea mays* L.) via enzyme catalyzed dehydration of citric acid.[178] Therefore, it is likely that the occurrence of *cis*-aconitic acid in corn stover extracts is due primarily to geometric isomerization at elevated temperature.[165] Data in Tables 4.4 and 4.7 collectively demonstrate that the dry-weight concentration of aconitic acid (i.e., the sum of *cis*- and *trans*-isomers) in tested feedstocks varied between 0.1 and 1 percent. These percentages suggest that a significant amount of aconitic acid could be recovered if biomass-to-ethanol processing reaches its projected potential (*e.g.*, consuming 60-80 million tons of corn stover on an annual basis). It is also likely that this strategy for reducing cost is not limited to corn stover, as previous work[166] has demonstrated that dry-weight concentrations of aconitic acid in early-season range grasses typically vary between 1 and 2.5 percent and were as high as 12.2 percent in the leaves of western larkspur (*Delphinium hesperium*).

Aromatic Acids

As demonstrated in Table 4.7, aromatic acids do not contribute significantly to the mass balance for organic acids. Aromatic monomers assessed in this study (Figure 4.4)

Table 4.7. Mass Percent of Organic Acids in Water Extracts of Corn Stover.[a]

	Sample ID				
Analyte	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
malic acid	5.70(2)	5.40(3)	1.59(2)	3.26(1)	2.37(4)
isocitric acid	3.52(1)	5.48(1)	2.09(1)	2.81(1)	2.12(5)
citric acid	1.45(1)	4.06(3)	1.81(2)	1.54(1)	2.36(4)
trans-aconitic acid	2.097(4)	3.622(9)	0.514(2)	0.672(4)	0.67(1)
fumraic acid	0.98(4)	1.01(4)	1.18(1)	0.536(7)	1.29(1)
cis-aconitic acid	1.01(1)	1.58(1)	0.231(4)	0.301(5)	0.29(1)
maleic acid	0.58(2)	0.046(1)	0.013(1)	0.020(1)	0.650(7)
Total Aliphatic acids	15.34	21.20	7.43	9.14	9.75
Total Aromatic acids	<0.06	<0.08	<0.06	<0.06	<0.1

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit.

are lignin-derived constituents of hydrolysates resulting from pretreatment of corn stover with dilute acid.[145,167] Significant hydrolysis of corn stover constituents upon Soxhlet extraction would also produce a variety of additional degradation products that are easily detected using the RPLC procedure employed for analysis of aqueous extracts.[145] Although careful inspection of the chromatogram shown in Figure 4.4 demonstrates that numerous compounds are present in these samples at trace levels, the negligible contribution of these components to the overall mass balance suggests that hydrolysis of lignocellulosic materials is not a significant contributor to the composition of water-soluble materials in corn stover. This is to be expected, as hot water pretreatments designed to initiate hydrolysis of corn stover[168-169] are typically conducted at elevated temperatures (e.g., 170-220 °C) and pressures relative to the conditions employed here.

Inorganic Ions

The mass percentages of inorganic ions in aqueous extracts are given in Table 4.8 and are in qualitative agreement with numerous studies of mineral content in plants. Cation composition was dominated by potassium and to a lesser extent calcium (and magnesium in one case). Potassium was the most abundant of the cations assayed, which is not unusual since potassium is normally applied to the cornfield as part of the fertilizer for growing corn. It is important to note that lithium was also monitored in this work but was not detected in any of the samples. Relative levels of detected anions were more balanced but typically decreased in the order chloride > phosphate > nitrate. Note that nitrite was also monitored but not detected in aqueous extracts. Charge-balance relationships were calculated for each aqueous extract and revealed a bias in favor of inorganic cations by as much as a factor of three. However, this is easily rationalized considering that di- and tri-functional aliphatic acids identified in aqueous extracts (*i.e.*, citric, malic, and aconitic acids) are widely recognized as chelating agents for cations (*e.g.*, Ca^{2+} and Mg^{2+}) in aqueous solution.

Tentative Identification of Constituents in the Red-Brown Fraction

Qualitative analysis of the red-brown fraction of water extracts suggested the presence of a complex oligomeric mixture. Recall that this fraction could be easily eluted from SPE cartridges with 1:1 water-acetonitrile, but neither water nor acetonitrile alone were successful as elution solvents. Since sugars are not soluble in acetonitrile, this observation suggested that the material contained sugar functionalities substituted on a relatively hydrophobic backbone. Furthermore, a near continuum of peaks, characterized

Table 4.8. Mass Percent of Inorganic Ions in Water Extracts of Corn Stover.[a]

	Sample ID				
Analyte	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
K^+	10.93	8.18	6.10	3.98	5.70
Ca^{2+}	1.16	1.40	1.50	1.21	1.73
Na^+	0.76	0.57	0.75	0.43	0.83
Mg^+	0.56	0.71	0.46	0.57	1.67
NH_4^+	0.21	0.15	0.17	0.12	0.62
Total Cations	13.62	11.01	8.98	6.31	10.55
Cl^-	1.78	0.43	3.24	1.72	3.00
PO_4^{3-}	0.83	1.31	1.05	1.01	1.50
NO_3^-	1.46	0.40	0.11	0.30	0.31
Total Anions	4.07	2.14	4.40	3.03	4.81

[a] Mass percentages represent the average of triplicate determinations. The standard deviation of reported means was < 0.001 in all cases.

by two broad distributions stretching from roughly 30 to 60 minutes, was observed in the chromatogram resulting from RPLC analysis of the crude red-brown residue (Figure 4.6A). The chromatogram resulting from HPAE-PAD analysis of a hydrolyzed residue (Figure 4.6B) was relatively clean and indicated significant quantities of hemicellulosic sugars (*i.e.*, arabinose, galactose, glucose, and xylose). RPLC analysis of this sample, following successive *n*-butanol and aqueous bicarbonate extractions, also resulted in a surprisingly clean chromatogram (Figure 4.6C), and confirmed the presence of ferulic acid along with notable amounts of *para*-coumaric and sinapic acid. These compounds represent the oxidized form of three lignin monomers (*i.e.*, coniferyl, coumaryl, and sinapyl alcohols). These results strongly suggest that the red-brown fraction is in part (if not primarily) composed of a diverse mixture of phenolic-glycosides. It is likely that this fraction of extractives is responsible for previously observed positive bias in Klason

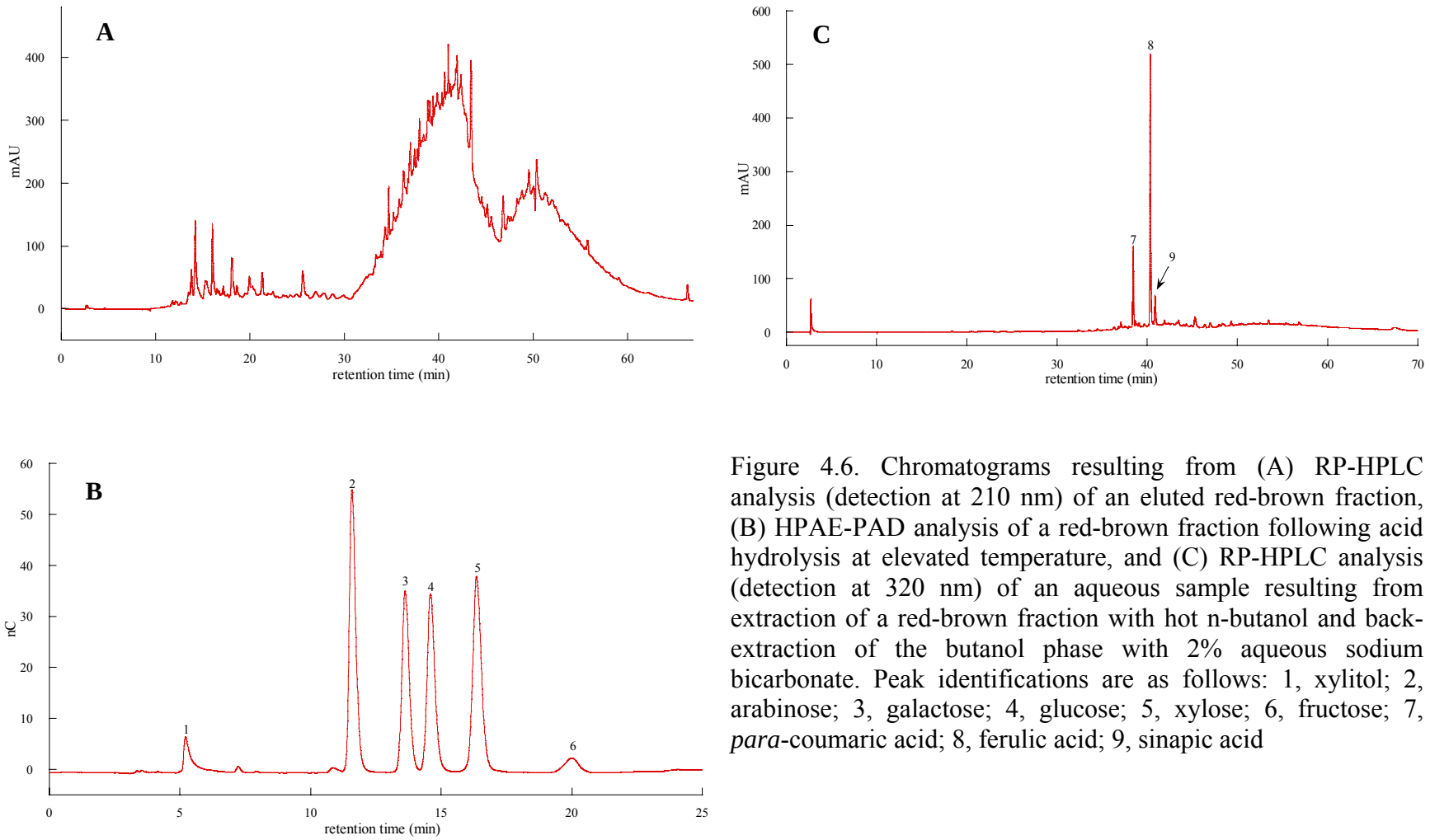


Figure 4.6. Chromatograms resulting from (A) RP-HPLC analysis (detection at 210 nm) of an eluted red-brown fraction, (B) HPAE-PAD analysis of a red-brown fraction following acid hydrolysis at elevated temperature, and (C) RP-HPLC analysis (detection at 320 nm) of an aqueous sample resulting from extraction of a red-brown fraction with hot n-butanol and back-extraction of the butanol phase with 2% aqueous sodium bicarbonate. Peak identifications are as follows: 1, xylitol; 2, arabinose; 3, galactose; 4, glucose; 5, xylose; 6, fructose; 7, *para*-coumaric acid; 8, ferulic acid; 9, sinapic acid

lignin determinations performed on native corn stover.[153] The presence of conjugated sugars in this fraction may also influence glucan composition of corn stover. However, it is unclear at this point how these constituents should be categorized or assessed in future compositional analyses of herbaceous feedstocks.

Contributors to the Unknown Fraction of Water Extracts

As reported in Table 4.5 and Figure 4.5, compositional analysis did not result in quantitative mass closure for water-soluble materials. Samples used to determine the monosaccharide content of extracts were also hydrolyzed with 4% sulfuric acid at elevated temperature, and total sugars (*i.e.*, free plus monomeric) were determined via HPAE-PAD. These values are reported in Table 4.9 along with calculated values for oligomeric sugar present in each sample. (Note that sucrose and fructose are not included here, since sucrose is rapidly hydrolysed to glucose and fructose, and fructose rapidly decomposes under these conditions.) Marginal increases (0-28%) were observed in mass percentages for total glucose relative to the free glucose values reported in Table 4.6. In contrast, mass percentages of total xylose, arabinose, galactose, and mannose typically differed from monomeric assessments by a factor of 2-3 (except sample 2302-079). Calculated differences in observed total and free sugars for each stover sample (*i.e.*, oligomeric sugar in Table 4.9) suggest that oligomeric sugars contribute an additional 4-12% to the overall mass balance for extractives in corn stover.

Additional insight into the composition of the unknown fraction of water extracts may be inferred from literature. The study conducted by Thammasouk et al.[153] provides an indirect assessment of water-soluble materials and implies that ash accounts for up to

Table 4.9. Total and Oligermeric Sugars in Water Extracts of Corn Sover.[a,b]

	Sample ID				
Analyte	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
Total Sugars					
glucan	20(3)	24(3)	30(3)	27(3)	14(1)
xylan	1.23(8)	0.9(1)	1.9(2)	0.32(2)	3.0(6)
arabinan	0.69(6)	0.44(5)	1.1(1)	0.17(2)	1.9(4)
galactan	0.72(3)	0.5(1)	1.2(2)	0.32(1)	1.8(5)
mannan	0.78(9)	8(2)	ND	0.45(7)	0.6(6)
Oligomeric sugars					
glucose	2	4	6	6	-1
xylose	0.70	0.50	1.2	0.20	0.8
arabinose	0.42	0.29	0.6	0.11	1.1
galactose	0.51	0.4	0.9	0.19	1.4
mannose	0.35	7	-0.27	0.22	0.3
Total oligermerics[c]	4	12	9	7	4

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit. [b] ND = not detected. [c] Calculated as the difference between total sugar entries tabulated here and the saccharide values given in Table 4.6.

30% of the mass balance for extractives in corn stover. Inorganic ions monitored in the present study represented roughly 10-18% of the mass balance. Thus, alternative inorganic materials may also be present in water extracts. Data reported in the same paper also imply that protein represents 6-15% of the mass balance for water-soluble materials in herbaceous feedstocks. Protein was not monitored in the present study; however, this observation suggests that inclusion of protein analysis in future compositional assessments of water extracts may improve mass closure.

APPENDICES

APPENDIX A

Accumulated Concentrations of Individual Degradation Products Identified at Three Levels of Reaction Severity

Table A.1. Analytical Concentrations (mM) Observed at Low Severity [a]

	Reaction Condition Log (Ro) = 2.66		
Analyte	180 ºC, 2min	170 ºC, 4min	160 ºC, 8min
formic acid	1.64(6)	1.3(1)	1.0(1)
lactic acid	20.9(5)	12.8(4)	9.7(3)
acetic acid	9.0(4)	6.4(2)	5.1(2)
maleic acid	0.27(4)	0.209(7)	0.168(8)
fumaric acid	0.43(1)	0.298(5)	0.224(5)
levulinic acid	2.75(1)	1.54(5)	1.23(4)
5-hydroxymethylfurfural	0.274(4)	0.184(4)	0.108(6)
furfural	7.6(2)	4.5(2)	3.0(2)
3,4-dihydroxybenzoic acid	0.0035(1)	0.0008(4)	0.0004(1)
4-hydroxybenzoic acid	0.0037(2)	0.0020(1)	0.0016(1)
4-hydroxybenzaldehyde	0.110(3)	0.084(2)	0.077(3)
vanillic acid	0.0125(4)	0.0074(4)	0.0072(6)
caffeic acid	0.009(1)	0.0060(2)	0.0051(4)
syringic acid	0.0077(3)	0.0048(2)	0.0037(2)
vanillin	0.0279(3)	0.0192(4)	0.0154(4)
para-coumaric acid	0.187(3)	0.136(2)	0.131(5)
syringaldehyde	0.029(3)	0.019(1)	0.0180(2)
ferulic acid	0.075(1)	0.051(1)	0.050(2)
3-hydroxy-4-methoxycinnamic acid	0.020(1)	0.014(2)	0.011(1)

[a] Concentrations represent the average of triplicate determinations. Values in parentheses represent plus or minus one standard deviation in the least significant digit.

Table A.2. Analytical Concentrations (mM) Observed at Moderate Severity [a]

Analyte	Reaction Condition Log (Ro) = 3.26				
	200 ºC, 2 min	190 ºC, 4 min	180 ºC, 8 min	170 ºC, 16 min	160 ºC, 32 min
formic acid	5.47(8)	4.72(6)	3.68(4)	2.1(2)	2.1(1)
lactic acid	38.6(4)	32.42(5)	23.3(6)	17.6(4)	17.1(4)
acetic acid	25.7(4)	21.72(4)	17.2(5)	11.4(3)	9.2(1)
maleic acid	0.68(3)	0.60(4)	0.438(5)	0.29(3)	0.26(1)
fumaric acid	2.05(4)	1.85(4)	1.43(1)	0.94(3)	0.86(3)
levulinic acid	11.93(3)	10.7(3)	5.91(7)	5.93(6)	5.3(1)
5-hydroxymethylfurfural	2.73(4)	1.77(3)	1.02(1)	0.55(2)	0.415(2)
furfural	29.4(4)	24.6(3)	19.9(4)	11.1(2)	9.4(1)
3,4-dihydroxybenzoic acid	0.008(1)	0.0045(1)	0.0021(3)	0.0012(1)	0.0016(1)
4-hydroxybenzoic acid	0.0102(7)	0.0084(5)	0.008(1)	0.0041(2)	0.0034(1)
4-hydroxybenzaldehyde	0.210(4)	0.167(3)	0.149(1)	0.108(2)	0.096(1)
vanillic acid	0.0344(6)	0.0302(7)	0.0234(9)	0.013(1)	0.0116(1)
caffeic acid	0.0323(8)	0.029(1)	0.0252(7)	0.0160(2)	0.015(1)
syringic acid	0.0186(1)	0.0157(1)	0.0134(5)	0.0078(2)	0.0071(2)
vanillin	0.064(1)	0.053(1)	0.043(2)	0.030(1)	0.0264(2)
para-coumaric acid	0.061(1)	0.051(1)	0.046(2)	0.036(1)	0.035(1)
syringaldehyde	0.0476(4)	0.036(1)	0.032(2)	0.026(1)	0.0197(3)
ferulic acid	0.039(2)	0.0321(2)	0.029(1)	0.030(1)	0.022(1)
3-hydroxy-4-methoxycinnamic acid	0.0460(8)	0.039(1)	0.039(2)	0.029(1)	0.026(1)

[a] Concentrations represent the average of triplicate determinations. Values in parentheses represent plus or minus one standard deviation in the least significant digit.

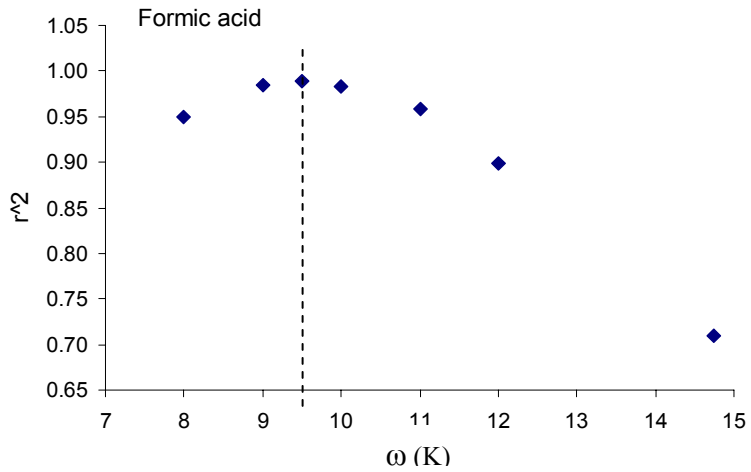
Table A.3. Analytical Concentrations (mM) Observed at High Severity [a]

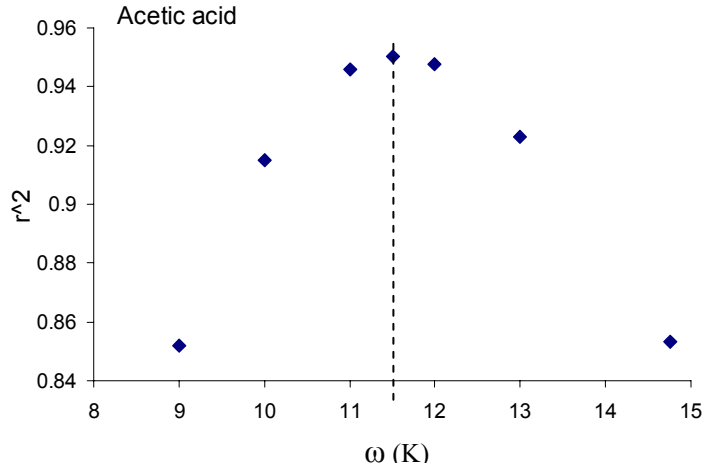
Analyte	Reaction Condition Log (Ro) = 3.86			
	200 ºC, 8 min	190 ºC, 16 min	180 ºC, 32 min	170 ºC, 64 min
formic acid	18.77(6)	11.64(7)	9.8(2)	7.0(3)
lactic acid	17.6(3)	14.4(5)	8.1(3)	6.4(2)
acetic acid	54.5(2)	61.0(5)	44.0(5)	37.0(2)
maleic acid	0.79(1)	0.52(1)	0.44(1)	0.30(3)
fumaric acid	5.71(3)	4.55(4)	3.85(6)	2.70(1)
levulinic acid	11.6(2)	23.0(4)	15.01(4)	13.82(3)
5-hydroxymethylfurfural	6.74(3)	3.89(5)	2.54(4)	1.50(3)
furfural	33.3(5)	23.2(2)	20.7(3)	15.6(2)
3,4-dihydroxybenzoic acid	0.0025(1)	0.0075(1)	0.006(1)	0.0038(2)
4-hydroxybenzoic acid	0.0173(2)	0.0117(5)	0.0113(1)	0.0073(6)
4-hydroxybenzaldehyde	0.203(4)	0.156(2)	0.129(1)	0.106(2)
vanillic acid	0.054(1)	0.0408(7)	0.0285(3)	0.0223(2)
caffeic acid	0.035(2)	0.0290(6)	0.0163(4)	0.029(1)
syringic acid	0.028(1)	0.0178(2)	0.0150(2)	0.0101(4)
vanillin	0.0963(5)	0.074(1)	0.057(1)	0.046(1)
para-coumaric acid	0.117(2)	0.080(1)	0.0703(1)	0.059(1)
syringaldehyde	0.055(1)	0.044(2)	0.035(2)	0.0359(4)
ferulic acid	0.0167(4)	0.014(3)	0.0103(4)	0.034(2)
3-hydroxy-4-methoxycinnamic acid	0.075(3)	0.048(1)	0.039(1)	0.052(3)

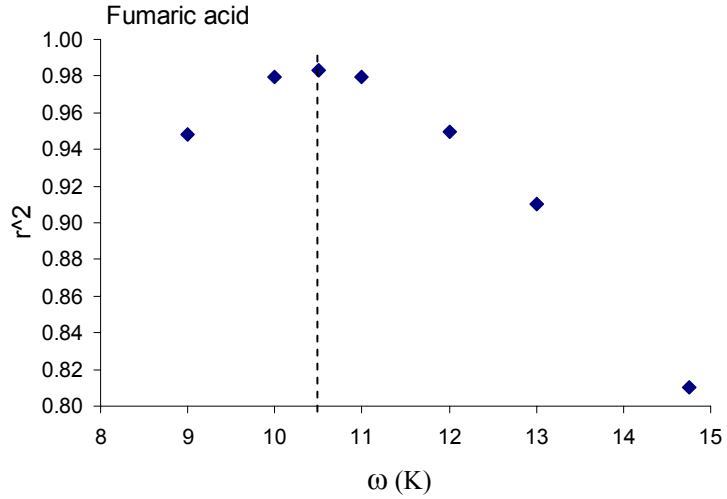
[a] Concentrations represent the average of triplicate determinations. Values in parentheses represent one plus or minus standard deviation in the least significant digit.

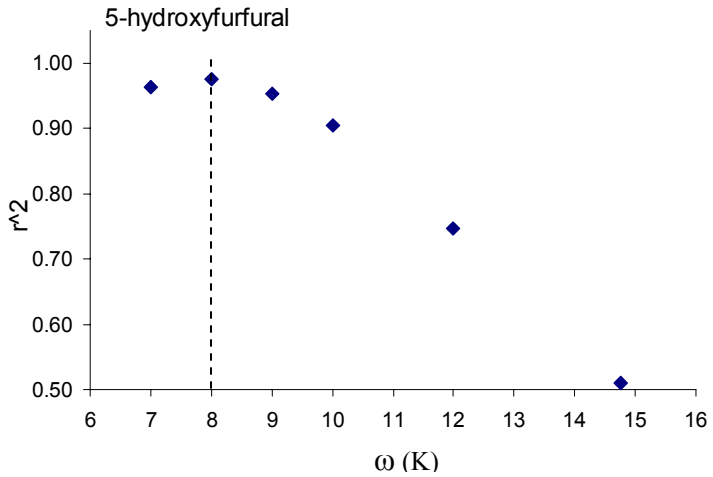
APPENDIX B

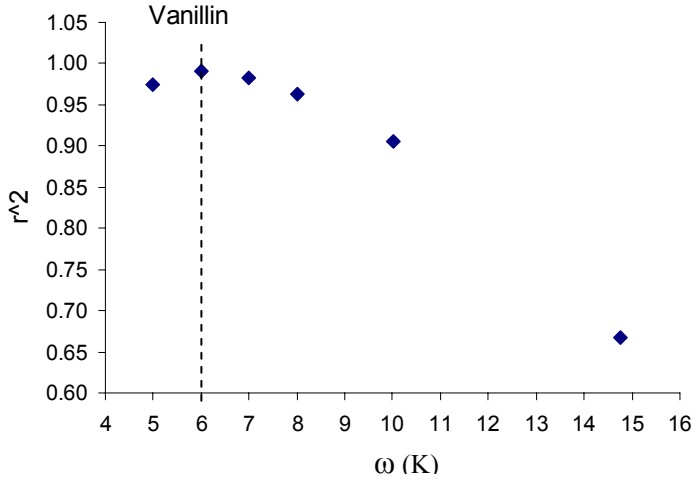
Optimization of Fitting Parameter ω of Individual Degradation Products

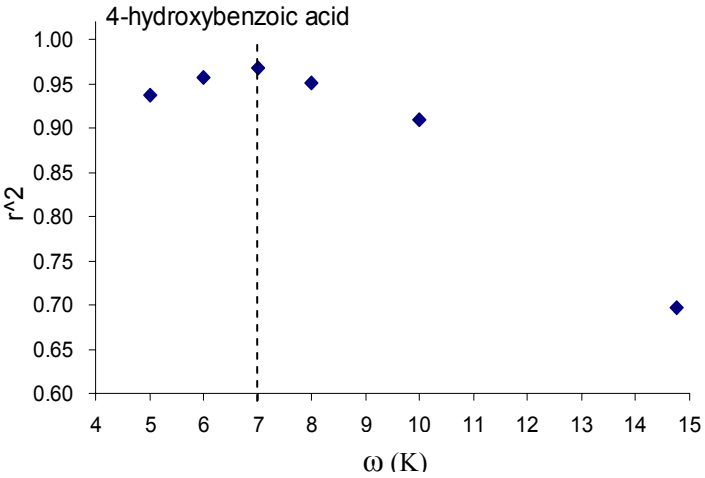

Formic acid
r^2
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
7
8
9
10
11
12
13
14
15
ω (K)

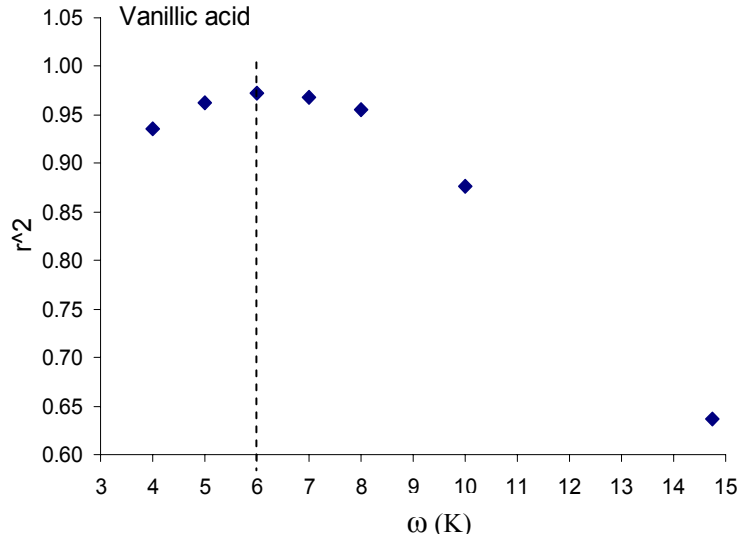

Acetic acid
r^2
0.96
0.94
0.92
0.9
0.88
0.86
0.84
8
9
10
11
12
13
14
15
ω (K)

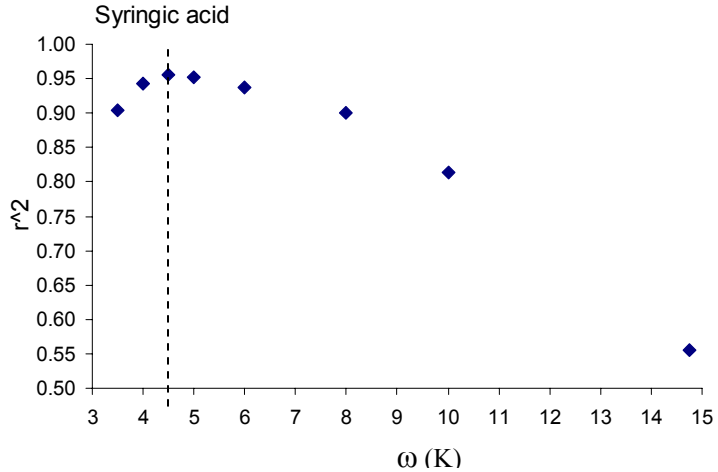

Fumaric acid
r^2
1.00
0.98
0.96
0.94
0.92
0.90
0.88
0.86
0.84
0.82
0.80
8
9
10
11
12
13
14
15
ω (K)

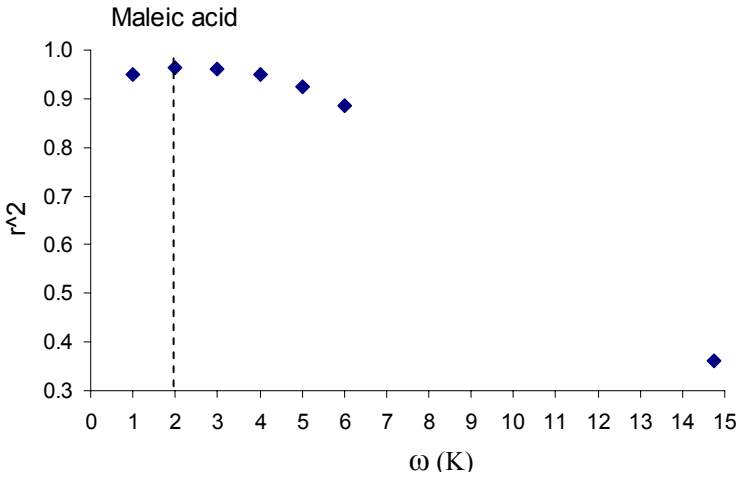

5-hydroxyfurfural
r^2
1.00
0.90
0.80
0.70
0.60
0.50
6 7 8 9 10 11 12 13 14 15 16
ω (K)

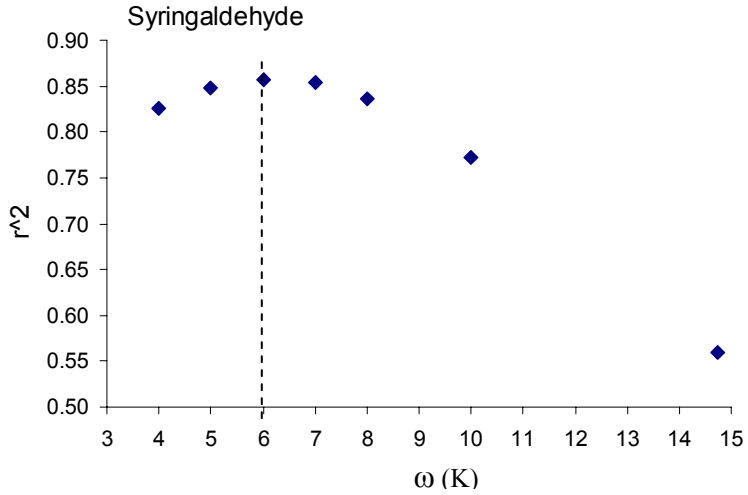

Vanillin
r^2
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
4 5 6 7 8 9 10 11 12 13 14 15 16
ω (K)

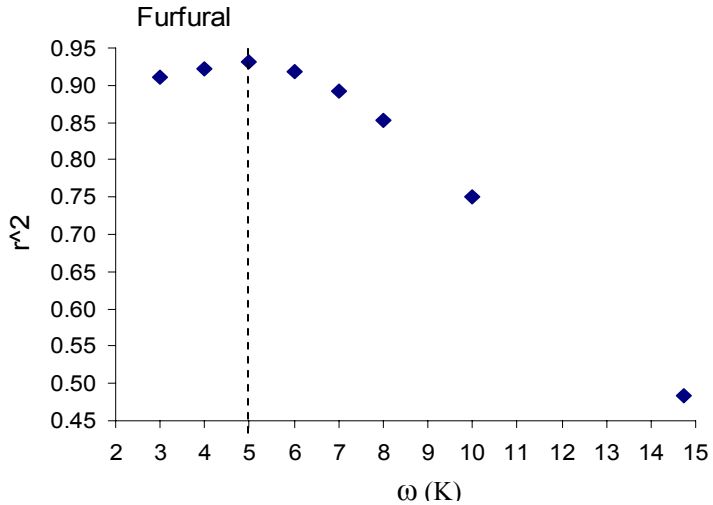

4-hydroxybenzoic acid
r^2
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
4 5 6 7 8 9 10 11 12 13 14 15
ω (K)

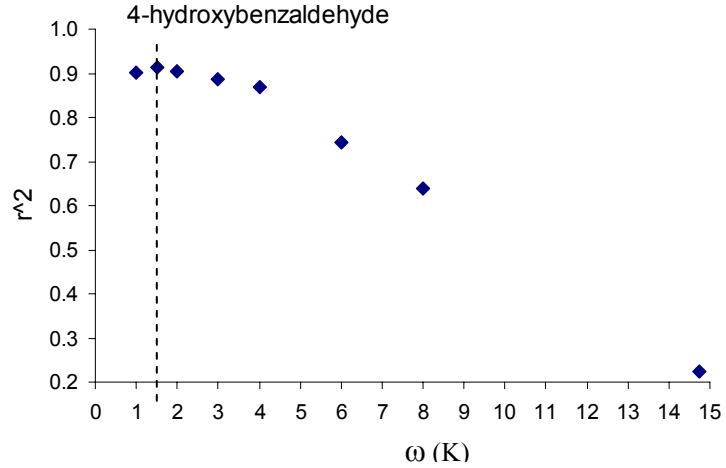

Vanillic acid
r^2
ω (K)

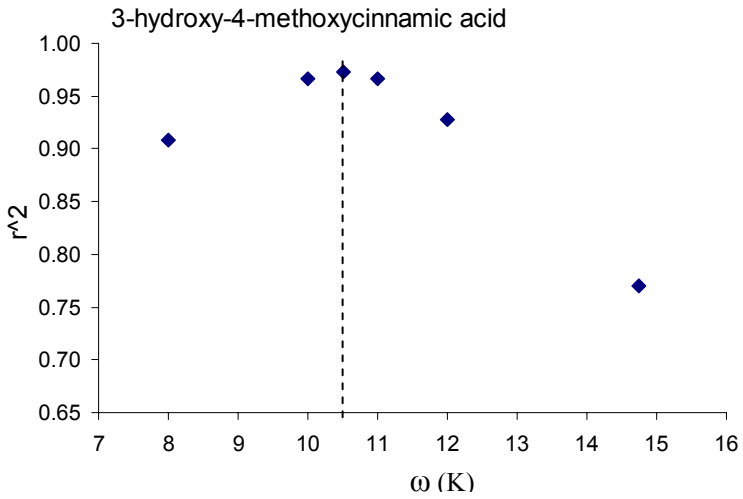

Syringic acid
r^2
ω (K)

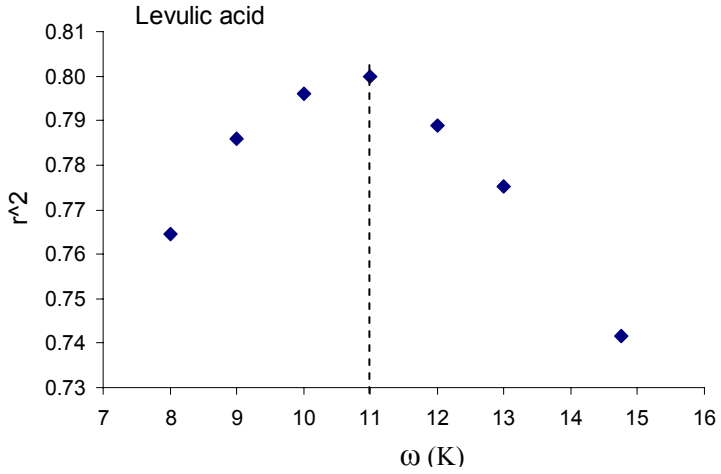

Maleic acid
r^2
ω (K)

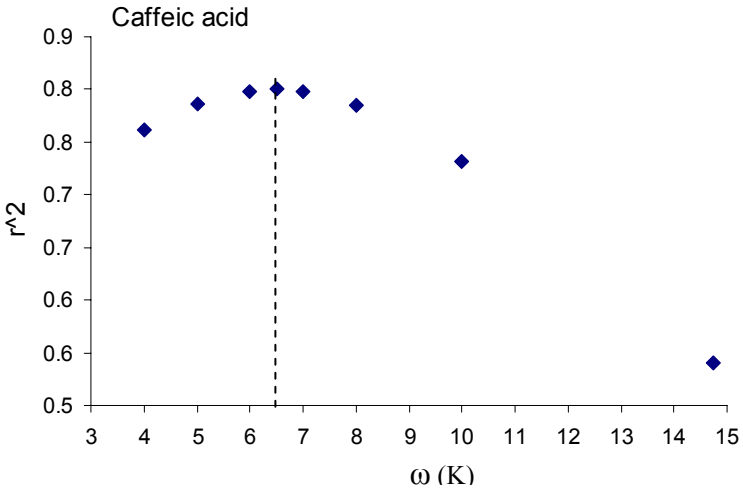

Syringaldehyde
r^2
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
3 4 5 6 7 8 9 10 11 12 13 14 15
ω (K)

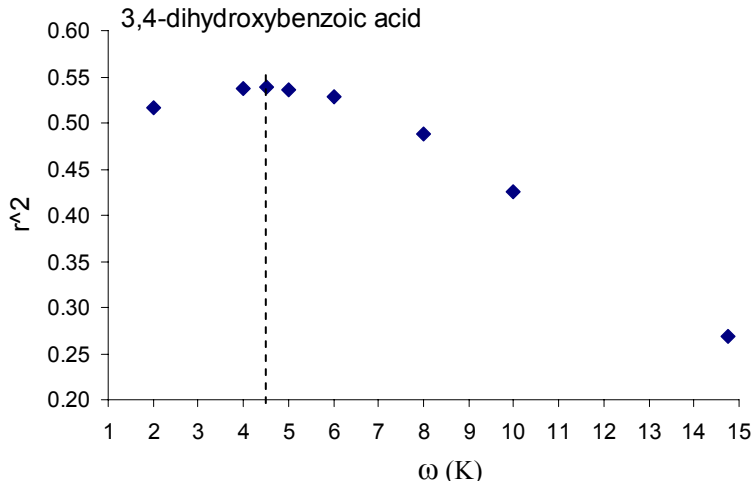

Furfural
r^2
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
2 3 4 5 6 7 8 9 10 11 12 13 14 15
ω (K)

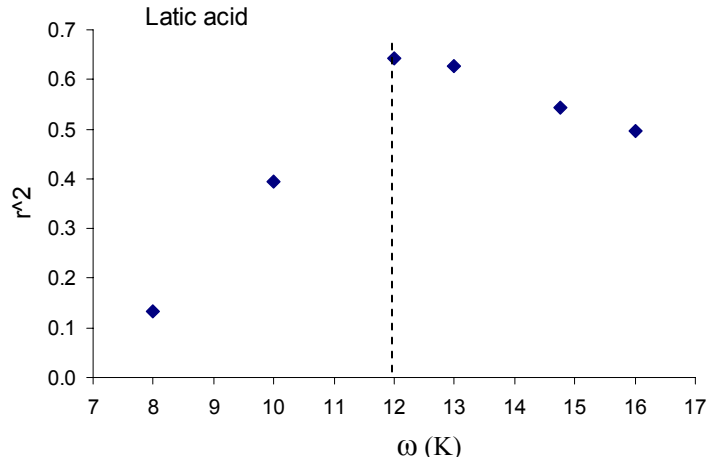

4-hydroxybenzaldehyde
r^2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ω (K)

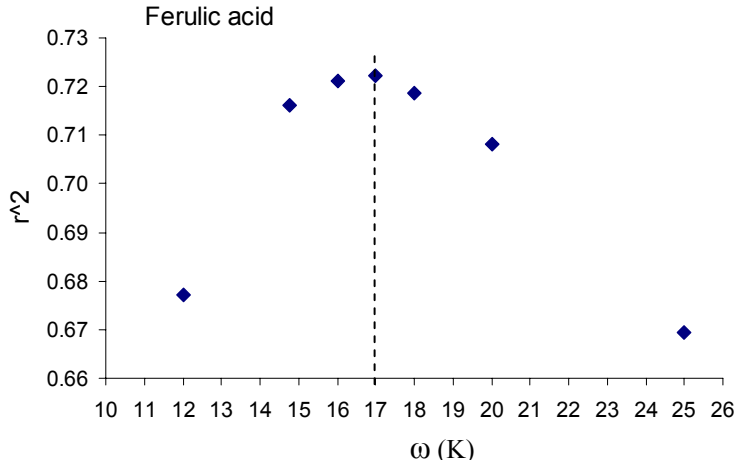

3-hydroxy-4-methoxycinnamic acid
r^2
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
7
8
9
10
11
12
13
14
15
16
ω (K)


Levulic acid
r^2
0.81
0.80
0.79
0.78
0.77
0.76
0.75
0.74
0.73
7
8
9
10
11
12
13
14
15
16
ω (K)


Caffeic acid
r^2
0.9
0.8
0.8
0.7
0.7
0.6
0.6
0.5
3
4
5
6
7
8
9
10
11
12
13
14
15
ω (K)


3,4-dihydroxybenzoic acid
r^2
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ω (K)


Latic acid
r^2
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
7 8 9 10 11 12 13 14 15 16 17
ω (K)


Ferulic acid
r^2
0.73
0.72
0.71
0.70
0.69
0.68
0.67
0.66
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
ω (K)

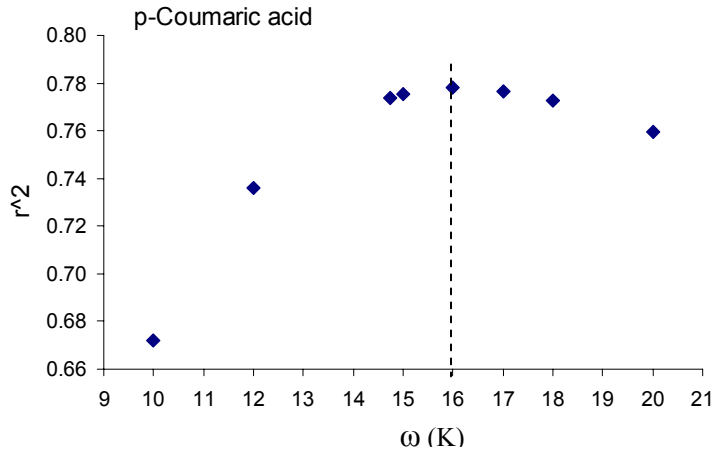
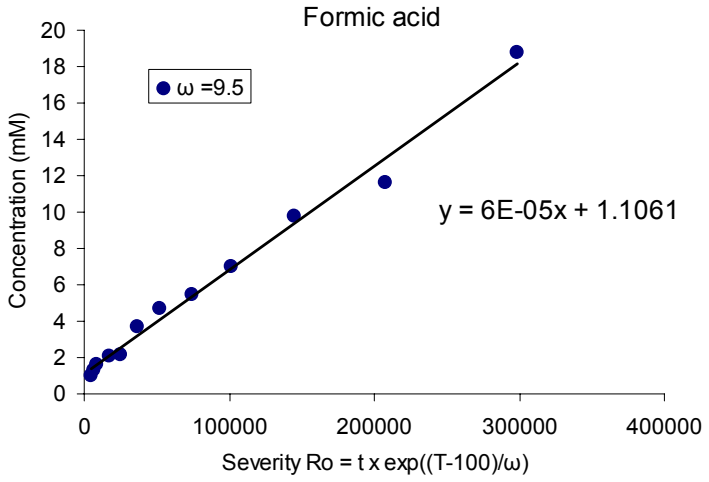
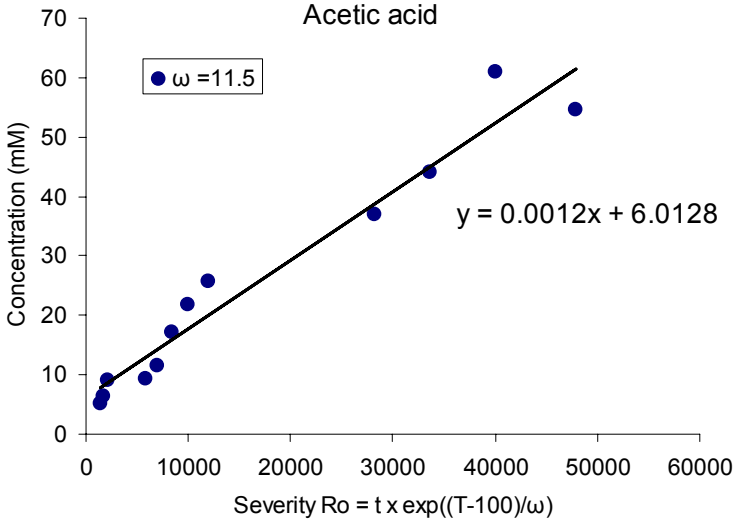


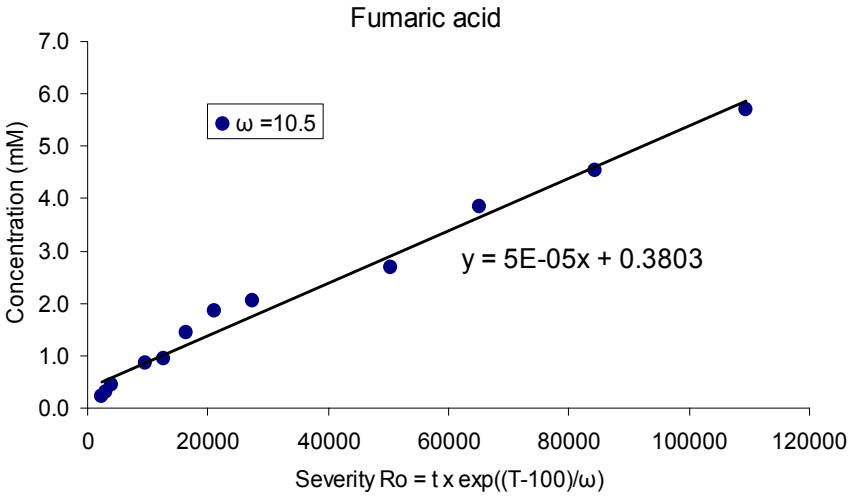
Figure B.1. Plots of r^2 versus ω, justifying the selection of optimized fitting parameters, for all identified compounds.

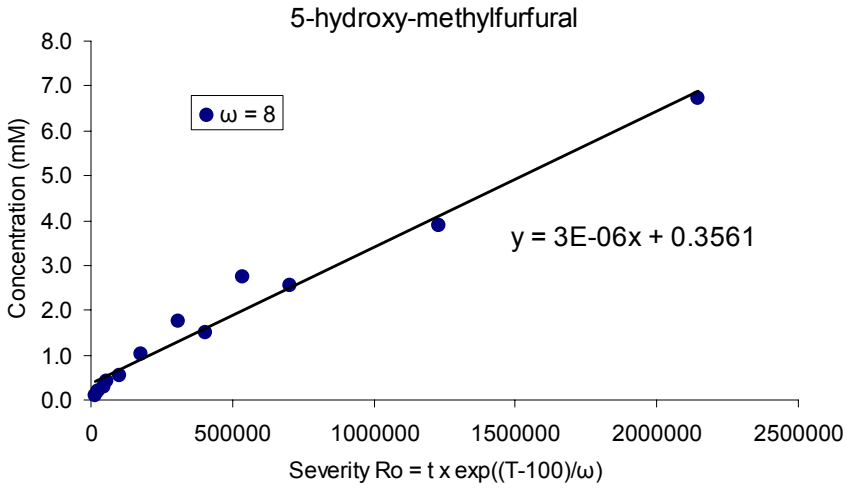
APPENDIX C

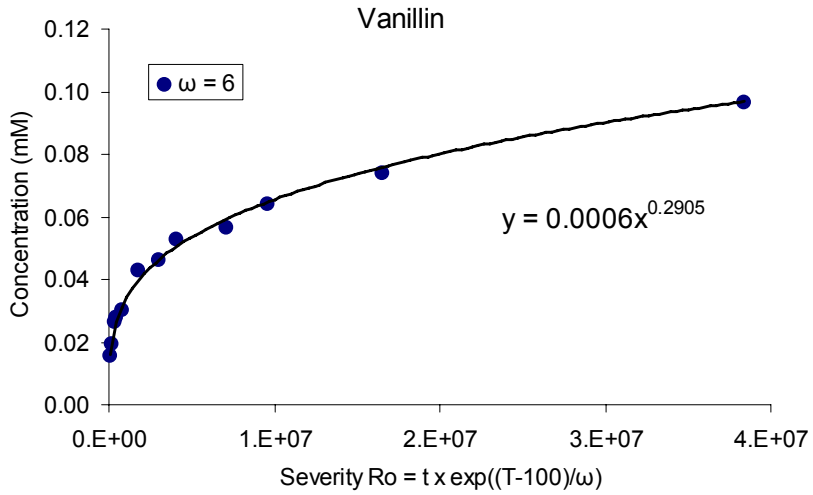
Specific Mathematical Relationship for Selected Degradation Products

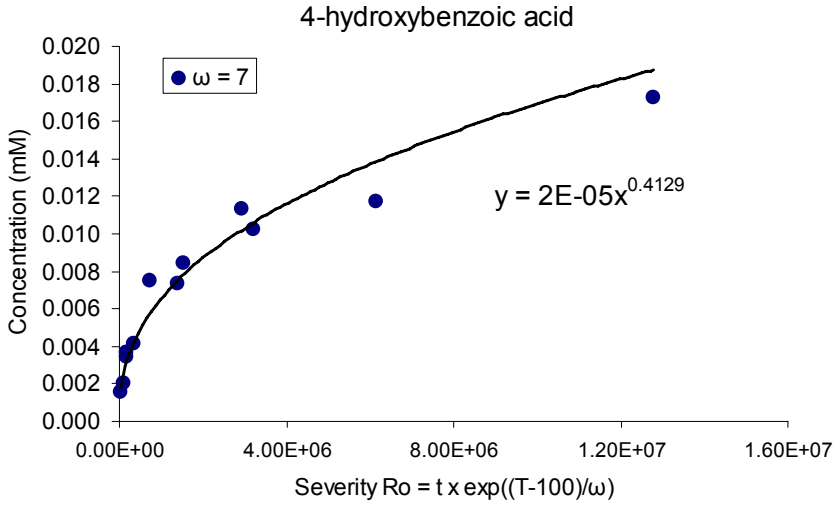

Formic acid
ω =9.5
y = 6E-05x + 1.1061
Concentration (mM)
Severity Ro = t x exp((T-100)/ω)
0
2
4
6
8
10
12
14
16
18
20
0
100000
200000
300000
400000

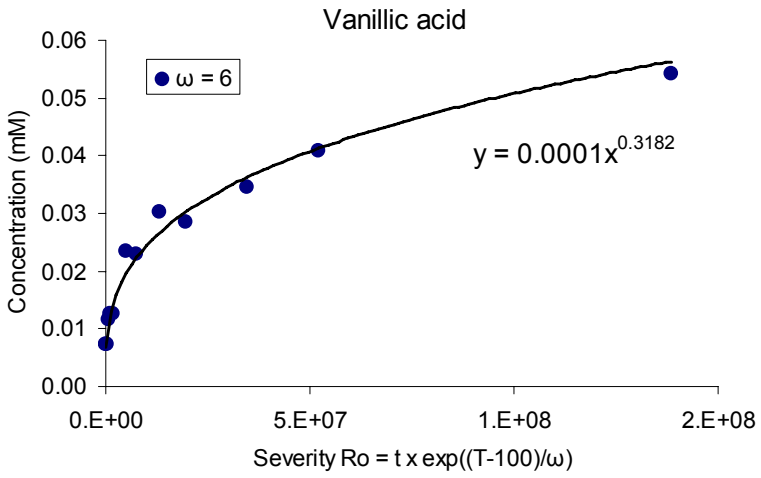

Acetic acid
ω =11.5
y = 0.0012x + 6.0128
Concentration (mM)
Severity Ro = t x exp((T-100)/ω)
0
10
20
30
40
50
60
70
0
10000
20000
30000
40000
50000
60000

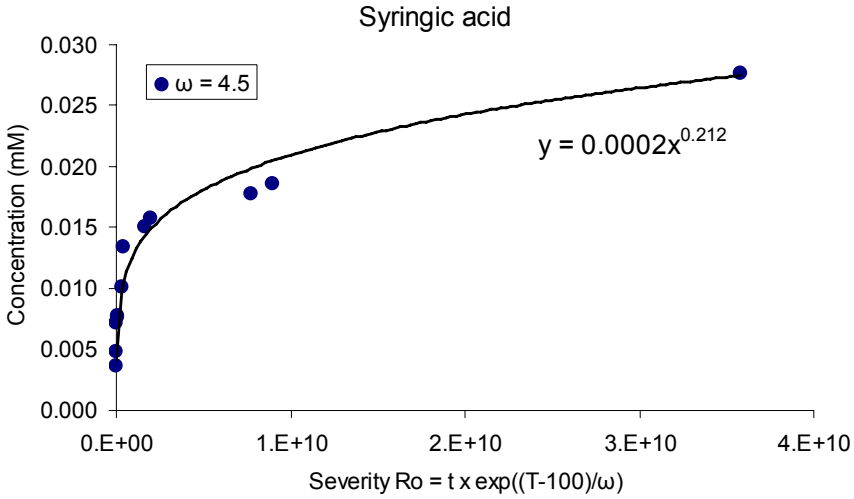

Fumaric acid
ω =10.5
y = 5E-05x + 0.3803
Concentration (mM)
Severity Ro = t x exp((T-100)/ω)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0
20000
40000
60000
80000
100000
120000

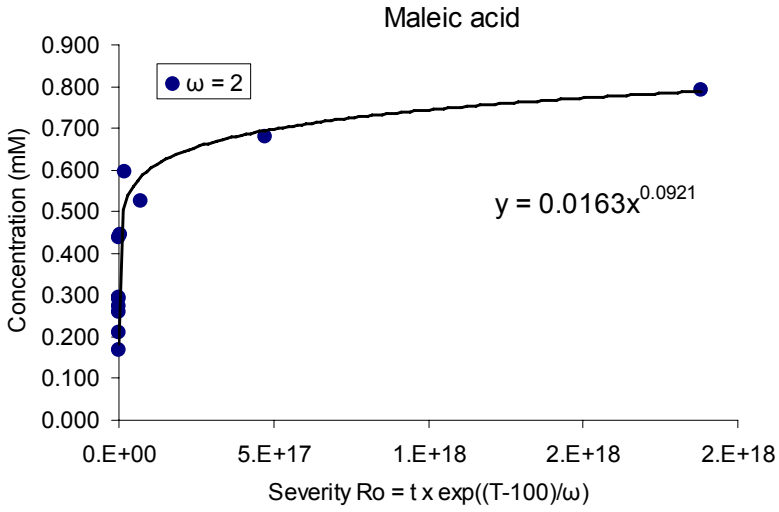

5-hydroxy-methylfurfural
ω = 8
y = 3E-06x + 0.3561
Concentration (mM)
Severity Ro = t x exp((T-100)/ω)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
0
500000
1000000
1500000
2000000
2500000

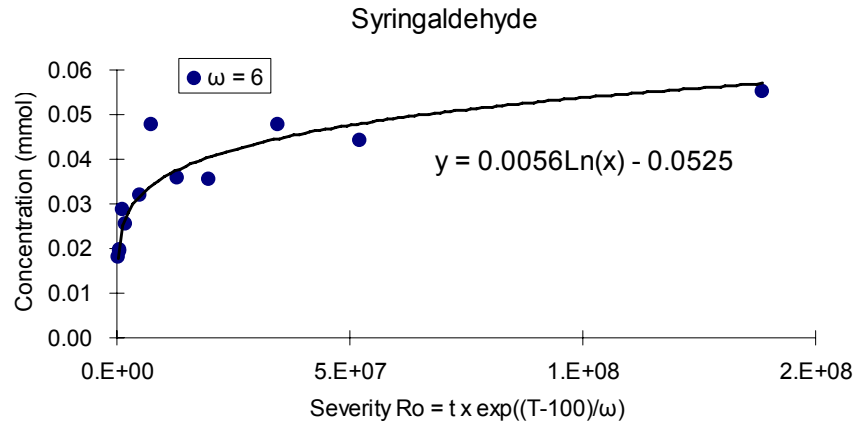

Vanillin
ω = 6
y = 0.0006x^0.2905
Concentration (mM)
Severity Ro = t x exp((T-100)/ω)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.E+00
1.E+07
2.E+07
3.E+07
4.E+07

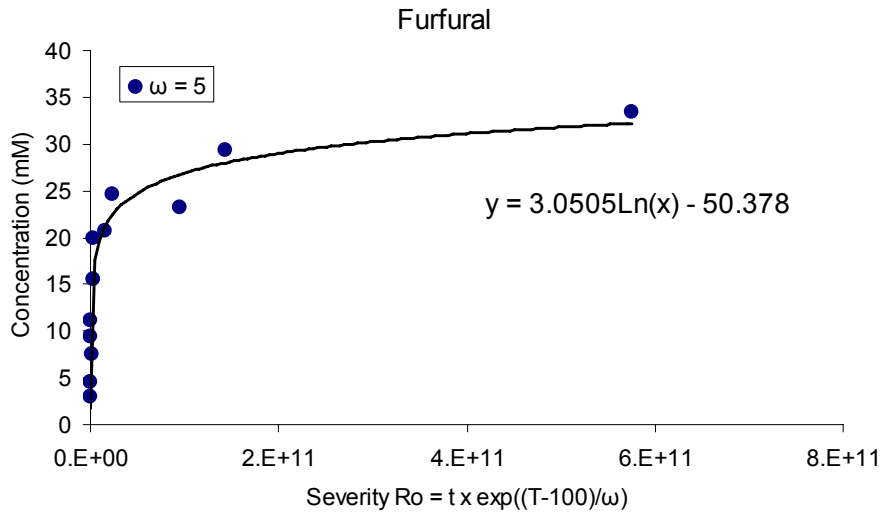

4-hydroxybenzoic acid
ω = 7
y = 2E-05x^0.4129
Concentration (mM)
Severity Ro = t x exp((T-100)/ω)
0.000
0.002
0.004
0.006
0.008
0.010
0.012
0.014
0.016
0.018
0.020
0.00E+00
4.00E+06
8.00E+06
1.20E+07
1.60E+07

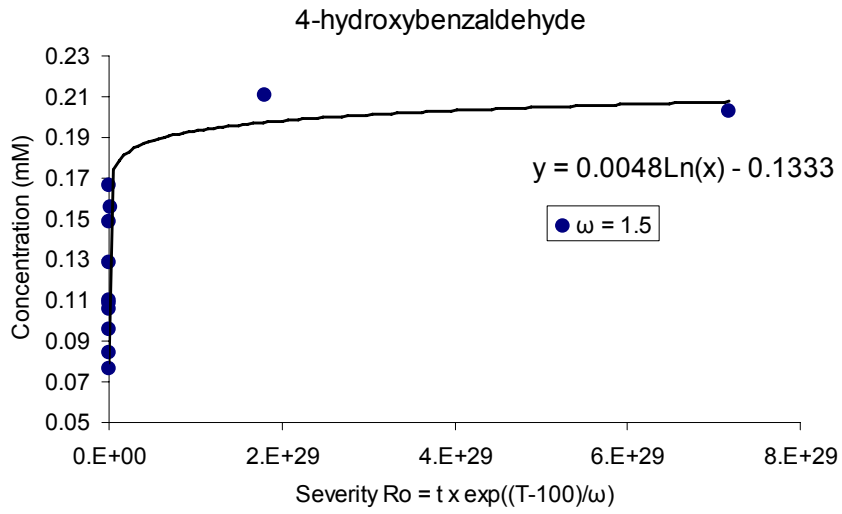

Vanillic acid
ω = 6
y = 0.0001x^0.3182
Concentration (mM)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.E+00
5.E+07
1.E+08
2.E+08
Severity Ro = t x exp((T-100)/ω)


Syringic acid
ω = 4.5
y = 0.0002x^0.212
Concentration (mM)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.E+00
1.E+10
2.E+10
3.E+10
4.E+10
Severity Ro = t x exp((T-100)/ω)


Maleic acid
ω = 2
y = 0.0163x^0.0921
Concentration (mM)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.E+00
5.E+17
1.E+18
2.E+18
2.E+18
Severity Ro = t x exp((T-100)/ω)


Syringaldehyde
ω = 6
y = 0.0056Ln(x) - 0.0525
Concentration (mmol)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.E+00
5.E+07
1.E+08
2.E+08
Severity Ro = t x exp((T-100)/ω)


Furfural
ω = 5
y = 3.0505Ln(x) - 50.378
Concentration (mM)
40
35
30
25
20
15
10
5
0
0.E+00
2.E+11
4.E+11
6.E+11
8.E+11
Severity Ro = t x exp((T-100)/ω)


4-hydroxybenzaldehyde
y = 0.0048Ln(x) - 0.1333
ω = 1.5
Concentration (mM)
0.23
0.21
0.19
0.17
0.15
0.13
0.11
0.09
0.07
0.05
0.E+00
2.E+29
4.E+29
6.E+29
8.E+29
Severity Ro = t x exp((T-100)/ω)

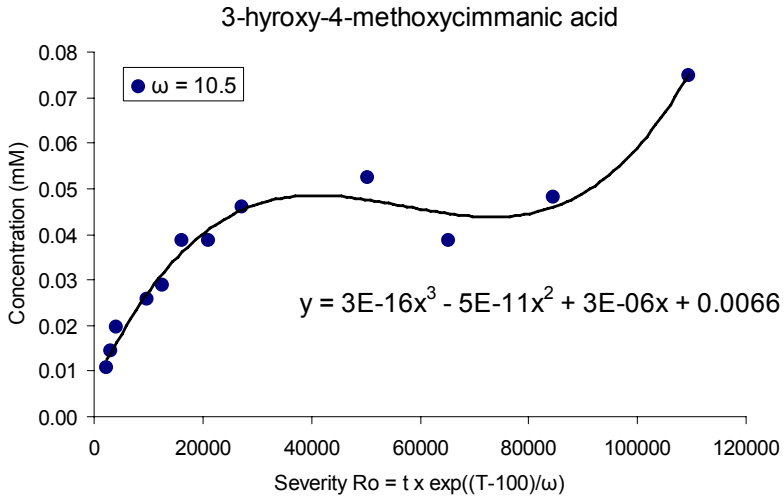


Figure C.1. Graphical representations of optimized correlations along with the specific mathematical relationship used to fit experimental data for each compound.

APPENDIX D

Copy Right Permissions

Copy Right Permission from American Chemical Society (*J. Agri. Food. Chem.*):

10/03/2007 15:18 FAX 2027768112 001/002

American Chemical Society

Publications Division
Copyright Office

1155 Sixteenth Street, NW
Washington, DC 20036
Phone: (1) 202-872-4368 or -4367
Fax: (1) 202-776-8112 E-mail: copyright@acs.org

VIA FAX: 254-710-4272 DATE: October 3, 2007

TO: Shou-Feng (Flora) Chen, Department of Chemistry & Biochemistry, Baylor University
One Bear Place, #97348, Waco, TX 76798-7348

FROM: C. Arleen Courtney, Copyright Associate C. Arleen Courtney

Thank you for your request for permission to include your paper(s) or portions of text from your paper(s) in your thesis. Permission is now automatically granted; please pay special attention to the implications paragraph below. The Copyright Subcommittee of the Joint Board/Council Committees on Publications approved the following:

Copyright permission for published and submitted material from theses and dissertations
ACS extends blanket permission to students to include in their theses and dissertations their own articles, or portions thereof, that have been published in ACS journals or submitted to ACS journals for publication, provided that the ACS copyright credit line is noted on the appropriate page(s).

Publishing implications of electronic publication of theses and dissertation material
Students and their mentors should be aware that posting of theses and dissertation material on the Web prior to submission of material from that thesis or dissertation to an ACS journal may affect publication in that journal. Whether Web posting is considered prior publication may be evaluated on a case-by-case basis by the journal's editor. If an ACS journal editor considers Web posting to be "prior publication", the paper will not be accepted for publication in that journal. If you intend to submit your unpublished paper to ACS for publication, check with the appropriate editor prior to posting your manuscript electronically.

If your paper has **not** yet been published by ACS, we have no objection to your including the text or portions of the text in your thesis/dissertation in **print and microfilm formats**; please note, however, that electronic distribution or Web posting of the unpublished paper as part of your thesis in electronic formats might jeopardize publication of your paper by ACS. Please print the following credit line on the first page of your article: "Reproduced (or 'Reproduced in part') with permission from [JOURNAL NAME], in press (or 'submitted for publication'). Unpublished work copyright [CURRENT YEAR] American Chemical Society." Include appropriate information.

If your paper has already been published by ACS and you want to include the text or portions of the text in your thesis/dissertation in **print or microfilm formats**, please print the ACS copyright credit line on the first page of your article: "Reproduced (or 'Reproduced in part') with permission from [FULL REFERENCE CITATION.] Copyright [YEAR] American Chemical Society." Include appropriate information.

Submission to a Dissertation Distributor: If you plan to submit your thesis to UMI or to another dissertation distributor, you should not include the unpublished ACS paper in your thesis if the thesis will be disseminated electronically, until ACS has published your paper. After publication of the paper by ACS, you may release the entire thesis **(not the individual ACS article by itself)** for electronic dissemination through the distributor; ACS's copyright credit line should be printed on the first page of the ACS paper.

Use on an Intranet: The inclusion of your ACS unpublished or published manuscript is permitted in your thesis in print and microfilm formats. If ACS has published your paper you may include the manuscript in your thesis on an intranet that is not publicly available. Your ACS article cannot be posted electronically on a publicly available medium (i.e. one that is not password protected), such as but not limited to, electronic archives, Internet, library server, etc. The only material from your paper that can be posted on a public electronic medium is the article abstract, figures, and tables, and you may link to the article's DOI or post the article's author-directed URL link provided by ACS. This paragraph does not pertain to the dissertation distributor paragraph above. 06/07/06

Copy Right Permission Elservier (*J. Chromatography. A.*):

ELSEVIER LIMITED LICENSE
TERMS AND CONDITIONS

Oct 02, 2007

This is a License Agreement between Shou-Feng Chen ("You") and Elsevier Limited ("Elsevier Limited"). The license consists of your order details, the terms and conditions provided by Elsevier Limited, and the payment terms and conditions.

License Number	1800930785290
License date	Oct 02, 2007
Licensed content publisher	Elsevier Limited
Licensed content publication	Journal of Chromatography A
Licensed content title	High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates
Licensed content author	Shou-Feng Chen, Richard A. Mowery, Vanessa A. Castleberry, G. Peter van Walsum and C. Kevin Chambliss
Licensed content date	3 February 2006
Volume number	1104
Issue number	1-2
Pages	8
Type of Use	Thesis / Dissertation
Portion	Full article
Format	Both print and electronic
You are an author of the Elsevier article	Yes
Are you translating?	No
Purchase order number	
Expected publication date	Dec 2007
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

Warranties: Publisher makes no representations or warranties with respect to the licensed material.

Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their

respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply to specific license types:

Translation: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

Website: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,

A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Author website with the following additional clauses: This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image, and
The permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Website (regular and for author): "A hyper-text must be included to the Homepage of the journal from which you are licensing at
http://www.sciencedirect.com/science/journal/xxxxx."

Thesis/Dissertation: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Other conditions: None

REFERENCES

(1) Cardona, C. A.; Sanchez, Q. J. *Bioresource Technology* **2007**, *98*, 2415-2457.

(2) Solomon, B. D.; Barnes, J. R.; Halvorsen, K. E. *Biomass and Bioenergy* **2007**, *31*, 416-425.

(3) Demirnas, A. *Energy Sources* **2005**, *27*, 327-337.

(4) Sun, Y.; Cheng, J. *Bioresource Technology* **2002**, *83*, 1-11.

(5) Mielenz, J. R. *Current Opinion in Microbiology* **2001**, *4*, 324-329.

(6) Saha, B. C.; Cotta, M. A. *Biotechnol. Prog.* **2006**, *22*, 449-453.

(7) Primental, D.; Patzek, T. W. *Natural Resources Research* **2005**, *14*, 65-76.

(8) Chum, H. L.; Overend, R. P. *Fuel Processing Technology* **2001**, *71*, 187-195.

(9) Wu, M.; Wu, Y.; Wang, M. *Biotechnol. Prog.* **2006**, *22*, 1012-1024.

(10) Chandel, A. K.; Chan, E. S.; Rudravaram, R.; Narasu, L. M.; Rao, V. L.; Ravindra, P. *Biotechnology and Molecular Biology Rev.* **2007**, *21*, 14-32.

(11) Kadam, K. L.; McMillan, J. D. *Bioresource Technology* **2003**, *88*, 17-25.

(12) Montross, M. D.; Crofcheck, C. L. *Bioresource Technology* **2004**, *92*, 269-274.

(13) Olsson, L.; Hahn-Hagerdal, B. *Enzyme and Microbial Technology* **1996**, *18*, 312-331.

(14) Palmqvist, E.; Hahn-Hagerdal, B. *Bioresource Technology* **2000**, *74*, 25-33.

(15) Klinke, H. B.; Thomesen, A. B.; Ahring, B. K. *Appl Microbiol Biotechnol.* **2004**, *66*, 10-26.

(16) Larsson, S.; Palmqvist, E.; Hahn-Hagerdal, B.; Tengborg, C.; Nilvergrant, N. *Enzyme and Microbial Technology* **1999**, *24*, 151-159.

(17) Laser, M.; Schulman, D.; Allen, G.; Lynd, L. R. *Bioresource Technology* **2002**, *81*, 33-44.

(18) Esteghlalian, A.; Hashimoto, A. G.; Fenske, J. J.; Penner, M. H. *Bioresource Technology* **1997**, *59*, 129-136.

(19) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Ladisch, M. *Bioresource Technology* **2005**, *96*, 673-686.

(20) Yourchisin, D. M.; van Walsum, G. P. *Applied Biochemistry and Biotechnology* **2004**, *113-116*, 1073-1086.

(21) Yang, B; Wyman, C. E. *Biotechnology and Bioengineering* **2004**, *86*, 89-95.

(22) Allen, S. G.; Schulman, D.; Lichwa, J.; Antal, M.J. *Ind. Eng. Chem. Res.* **2001**, *40*, 2934-2941.

(23) Kabel, M.; Bos, G.; Zeevalking, J.; Voragen, A.; Sshols, H. *Bioresource Technology* **2007**, *98*, 2034-2042.

(24) Ohgren, K.; Bura, R.; Saddler J.; Zacchi, G. *Bioresource Technology* **2007**, *98*, 2503-2510.

(25) Klinke, H. B.; Ahring, B. K.; Thomsen, A. S. *Bioresource Technology* **2001**, *82*, 15-26.

(26) Luo, C.; Brink, D. L.; Blanch, H. W. *Biomass and Bioenergy* **2002**, *22*, 125-138.

(27) Malester, I.; Green, M.; Shelef, G. *Ind. Eng. Chem*. **1992**, *31*, 1998-2003.

(28) Fagan, R.; Converse, O.; Grethlein, H.; Porteous, A. *Enviro. Sci. Technol.* **1971**, *5(6)*, 545-547.

(29) Church, J; Wooldridge, D. *Ind. Eng. Chem. Prod. Rev. Dev.* **1981**, *20(2)*, 371-378.

(30) McParland, J.; Grethlein, H.; Converse, A. *Solar Energy.* **1982**, *28(1)*, 55-63.

(31) Bhandari, N.; MacDonald, D.; Bakhshi, N. *Biotechnol. Bioeng.* **1984**, *26*, 320-327.

(32) Sidiras, D.; Kaukios, E. *Biomass* **1989**, *19(4)*, 289-306.

(33) Karr, W. E.; Brink, D. L. *J. Wood Chem. Technol.* **1991**, *11*, 447-463.

(34) Tran, A. V.; Chambers, R. P. *Appl. Microbio. Biotechnol.* **1986**, *23*, 191-197.

(35) Palmqvist, H.; Hahn-Hagerdal, B.; Galbe, M.; Zacchi, G. *Enzyme Microbio. Technol.* **1996**, *19*, 407-476.

(36) Theander, O. *Anim. Feed Sci. Technol.* **1991**, *32*, 35-44.

(37) Theander, O.; Westerlund, E. *J. Agric. Food Chem.* **1986**, *34*, 330-336.

(38) Chatzimichalakis, P. F.; Samanidou, V. F.; Papadoyannis, I. N. *Journal of Chromatography B* **2004**, *805*, 289-296.

(39) Shui, G.; Leong, L. P. *Journal of Chromatography A* **2002**, *977*, 89-96.

(40) Chen, H.; Zou, Y.; Deng, Y. *Journal of Chromatography A* **2001**, *913*, 387-395.

(41) Muenter, M. M.; Stokes, K. C.; Obie, R. T.; Jezorek, J. R. *Journal of Chromatography A* **1999**, *844*, 30-51.

(42) Suarez, B.; Palacios, N.; Fraga, N.; Rodriguez, R. *Journal of Chromatography A* **2005**, *1066*, 105-110.

(43) Escarpa, A.; Gonzalez, M. C. *Journal of Chromatography A* **1999**, *830*, 301-309.

(44) Robards, K.; Haddad, P. R.; Jackson, P. E. *Principles and Practice of Modern Chromatographic Methods*, Academic Press, Inc.: San Diego, CA. **1994**, 331-344.

(45) Swadesh, J. K. *HPLC: Practical and Industrial Applications, 2nd Edition*, CRC Press, LLC: Boca Raton, FL. **2001**, 141-200.

(46) Miller, J. M. *Chromatography: Concepts and Contrasts, 2nd Edition*, John Wiley & Sons, Inc.: Hoboken, NJ. **2005**, 249-262.

(47) Snyder, L. R.; Kirkland, J. J; Giajch, J. L. *Practical HPLC Method Development, 2nd Edition*, John Wiley & Sons, Inc.: New York, NY. **1997**, 234-264.

(48) Fifield, F. W.; Kealey, D. *Analytical Chemistry: principles and practice, 5th Edition*, Blackwell Science, Ltd.: Malden, MA. **2000**, 118-131.

(49) Melander, W. R.; Horvath, C. *High-Performance Liquid Chromatography: Advances and Perspectives*, Academic Press, Inc.: New York, NY. **1980**, 113-145.

(50) Snyder, L. R.; Kirkland, J. J; Giajch, J. L. *Practical HPLC Method Development, 2nd Edition*, John Wiley & Sons, Inc.: New York, NY. **1997**, 234-264.

(51) Satinder, A. *Chromatography and Separation Science*, Academic Press, Inc.: New York, NY. **2003**, 455-466.

(52) Scott, R. P. W. *Chromatographic Detectors: Design, Function, and Operation*, Marecl Dekker, Inc.: New York, NY. **1996**, 177-210.

(53) Huber, L.; George, S. A. *Diode Array Detection in HPLC: Chromatographic Science Series volume 62*, Marecl Dekker, Inc.: New York, NY. **1993**, 23-49

(54) Pohl, C. A.; Johnson, E. L. *Journal of Chromatographic Science* **1980**, *18*, 422-452.

(55) Document No. 031367-08: ASRS ULTRA Manual *DIONEX Corporation* **2002**.

(56) Application Note 106 *DIONEX Corporation* http://www1.dionex.com/en-us/webdocs/4642_106.pdf

(57) Smith, R. E. *Ion Chromatography Applications*; CRC Press, Inc.: Boca Raton, FL. **1988**, 37-46.

(58) Gjerde, D. T.; Fritz, J. S *Ion Chromatography, 2nd Edition*; Huthig: Heidelberg, NY. **1987**, 93-108.

(59) Weiss, J. *Ion Chromatography, 2nd Edition*; VCH Publisher Inc.: New York, NY. **1995**, 32-80.

(60) Schiller, M.; von der Heydt, H.; Marz, F.; Schmidt, P. *Journal of Chromatography A* **2002**, *968*, 101-111.

(61) Technical Note 21 *DIONEX Corporation* http://www1.dionex.com/en-us/webdocs/5050_TN21_16May07_LPN034889-03.pdf

(62) LaCourse, W. R.; Johnson, D. C. *Anal. Chem.* **1993**, *65*, 50-55.

(63) Jensen, M. B.; Johnson, D. C. *Anal. Chem.* **1997**, *69*, 1776-1781.

(64) Rocklin, R. D.; Clarke, A. P.; Weitzhandler, M. *Anal. Chem.* **1998**, *70*, 1496-1501.

(65) Ding, Y.; Yu, H.; Mou, S. *Journal of Chromatography A* **2004**, *1039*, 39-43.

(66) Cai, Y.; Cheng, J.; Mou, S.; Yiqiang, L. *Journal of Chromatography A* **2005**, *1085*, 124-130.

(67) Application Note 87 *DIONEX Corporation* http://www1.dionex.com/en-us/webdocs/5041_an87.pdf

(68) Campbell, C. J.; Laherrere, J. H.; Mou, S. *Sci. Am.* **1998**, *3*, 78-83.

(69) Dresselhaus, M. S.; Thomas, I. L. *Nature* **2001**, *414*, 323-337.

(70) Wyman, C. E. *Bioresource Technology* **1994**, *50*, 3-16.

(71) Sheehan, J.; Himmel, M. *Biotechnol. Prog.* **1999**, *15*, 817-827.

(72) Chum, H. L.; Overend, R. P. *Fuel Process. Technol.* **2001**, *71*, 187-195.

(73) Hansen, A. C.; Zhang, Q.; Lyne, P. W *Bioresource Technology* **2005**, *96*, 277-393.

(74) Mielenz J. R. *Curr. Opin. Microbiol* **2001**, *4*, 324-329.

(75) Tolen, J. S. *Clean Technol. Environ. Policy.* **2002**, *3*, 339-345.

(76) Sun, Y.; Cheng, J. *Bioresource Technology* **2002**, *83*, 1-14.

(77) Saha, B. C. *J. Indus. Microbiol. Biotechnol* **2003**, *30*, 279-291.

(78) Klinke, H. B.; Thomsen, A. B.; Ahring, B. K. *Appl. Microbiol. Biotechnol* **2004**, *66*, 10-26.

(79) Klinke, H. B.; Ahring, B. A.; Thomsen, A. B. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 631-638.

(80) Cantarella, M.; Cantarella, L.; Gallifuoco, A.; Spera, A *F. Alfani, Biotechnol. Prog.* **2004**, *20*, 200-206.

(81) Thomasser, C.; Danner, H.; Neureiter, M.; Saidi, B.; Braun, R. *Appl. Biochem. Biotechnol.* **2002**, *98-100*, 765-774.

(82) Karagoz, S.; Bhaskar, T.; Muto, A.; Sakata, Y. *Fuel* **2004**, *83*, 2293-2299.

(83) Luo, C.; Brink, D. L.; Blanch, H. W. *Biomass and Bioenergy* **2002**, *22*, 125-138.

(84) Fenske, J. J.; Griffin, D. A.; Penner, M. H. *J. Indus. Microbiol. Biotechnol.* **1998**, *20*, 364-368.

(85) DiNardo, R. C.; Larson, R.A. *Bioresource Technology* **1994**, *48*, 135-141.

(86) Niemela, K. *Holzforschung* **1998**, *42*, 169-173.

(87) Pecina, R.; Burtscher, P.; Bonn, G.; Bobleter, O. *Fresenius Z. Anal. Chem.* **1986**, *325*, 461-465.

(88) Niemela, K.; Sjostrom, E. *Holzforschung* **1986**, *40*, 361-368.

(89) Alen, R.; Niemela, K.; Sjostrom, E. *J. Chromatogr.* **1984**, *301*, 273-276.

(90) Lapierre, C.; Rolando, C.; Monties, B. *Holzforschung* **1983**, *37*, 189-198.

(91) Gey, M *Acta Biotechnol* **1988**, *8*, 197-205.

(92) Klink, H. B.; Ahring, B. K.; Schmidt, A. S; Thmosen, A. B. *Bioresource Technology* **2002**, *82*, 15-26.

(93) Persson, P.; Andersson, J.; Gorton, L.; Lasson, S.; Nilvebrant, N.; Jonsson, L. *J. Agric. Food. Chem.* **2002**, *50*, 5318-5325.

(94) Bonn, G.; Oefner, P. J.; Bobleter, O. *Fresenius Z. Anal. Chem.* **1988**, *331*, 46-50.

(95) Bipp, H. P.; Fischer, K.; Bienkek, D.; Kettrup, A. *Fresenius Z. Anal. Chem.* **1997**, *357*, 321-325.

(96) Zeanann, A. J.; Bobleter, O. *Adv. Therochem. Biomass Convers.* **1994**, *2*, 953-956.

(97) Burtscher, E.; Bobleter, O.; Schwald, W.; Concin, R.; Binder, H. *J. Chromatogr.* **1987**, *390*, 401-412.

(98) Bonn, G.; Bobleter, O. *Chromatographia.* **1984**, *18*, 445-448.

(99) Shui, G.; Leong, L. P. *Journal of. Chromatography A* **2002**, *977*, 89-96.

(100) Lewis, R. J; Johnson, R. D.; Angier, M. K.; Ritter, R. M *Journal of Chromatography B* **2004**, *806*, 141-150.

(101) Palmqvist, E.; Hann-Hagerdal, B. *Bioresource Technology* **2000**, *74*, 17-24.

(102) Himmel, M. E.; Baker, J. O.; Overened, R. P. *ACS Symposium Series* **1994**, *566*, 292-324.

(103) Gray, M.; Converse, A. O.; Wyman, C. E. *Appl. Biochem. Biotechnol.* **2003**, *105-108*, 179-193.

(104) Lee, Y. Y.; Wu, Z; Torget, R. W. *Bioresource Technology* **2000**, *71*, 29-39.

(105) Lloyd, T.; Wyman, C. *Appl. Biochem. Biotechnol.* **2003**, *105-108*, 53-67.

(106) Torget, R. W.; Kim, J. S.; Lee, Y. Y. *Ind. Eng. Chem. Res.* **2000**, *39*, 2817-2825.

(107) Yang, B.; Wyman, C. E. *Biotechnol. Bioeng.* **2004**, *86*, 88-98.

(108) Jacobsen, S. E.; Wyman, C. E. *Appl. Biochem. Biotechnol.* **2000**, *84-86*, 81-96.

(109) Jacobsen, S. E.; Wyman, C. E. *Ind. Eng. Chem. Res.* **2002**, *41*, 1454-1461.

(110) Lynd, L. R.; Cushman, J. H.; Nichols, R. J. *Science* **1991**, *251*, 1223-1318.

(111) Lynd, L. R. *Annu. Rev. Energy Evniron.* **1996**, *21*, 403-465.

(112) Ancillotti, F.; Fattore, V. *Fuel Process. Technol.* **1998**, *57*, 163-194.

(113) Aristodou, A.; Penttila, M. *Current Opinion in Biotechnology* **2000**, *11*, 187-198.

(114) Bothast, R. J.; Schlicher, M. A. *Appl. Microbio. Biotechnol.* **2005**, *67*, 19-25.

(115) Gnansounou, E.; Dauriat, A.; Wyman, C. E. *Bioresource Technology* **2005**, *96*, 985-1002.

(116) Roberto, I. C.; Mussatto, S. I.; Rodrigues, R. C. L. B. *Indus. Crops Prod.* **2003**,*17*, 171-176.

(117) Saha, B. C; Iten, L. B.; Cotta, M. A.; Wu, Y. V. *Biotechnol. Prog.* **2005**, *21*, 816-822.

(118) Schell, D. J.; Walter, P. J.; Johnson, D. K. *Appl. Biochem. Biotechnol.* **1992**, *34-35*, 659-665.

(119) Wyman, C. E.; Dale, B. E.; Elander, R. T.; Holtzapple, M.; Ladisch, M. R.; lee, Y. Y. *Bioresource Technology* **2005**, *96* 1959-1966.

(120) Liao, W.; Liu, Yan.; Zhiyou, W.; Frear, C.; Chen, S. *Bioresource Technology* **2007**, *98*, 1992-1999.

(121) Liao, W.; Liu, Yan.; Zhiyou, W.; Frear, C.; Chen, S. *Bioresource Technology* **2004**, *94(1)*, 33-41.

(122) Liao, W.; Liu, Yan.; Zhiyou, W.; Frear, C.; Chen, S. *Appl. Biochem. Biotechnol.* **2005**, *121-124*, 765-774.

(123) Yourchisin, D.; van Walsum, G. P. *Appl. Biochem. Biotechnol.* **2004**, *113-115*, 1073-1086.

(124) Abatzoglou, N.; Chornet, E.; Overend, R. P. *Chemical Engineering Science* **1992**, *47(5)*, 1109-1122.

(125) Bouchard, J.; Nguyen, T. S.; Chornet, E.; Overned, R.P. *Bioresource Technology* **1991**, *36*, 121-131.

(126) Maloney, I. A.; Green, M.; Shelef, G. *Ind. Eng. Chem. Res.* **1992**, *31*, 1998-2003.

(127) Cheng, K-K.; Liu, H-J.; Liu, D-H. *Biotechnology Letters* **2005**, *27(1)*, 19-22.

(128) Garrote, G.; Dominguez, H.; Parajo, J. C. *Process Biochemistry* **2002**, *37*, 1067-1073.

(129) Ian, F.; Cullis, J. N. S.; Shawn, D. *Biotechnol. Bioeng.* **2004**, *85(4)*, 413-421.

(130) Jollez, P.; Chornet, E.; Overend, R. P. *Advance in Thermochemical Biological Conversion* **1994**, *50*, 156-159.

(131) McWillams, R. C.; van Walsum, G. P. *Current Opinion in Microbiology* **2002**, *98-100*, 109-134.

(132) Montane, D.; Salvado, J.; Farriol, X.; Jollez, P.; Chornet, E. *Wood Science and Technology* **1994**, *28*, 387-402.

(133) Overend, R. P.; Chornet, E. *Phil. Trans. R. Soc. Lond.* **1987**, *A321*, 523-536.

(134) Soderstrom, J.; Plicher, L.; Galbe, M.; Zacchi, G. *Biomass and Bioenergy* **2003**, *24(3)* 475-486.

(135) van Walsum, G. P. *Appl. Biochem. Biotechnol.* **2001**, *91-93*, 317-329.

(136) van Walsum, G. P.; Shi, H. *Bioresource Technology* **2004**, *93*, 217-226.

(137) Brasch, D. J.; Free, K. W. *Tappi. Journal.* **1965**, *48(4)*, 245-248.

(138) Chum, H. L.; Johnson, D. K.; Black, S. K.; Overend, R. P. *Appl. Biochem. Biotechnol.* **1990**, *24-25*, 1-14.

(139) Tongborg, C.; Stenberg, K.; Galbe, M.; Zacchi, G.; Larrson, S.; Hahn-Hagerdal, B. *Appl. Biochem. Biotechnol.* **1998**, *70-72*, 3-15.

(140) Larsson, S.; Palmqvist, E.; Hahn-Hagerdal, B.; Tengborg, C.; Stengerg, K.; Zacchi, G.; Niolvebrant, N.O. *Enz. Microbiol. Technol.* **1998**, *24*, 151-159.

(141) Baugh, K. D.; Levy, J. A.; McCarty, P. L. *Biotechnology & Bioengineering* **1988**, *31*, 62-70.

(142) Baugh, K. D.; McCarty, P. L. *Biotechnology & Bioengineering* **1988**, *31*, 50-61.

(143) Persson, P.; Larsson, S.; Jonsson, L. J. *Biotechnology & Bioengineering* **2002**, *79(6)*, 694-700.

(144) Maloney, M. T.; Chapman, T. W.; Baker, A. J. *Biotechnology & Bioengineering* **1985**, *27*, 355-361.

(145) Chen, S-F.; Mowery, R. A.; Castleberry, V. A.; van Walsum, G. P.; Chambliss, C. K. *Journal of Chromatography A* **2006**, *1104*, 54-61.

(146) Wyman, C. E. Application of corn stover and fiber. In Corn, 2nd edition. *American Association of Cereal Chemists:St. Paul, MN.,* **2003**, 723-750.

(147) Pordesimo, L. O.; Hames, B. R.; Sokhansanj, S.; Edens, W.C. *Biomass and Bioenegy.*, **2005**, *28*, 366-374.

(148) Sokhansanj, S.; Turhollow, A.; Cushman, J.; Cundiff, J. *Biomass and Bioenergy* **2002**, *23*, 347-355.

(149) National Renewable Energy Laboratory. Standard Biomass Analytical Procedures. http://www1.eere.energy.gov/biomass/analytical_procedures.html

(150) Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *NREL.* **2006**, http://devafde.nrel.gov/pdfs/9345.pdf

(151) Hames, B. R.; Thomas, S. R.; Sluiter, A. D.; Roth, C. J.; Tempeton, D. W. *Appl. Biochem. Biotechnol* **2003**, *105-108*, 5-16.

(152) Sanderson, M. A.; Agblevor, F.; Collins, D. K. *Biomass and Bioenergy* **1996**, *11*, 365-370.

(153) Thammasouk, K.; Tandjo, D.; Penner, M. H. *J. Agric. Food Chem.* **1997**, *45*, 437-443.

(154) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *NREL.* **2006**, http://devafde.nrel.gov/pdfs/9361.pdf

(155) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *NREL.* **2006**, http://devafde.nrel.gov/pdfs/9462.pdf

(156) Porter, L. J.; Hrstich, L. N.; Chan, B. G. *Phytochemistry* **1986**, *25*, 223-230.

(157) Galletti, G. C.; Reeves III, J. B.; Bocchnin, P.; Muscarella, C. I. *J. Agric. Food Chem.* **1997**, *45*, 1715-1719.

(158) Pordesimo, L. O.; Hames, B. R.; Sokhansanj, S.; Deens, E. C. *Biomass and Bioenergy* **2005**, *28*, 366-374.

(159) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *NREL.* **2006**, http://devafde.nrel.gov/pdfs/9572.pdf

(160) Newth, F. H. The formation of furan compounds from hexoses. *Advances in Carbohydrate Chemistry* Academic Press, Inc.: New York, **1951**, *Vol. 6*, 83-106.

(161) Klinke, H. B.; Olsson, L. K.; Thomsen, A. B.; Ahring, B. K. *Biotechnology & Bioengineering* **2003**, *81*, 783-747.

(162) Top Value Added Chemicals from Biomass: Volume 1, Werpy, T.; Petersen, G., Eds.; *Report No. DOE/GO102004-1992* **2004**, *NREL*.

(163) Regna, E. A.; Bruins, P. F. *Ind. Eng. Chem.* **1956**, *48*, 1268-1277.

(164) Brauer, D.; Teel, M. R. *Plant Physiol.* **1982**, *70*, 723-727.

(165) Walford, S. N. *South African Sugar Technologists Association.* **1998**, *72*, 234-241.

(166) Burar, R.; Stout, P. R. *Science* **1965**, *150*, 766-767.

(167) Fenske, J. J.; Griffin, D. A.; Penner, M. H. *J. Inds. Microbiol. Biotechnol.* **1998**, *20*, 364-368.

(168) Mosier, N.; Hendrickson, R.; Ho, N.; Sedlak, M. *Bioresource Technology* **2005**, *96*, 1986-1993.

(169) Liu, C.; Wyman, C. E. *Ind. Eng. Chem. Res.* **2003**, *42*, 5409-5416.