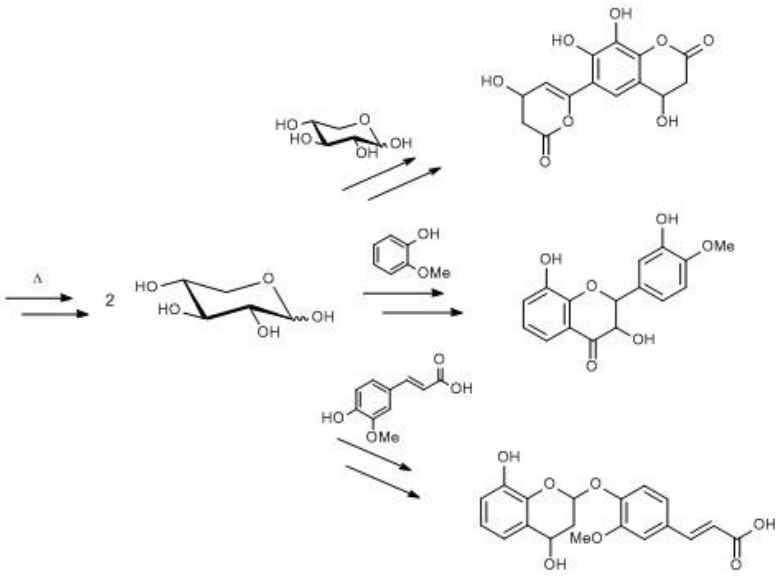
Carbohydrate degradation mechanisms and compounds from pretreated biomass

Rasmussen, Helena

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Carbohydrate degradation mechanisms and compounds from pretreated biomass



Ph.D. Thesis

Helena Rasmussen

DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

DONG energy

Preface

This industrial Ph.D. project was carried out from October 2012 to June 2016 (including 2 leaves of absence of in total 9 months) for DONG Energy in collaboration with DTU, Center for BioProcess Engineering and as a part of the B21st project - Biofuels for the 21st Century funded by Innovation Fund Denmark.

The project was supervised by Professor Anne S. Meyer, Center for BioProcess Engineering and Head of Technology Hanne R. Sørensen, DONG Energy.

I would like to thank my supervisors for their support and for giving me the opportunity to work on this Ph.D. project. I would also like to thank David Tanner for discussion of reaction mechanisms with me.
Thank you Kit, Frida and Martin for providing me with data from pilot plant experiments. Thanks to Duy for analysis of glycol ether pretreated wheat straw fiber fraction and Jane for proof reading of MS fragments.
Thank you to all my nice colleagues in Dong and BioEng, DTU.

Most of all, I would like to thank my family.

Helena Rasmussen, Farum, June 2016

Summary

The formation of inhibitors during pretreatment of lignocellulosic feedstocks is a persistent problem, and notably the compounds that retard enzymatic cellulose conversion represent an obstacle for achieving optimal enzymatic productivity and high glucose yields. Compounds with many chemical functionalities are formed during biomass pretreatment, which gives possibilities for various chemical reactions to take place and hence formation of many new potential inhibitor compounds. This somehow overlooked contemplation formed the basis for the main hypothesis investigated in this work:

Hypothesis 1) Liquors from biomass pretreatment contain an array of hitherto unidentified cellulase* inhibitors that are believed to be reaction products from carbohydrate degradation.

(*cellulases include endo-cellulases, cellobiohydrolases, LPMO, and beta-glucosidase enzyme activities)

Furthermore the two following two hypotheses were tested.

Hypothesis 2). Formation of these inhibitor compounds can be prevented by protection of reactive chemical functionalities as revealed from their mechanisms for formation.

Hypothesis 3) Process parameters influence the amount and type of reaction products (from hypothesis 1) that are formed and in turn change inhibition.

In order to point out potent cellulase inhibitors, a solvent extraction based fractionation method was developed to separate compounds in liquid from pilot plant hydrothermal pretreatment of wheat straw. *Via* 2-butanone extraction a group of potent cellulase inhibitors were identified with LC-MS/MS to be oligophenolic compounds. 26 of the compounds were new and by considering the reaction mechanisms and synthesis routes for their formation it was revealed that xylose was heavily involved in their formation. The new oligophenolic cellulase inhibitors were suggested to be formed during hydrothermal pretreatment by xylose self-condensation reactions involving aldol condensations, 1,4 additions to α,β unsaturated carbonyl compounds, 3-keto acid decarboxylations and oxidations. In addition xylose reactions with phenolic lignin components were suggested.

The identification of the central role of xylose in the reaction routes for oligophenolic inhibitor formation led to the solution to protect the reactive anomeric center in xylose. Protection of the anomeric center in *in situ* generated xylose with ethylene glycol monobutyl ether, during pretreatment of wheat straw, reduced the level of oligophenolic compounds with 73 % compared to the original pretreatment and 41 % compared to the control.

When pretreatment severity was increased the amount of xylooligosaccharides decreased whereas the amount of oligophenolic compounds increased. No new degradation compounds were formed although the profile of the oligophenolic inhibitors changed.

New dipentoses with hydroxylated oxane bicyclic moieties and feruloylated tripentoses are suggested also to play a role in inhibition, because LC-MS/MS analysis revealed the presence of these components in the liquid from hydrothermal pretreated wheat straw after enzymatic treatment.

It was found that formation of the oligophenolic degradation compounds were common across biomass sources as sugar cane bagasse and oil palm empty fruit bunches. These findings were in line with that the oligophenolic compounds arise from reactions involving xylose from hemicellulose in the biomass. Even

though oligophenolic degradation compounds were common across biomasses, variations were found in biomass structural elements that were released during pretreatment. Pentoseoligosaccharides from sugar cane bagasse had a more acetylated substitution pattern than wheat straw, and in oil palm empty fruit bunches 4-hydroxybenzoic acid was identified to be a variation from a lignin structural elements released during pretreatment.

In conclusion it was found that the reactions taking place during pretreatment of biomass are complex and involve both degradation compounds and biomass structural elements. The present work has shed some light over the reactions and from this new insight a new type of pretreatment with anomeric protection was proposed and tested. The results open up for implementation of new types of pretreatments that hinder monosaccharide degradation to inhibitor compounds in lignocellulosic biomass processing.

Dansk sammenfatning

Dannelsen af inhibitorer under forbehandling af lignocelluose biomasser er et problem - specielt stoffer der inhiberer den enzymatiske cellulose konvertering udgør en forhindring i forhold til at opnå optimal enzymatisk katalyse og højt glukose udbytte. Stoffer med mange kemiske funktionaliteter dannes under forbehandlingen, hvilket giver mulighed for, at der kan finde reaktioner sted, hvorved der dannes nye potentielle inhibitorer. Denne lidt oversete betragtning gav anledning til den primære hypotese, der blev undersøgt i dette arbejde:

Hypotese 1) Væske fra biomasse forbehandling indeholder en række hidtil uidentificerede cellulase* inhibitorer, som tænkes at være reaktions produkter fra kulhydrat nedbrydning.

(*cellulase inkluderer endo-cellulase, cellobiohydrolase, LPMO, and beta-glucosidase enzym aktiviteter)

Derudover blev følgende to hypoteser undersøgt.

Hypotese 2) Dannelsen af disse inhibitorer kan forhindres ved at beskytte reaktive kemiske funktionaliteter, som udledt fra reaktionsmekanismerne for deres dannelse.

Hypotese 3) Proces parametre har indflydelse på mængden og typen af reaktionsprodukter (fra hypotese 1), der dannes og dermed påvirkes inhiberingen.

For at udpege potente cellulase inhibitorer blev en solvent ekstraktions baseret metode udviklet for at separere stoffer i væske fra hydrotermisk forbehandling af hvedestrå foretaget i et pilot anlæg. *Via* 2-butanone ekstraktion blev en gruppe af potente cellulase inhibitorer identificeret med LC-MS/MS til at være oligophenol forbindelser. 26 af forbindelserne var nye og ved at undersøge reaktionsmekanismer og syntese ruter for dannelsen af stofferne blev det klart, at xylose var kraftigt involveret i reaktionerne og dermed dannelsen af stofferne. Dannelsen af de nye oligophenol cellulase inhibitorer blev foreslået til at finde sted under den hydrotermiske forbehandling ved xylose selv-kondenseringsreaktioner, inklusive aldol kondensationer, 1,4 additioner til α,β umættede carbonyl forbindelser, 3-keto syre decarboxyleringer og oxidationer. Hertil kommer også reaktion af xylose med phenol forbindelser fra lignin.

Identifikationen af xyloses centrale rolle i reaktionerne for dannelsen af oligophenol inhibitorerne førte til løsningen at beskytte det reaktive anomeriske center i xylose. Beskyttelse af det anomeriske center i *in situ* dannet xylose med ethylene glycol monobutyl ether under en forbehandling af hvedestrå, reducerede niveauet af oligophenol forbindelser med 73 % sammenlignet med den originale forbehandling og med 41 % sammenlignet med kontrol forsøget.

Ved hårdere forbehandling faldt mængden af xylooligosaccharider, mens mængden af oligophenol forbindelser steg. Der blev ikke dannet nye typer af nedbrydningsprodukter - dog ændredes profilen af oligophenol forbindelser, idet der blev dannet relativt mere af nogle forbindelser.

Nye dipentoser med hydroxylerede oxane bicykliske strukturer samt feruloylerede tripentoser blev foreslået til også at spille en rolle i inhiberingen, fordi disse blev fundet med LC-MS/MS i væske fra hydrotermisk forbehandling af hvedestrå efter den enzymatiske behandling.

Resultaterne viste, at dannelsen af oligophenol forbindelser var fælles for hvedestrå, sukkerrør bagasse og olie palme "empty fruit bunches". Disse resultater passede med, at oligophenol forbindelser stammer fra reaktioner, der involverer xylose fra hemicellulosen i biomassen. Selvom oligophenol forbindelserne var

fælles for de undersøgte biomasser, var der også variationer i de biomasse strukturelle elementer, der blev frigjort under forbehandlingen. Pentoseoligosaccharider fra sukkerrør bagasse havde et mere acetyleret substitutions mønster end hvedestrå og i olie palme "empty fruit bunches" blev 4-hydroxybenzoe syre identificeret til at være et eksempel på variation fra lignin delen af biomassen.
Den overordnede konklusion på arbejdet er, at reaktionerne der finder sted under forbehandling af biomasse er komplekse og involverer både nedbrydningsprodukter og biomasse strukturelle elementer. Arbejdet har kastet lys over reaktionerne og ud fra denne nye indsigt blev en ny type af forbehandling med anomerisk beskyttelse foreslået og testet. Resultaterne åbner op for implementeringen af nye typer af forbehandling, som forhindrer monosaccharid nedbrydning til inhibitor forbindelser under lignocellulose biomasse forbehandling.

List of abbreviations

BPC	Base Peak Chromatogram
DP	Degree of Polymerisation
EFB	oil palm Empty Fruit Bunches
4-HBA	4-Hydroxybenzoic acid
HCD	Higher-energy collisional dissociation
HMF	5-(hydroxymethyl)-2-furaldehyde
HPAEC	High Pressure Anion Exchange Chromatography
HPLC	High Pressure Liquid Chromatography
LC-MS/MS	Liquid Chromatography mass spectrometry with fragmentation
LfHP	Liquid from Hydrothermal Pretreatment
LPMO	Lytic Polysaccharide Monooxygenase
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
RI	Refractive Index
SCB	Sugar Cane Bagasse
TIC	Total Ion Chromatogram
WF	Water Fraction

Carbohydrate degradation mechanisms and compounds from pretreated biomass

1 Introduction

Lignocellulosic conversion processes have the potential to provide the future society with environmentally friendly energy and supply starting materials for platform chemicals (Climent *et al* 2014, Han *et al* 2014, Luterbacher *et al* 2014, Matson et al 2011, Rinaldi 2014). When lignocellulosic biomass is processed into biofuels, and potentially other biorefinery products, the biomass is usually pretreated in order to make the cellulose and hemicellulose amenable to enzymatic depolymerization. Although such pretreatment may be done according to a number of regimes (Behera *et al* 2014, Silveira *et al* 2015, Pedersen and Meyer 2010, Larsen *et al* 2008), current large scale pretreatment processes of lignocellulosic biomass usually involves some kind of high temperature treatment. The biomass pretreatment, especially pretreatment involving acid and/or temperatures above 160-180 °C, induces formation of degradation products that may inhibit the cellulolytic enzymes (Table 1) and/or the ethanol producing microorganisms (notably yeast, *Saccharomyces cerevisiae*) (Table 2) that are required for the subsequent sugar conversion (Jönsson and Martín 2016).

Table 1. Inhibition of cellulolytic enzyme activity (commercial cellulase preparations) by lignocellulosic biomass degradation products. Activity (%) compared to reference hydrolysis with no inhibitor present (data from a (Kim *et al* 2011, b (Panagiotou and Olsson 2007), c (Tejirian and Xu 2011), d (Ximenes *et al* 2011), e (Qing *et al* 2010).

Inhibitor compound	Conc.(mM)	Commercial cellulases (% activity relative to reference)
Furfural and HMF total	19	~ 100[a]
Syringaldehyde	5/10	~100[b]/~30[c]
Ferulic acid	10	~100[d]
Tannic acid	1	~40[d]
Vanillin	13	~100[d]
p-Coumaric acid	12	~100[d]
4-Hydroxybenzoic acid	14	~100[d]
Phenolic and/or lignin model compounds	1	~20-30[c]
Acetic acid	218	95-100[a]
Formic acid	326	5-20[b]
Xylooligomers (mixed sizes)	55 (xylose based)	18[e]

5-(hydroxymethyl)-2-furaldehyde (HMF) and furan-2-carbaldehyde (furfural) are considered the key primary degradation compounds from the carbohydrate fraction, but several other products, notably other aldehydes, aliphatic acids (Flannelly *et al* 2016) and phenolic compounds (Popoff and Theander 1976, Popoff and Theander 1971, Mitchell *et al* 2014) may also form and they have different inhibitory impact on the enzymes and yeast (Table 1 and Table 2).

Table 2. Ethanol yields (g ethanol/g consumed glucose) relative to reference fermentation. Data adapted from (Klinke *et al* 2004, Larsson *et al* 1999).

Inhibitor compound	Conc. (mM)	*S.cerevisiae* (Baker's yeast)
Furfural	21-65	82-50
HMF	8-57	50
4-Hydroxybenzaldehyde	4-17	50
Vanillin	1-18	~50-100
Syringaldehyde	1-25	50
Levulinic, acetic and formic acids	Total < 100	Increase in yield
	Total > 100	Decrease in yield

Knowledge of the degradation routes is therefore an important foundation for controlling the inhibitor formation and in turn for improving the current cellulosic ethanol processes - notably:

1. The degradation products and their routes of formation.
2. The influence from process parameters on reaction mechanisms and routes of formation and in turn which degradation products that are formed.

1.1 Biomass degradation during hydrothermal treatment

1.1.1 Cellulose, hemicellulose and lignin

The glucose originating from cellulose can be thermally degraded directly during the biomass pretreatment. In one type of degradation, glucose is firstly dehydrated to HMF, which can then further degrade to formic acid and levulinic acid (Flanelly *et al* 2016, Yang *et al* 2012) (Figure 1). As the monomeric substituents in hemicellulose include both hexoses and pentoses, hemicellulose may also give rise to HMF, formic acid, and levulinic acid (Figure 1). In contrast, furfural is formed exclusively from pentoses, i.e. mainly from xylose and arabinose released from the hemicellulose (Figure 1).

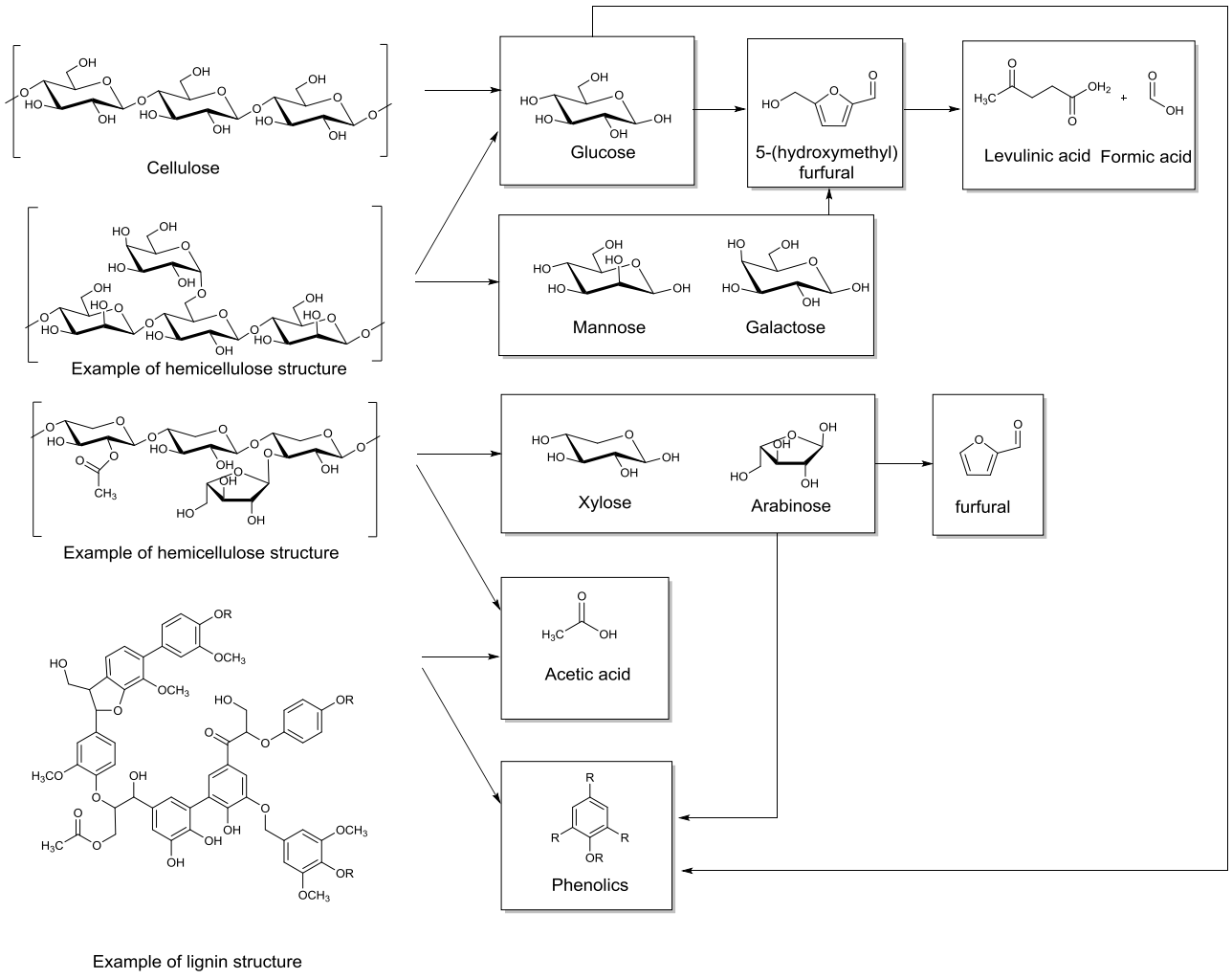


Figure 1. Suggested products and summary reaction routes for degradation of biomass saccharides (cellulose and hemicelluloses structures) plus lignin during hydrothermal treatment.

Hydrothermal biomass pretreatment can also lead to cleavage of the acetyl linkages in xylan and presumably also induce hydrolytic cleavage of the acetyl substitutions in e.g. acetylated galacto-glucomannans, leading to formation of free acetic acid (Yelle *et al* 2013, Chen *et al* 2012) (Figure 1). Although phenolic compounds found in biomass liquors can stem from lignin (Mitchell *et al* 2014), early reports show that various phenolic compounds may also form as degradation products from glucose, xylose and arabinose (Forsskåhl *et al* 1976, Popoff and Theander 1976, Popoff and Theander 1972 (Figure 1). Recently this formation of phenolics has been confirmed by demonstration of pseudo-lignin generation from model substrates of cellulose (i.e. Avicel), xylan and xylose (Kumar *et al* 2013).

Apart from monosaccharides from cellulose and hemicellulose, also xylooligosaccharides, that are biomass structural element liberated from xylan during pretreatment have shown importance in cellulase inhibition (Kont *et al* 2013, Zhang and Viikari 2012, Baumann *et al* 2011, Qing *et al* 2010). Xylooligosaccharides may in addition to substitution with different saccharides (Figure 1), also be substituted with for example ferulic acid (Appeldoorn *et al* 2013).

The aromatic residues in lignin can be degraded to different types of phenolic structures depending on the type of structural monomeric unit in the lignin (Mitchell *et al* 2014). Furthermore it cannot be excluded that

acetylated lignin (Rio *et al* 2007, Rio *et al* 2015) liberates acetic acid when thermally treated (Yelle *et al* 2013) (Figure 1).

1.1.2 Degradation product inter-reactions

The degradation compounds shown in Figure 1 are moreover able to undergo various reactions with each other. Such intermolecular reactions can lead to several new products and polymerization reactions.

Pseudo lignin

Pseudo lignin is an aromatic material containing hydroxyl and carbonyl functional groups. In this way pseudo lignin resembles native lignin, but is not derived thereof. Sannigrahi *et al* (Sannigrahi *et al* 2011) proposes the broad definition of pseudo-lignin to be an aromatic material that yield a positive Klason lignin value and is not derived from native lignin. The existence of pseudo lignin has been recognized because the amount of Klason lignin in pretreated biomass has often been found to be higher than in untreated biomass and has furthermore been reported to increase with pretreatment severity (Sannigrahi *et al* 2011). Recent progress has confirmed that pseudo-lignin can form solely *via* carbohydrate degradation (Kumar *et al* 2013). It has furthermore been proposed (Hu *et al* 2012) that pseudo-lignin arises from polymerization and/or condensation reactions from the key intermediates 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol derived from furfural and HMF respectively. However, these reactions need experimental support regarding at least three aspects: 1) It is uncertain whether 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol are in fact “key intermediates” in the sense that it is unclear whether the pseudo lignin formation also involves other compounds. 2) The formation of 3,8-dihydroxy-2-methylchromone from furfural has not been explicitly demonstrated. 3) It has not been unequivocally proven that 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol give rise to pseudo lignin formation.

Popof and Theander (Popoff and Theander 1972) showed that degradation of different monosaccharides at low pH (pH 3.5 and pH 4.5) at 96 °C produced 3,8-dihydroxy-2-methylchromone as a major degradation product, but they did not show that its formation took place through furfural. As well, the formation of 1,2,4-benzenetriol through HMF has been found to occur at 290-400 °C and high pressure (27.5 MPa) (Luijkx *et al* 1993), but this reaction remains to be demonstrated to occur at more typical lignocellulosic biomass pretreatment conditions.

Pseudo lignin has in several reports (Kumar *et al* 2013, Sannigrahi *et al* 2011, Hu *et al* 2012) been demonstrated to deposit as droplets on the surface of pretreated lignin free biomass and carbohydrate model substrates and is in this way thought to retard the enzymatic hydrolysis by enzyme inhibition and/or reduced substrate accessibility (Kumar *et al* 2013).

Humins

Humins are dark coloured substances that are formed during thermal hydrolysis of glucose. Formation of humins has been shown to arise from reaction of HMF with glucose (Dee and Bell 2011) and/or *via* reaction of HMF with 2,5-dioxo-6-hydroxy-hexanal (hydrated HMF) (Patil and Lund 2011) and subsequent polymerization (Figure 2). The formation of humins *via* HMF reactions have been reported relatively recently

(Dee and Bell 2011, Patil and Lund 2011), and whether, and to which extent, humins impact cellulolytic enzymes and/or yeast during cellulosic ethanol production is at present uncertain.

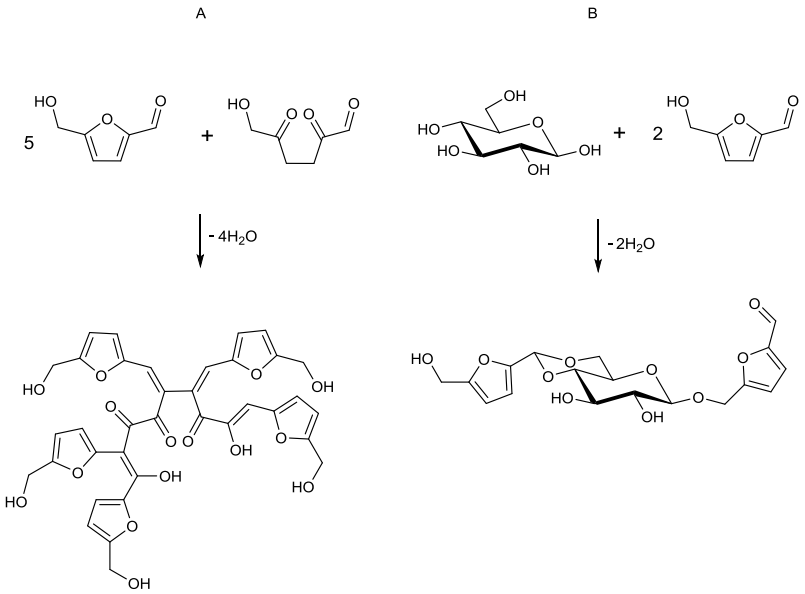


Figure 2. Examples of humins formation. A: From HMF reaction with hydrated HMF (2,5-dioxo-6-hydroxy-hexanal. B: From HMF and glucose (Patil and Lund 2011) and (Dee and Bell 2011).

Maillard products

Maillard products are a wide array of reaction products that are formed from the reaction of carbohydrates with amino containing compounds at elevated temperature. They are relevant to consider as reaction products in pretreatments of biomass because carbohydrate constituents may react with amino acids in proteins from the biomass (Echavarría at al 2012, Wang et al 2011). The initial steps of the Maillard reactions are reaction of the reducing carbohydrate with the amino containing compound followed by an amadori rearrangement to the ketoamine (Echavarría at al 2012, Wang et al 2011) as illustrated with glucose (Figure 3). From here a wide array of less characterised reactions can take place to form a plethora of compounds including small heterocyclic aroma compounds and low to high molecular melanoidins (Figure 3).

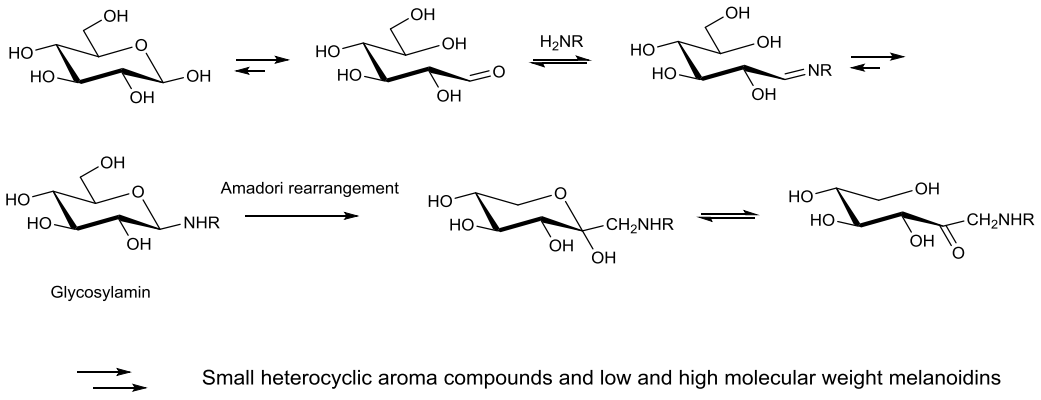


Figure 3. The initial well-known steps of Maillard reactions.

Although Maillard products have shown to inhibit protein degrading enzymes as carboxypeptidases (Ibarz *et al* 2008) and have been suggested as inhibitors for lignocellulytic enzymes (Humpula *et al* 2014, Chundawat *et al* 2010), the effect still has to be proven.

1.1.3 Influence from pretreatment process parameters on degradation products

Based on the knowledge about the many different degradation products formed during pretreatment it is relevant to consider the influence from pretreatment process parameters. The presence of sulphuric acid and in turn low pH during hydrothermal pretreatment of corn stover affect the level of several degradation compounds (Table 3). In general, the formation of degradation products were, not surprisingly, higher in the dilute acid pretreatment than with no addition of acid. The most distinct difference is the increase in the levels of levulinic, acetic and formic acids together with HMF and furfural with dilute acid pretreatment. The increase in levulinic and formic acids corresponds well with the increase in HMF as described in Figure 1. The increase in furfural and acetic acid after dilute acid pretreatment is in accord with more pronounced degradation of pentoses and acetylated xylan, i.e. degradation of pentose rich hemicellulose as outlined in Figure 1.

Table 3. [a]0.7 % (w/w) H_2SO_4, 180 °C, 8 min. solids conc. 10 g/L. [b]180 °C, 8 min., solids conc. 10 g/L (Du *et al* 2010).

Degradation product	Corn Stover Dilute acid pretreatment[a]	Corn Stover Hydrothermal pretreatment[b]
	μM	
Levulinic acid	350	0
Acetic acid	2830	570
Ferulic acid	30	10
HMF	350	20
Furfural	2290	80
Total phenolics	210	170

The data from pretreatment of corn stover (Table 3) clearly show that reaction conditions influence degradation product profile.

1.2 Thesis outline and hypotheses

This work focuses on hydrothermal pretreatment of wheat straw and the subsequent enzymatic hydrolysis as this is considered the core processes for Inbicon (Figure 4), which is the industrial partner of the PhD project. Sugar cane bagasse and oil palm empty fruit bunches are also considered as biomass feedstocks, to a certain extent, to consider variations from biomass.

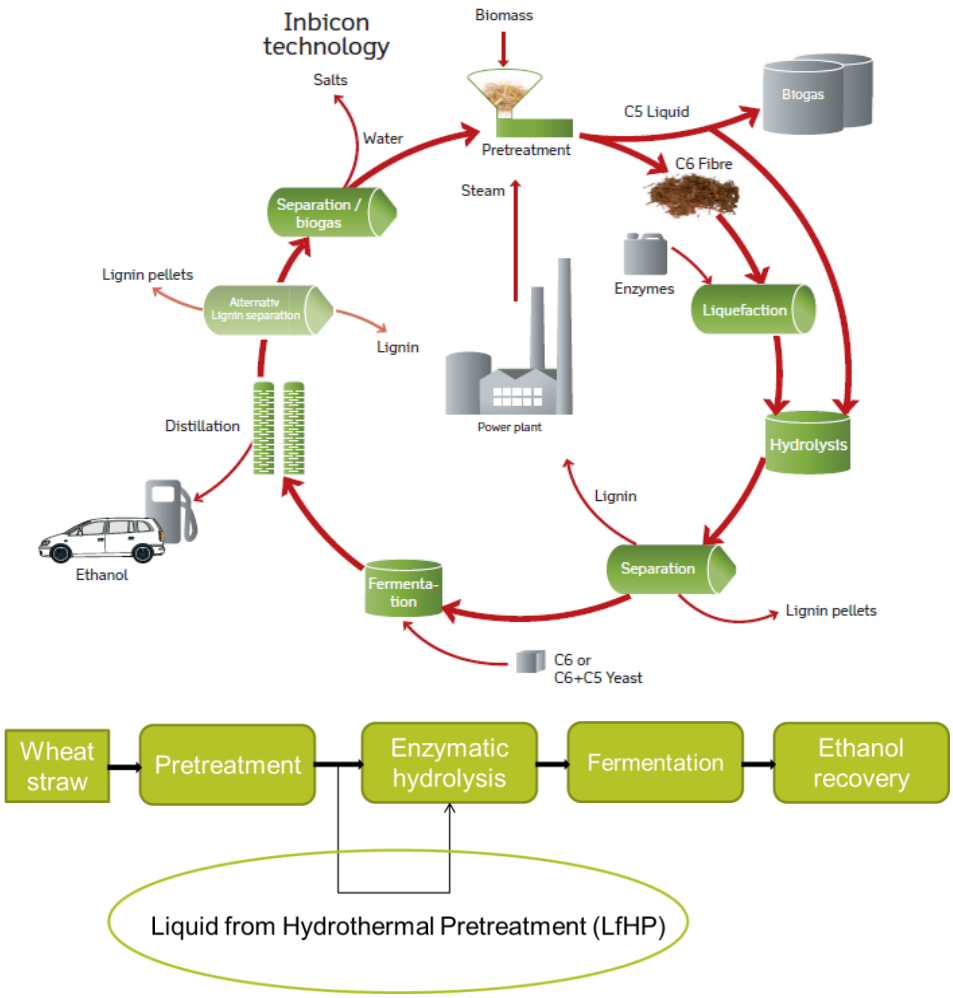


Figure 4. Top: The overall Inbicon process. Bottom: Liquid from hydrothermal pretreatment (LfHP) from the Inbicon process considered in this work.

The main goal of this work was to reveal the chemical identity of potent cellulase inhibitors originating from the carbohydrate fraction in liquors from hydrothermal pretreatment of wheat straw (Figure 4). Furthermore to consider their synthesis routes in order to understand the origin of the compounds and, if possible, hinder their formation.

As outlined above many different degradation products are formed during pretreatment of biomass and many of these are enzyme inhibitors. This include biomass structural elements as xylooligosaccharides that are liberated from xylan during pretreatment, as well as compounds that originate from chemical reactions taking place during pretreatment as some phenolic compounds. It can be hypothesised, that other still unidentified compounds can be potent inhibitors as well, since compounds with many chemical functionalities are formed during biomass pretreatment and give rich possibilities for various chemical reactions to take place and hence form many new potential inhibitor compounds. This gives rise to the following hypotheses:

Hypothesis 1) Liquors from biomass pretreatment contain an array of hitherto unidentified cellulase* inhibitors that are believed to be reaction products from carbohydrate degradation.
(*cellulases include endo-cellulases, cellobiohydrolases, Lytic Polysaccharide Monooxygenases (LPMO), and beta-glucosidase enzyme activities)

Hypothesis 2) The disclosure of the mechanisms for formation of these inhibitor compounds can lead to new processing strategies that avoid their formation.
During the PhD project, notably after unveiling the reaction mechanisms for xylose degradation during hydrothermal biomass pretreatment, hypothesis 2 was advanced:
Hypothesis 2). Formation of these inhibitor compounds can be prevented by protection of reactive chemical functionalities as revealed from their mechanisms for formation.

Hypothesis 3) Process parameters influence the amount and type of reaction products (from hypothesis 1) that are formed and in turn change inhibition.

In order to test the hypotheses, the specific objectives was:
In relation to hypothesis 1) To develop a method to i) fractionate compounds in liquors from hydrothermal pretreatment and ii) test and quantify their inhibitor potency towards cellulytic enzymes and iii) account for all inhibition from liquid from hydrothermal pretreatment. To elucidate the structures of the compounds in the fractions and consider their synthesis routes in order to determine if they are reaction products or native biomass structural elements that are released during hydrothermal pretreatment.
In relation to hypothesis 2) To consider the reaction mechanism for representative compounds in detail and from this evaluate protection reactions.
In relation to hypothesis 3) To subject a liquid from hydrothermal pretreatment conducted at higher severity to the fractionation and inhibitor potency test and account for all inhibition from liquid from hydrothermal pretreatment and compare it to the original pretreatment. To elucidate the structures of the compounds in the fractions.

2 Mechanistic considerations

Mechanistic and retrosynthesis considerations are important in relation to hypothesis 1 and hypothesis 2 in regard to two aspects: 1) Determination of the origin of any new compounds i.e. if they are reaction products generated during hydrothermal pretreatment or native biomass structural elements that are liberated during hydrothermal pretreatment. 2) Identification of reactive sites that possibly can be protected to hinder reaction.

In the literature it is vividly discussed which degradation/synthesis routes and reaction mechanisms biomass monosaccharides undertake, with the reaction mechanisms for xylose degradation to furfural and for glucose degradation to HMF appearing to be particularly discussed (Ahmad *et al* 1995, Antal *et al* 1991, Yang *et al* 2012, Kimura *et al* 2011, Qian 2012). It is also discussed which degradation products xylose and glucose actually lead to. During a literature review (Rasmussen *et al* 2014) it became clear that the stated viewpoints may in fact all be correct, because several degradation routes and reaction mechanisms are possible and they are influenced by reaction conditions. It is an important retrosynthesis consideration because one reactant can lead to different products depending on reaction conditions.

This is exemplified for xylose below (and for glucose in Rasmussen *et al* 2014).

2.1.1 Degradation of xylose to furfural

Both a ring opening of xylose i.e. an acyclic mechanism and two different types of direct cyclic mechanisms (Figure 5) have been suggested for the degradation of xylose to furfural. There is experimental support of both mechanisms (Ahmad *et al* 1995, Antal *et al* 1991) indicating that the degradation of xylose is complex and may not occur according to one mechanism only. Moreover, several different organic acids, aldehydes, and ketones have been reported as products from xylose degradation (Möller and Schröder 2013, Antal *et al* 1991, Oefner *et al* 1992).

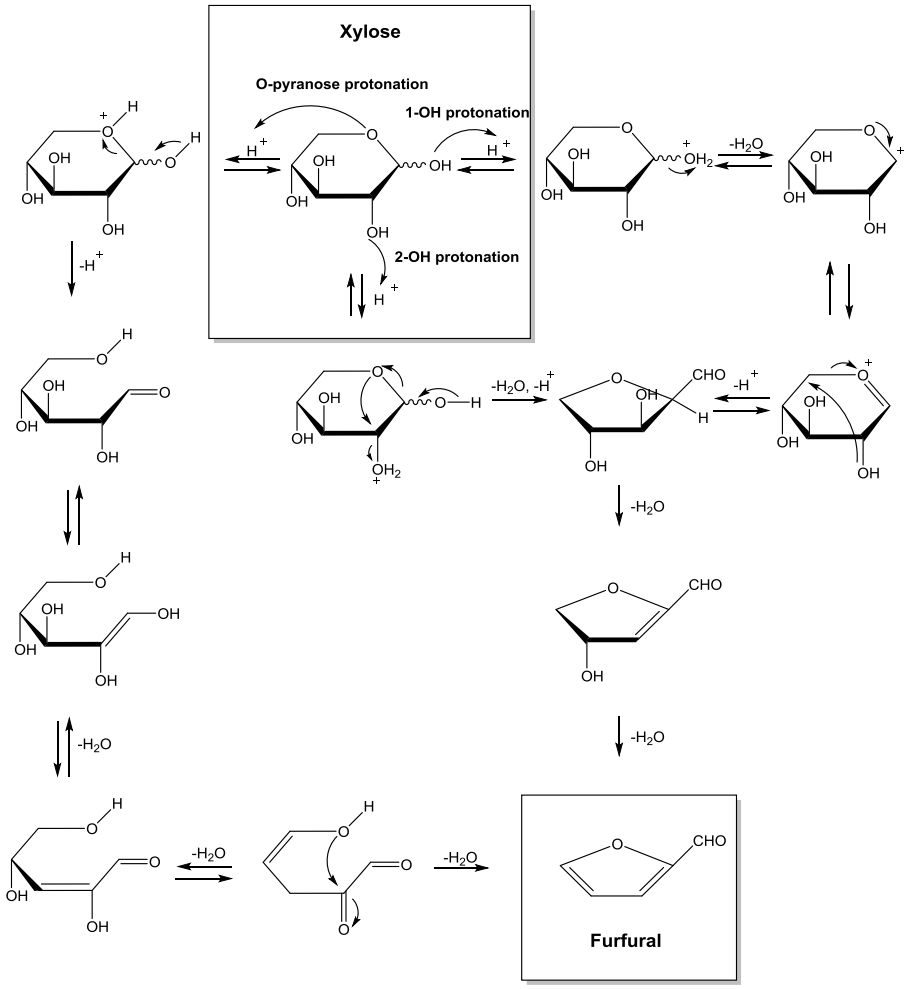


Figure 5. Suggested acyclic and cyclic mechanisms for furfural formation from xylose resulting from protonation at either *O*-pyranose, 1-OH or 2-OH. Reaction scheme adapted and summarised from schemes proposed by (Ahmad *et al* 1995 and Antal *et al* 1991).

Via ab initio molecular dynamics simulations it has been shown that the rate limiting step in the reaction of xylose is protonation of hydroxyl groups on the xylose or direct protonation of the pyranose oxygen. Hence, the subsequent degradation product formed depends on the hydroxyl site of protonation (Qian *et al* 2005). The significance of the initial hydroxyl group protonation for the subsequent reactions is supported by calculation of activation energies by using quantum mechanics modelling in combination with NMR studies (Nimlos *et al* 2006). For *ab initio* molecular dynamics simulations and quantum mechanics modelling combined with NMR with no solvent effects (in vacuum) the reaction schemes are summarised in Figure 6 together with the proposed reaction mechanisms from Figure 5.

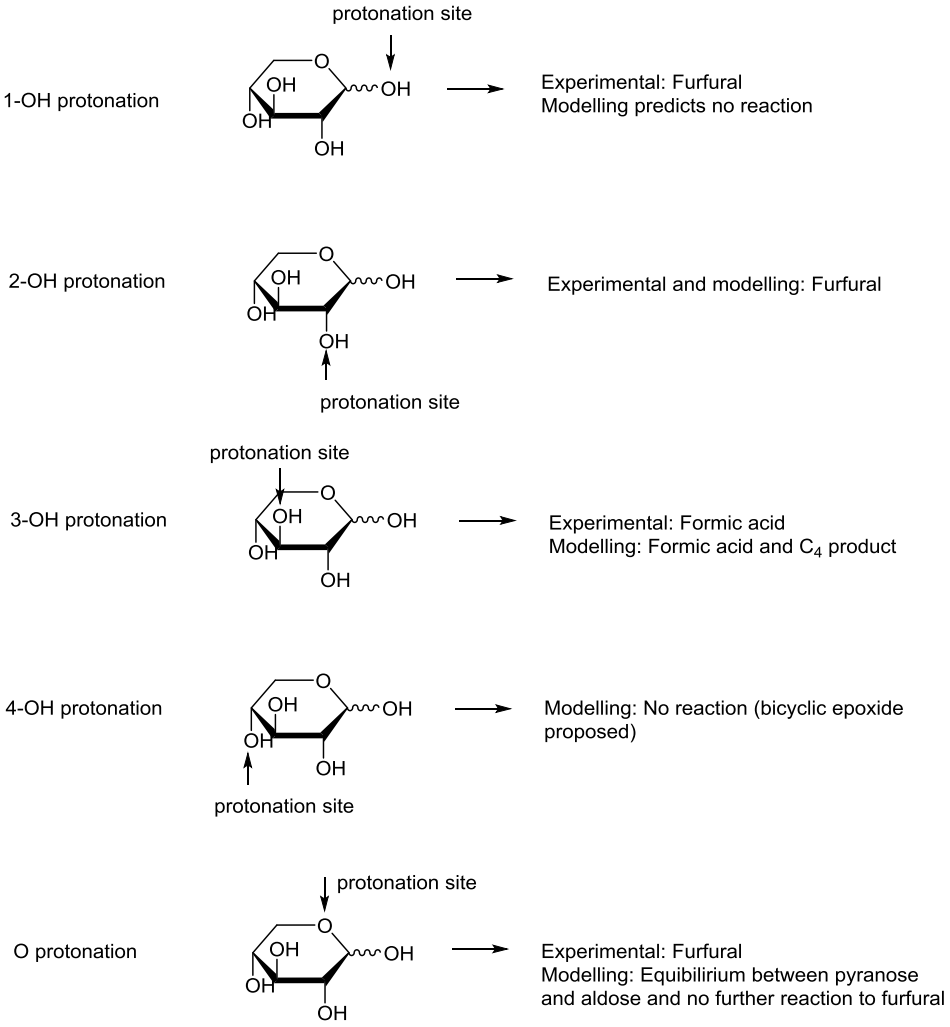


Figure 6 Xylose: Site of protonation and subsequent degradation products determined by *ab initio* molecular dynamics simulations in the gas phase and quantum mechanics modelling with no solvent water. Summarised from (Qian *et al* 2005 and Nimlos *et al* 2006) and Figure 5.

It is worth noting that in the models and simulations (summarised in Figure 6), the furfural formation from the ring opened xylose, i.e. the acyclic mechanisms followed by *O*-pyranose protonation, shown in Figure 5, does not occur. Protonation of the pyranose oxygen, which leads to ring opening, only leads to the equilibrium between pyranose and aldose and not to further reaction to furfural according to the models. Hence the models contradict with the experimental data which support furfural formation *via* the acyclic mechanism (Figure 5). Furthermore the ring closed mechanism resulting from protonation at 1-OH (as suggested in Figure 5) does not give any observable reaction when modeled by *ab initio* molecular dynamics simulations and quantum mechanics. Hence *via* this modeling the furfural formation appears to arise exclusively from the 2-OH protonation of xylose (Figure 5).

The solvent water structure is crucial for the protonation site since water molecules compete for protons and hydrogen bond to the hydroxyl groups. In addition, reaction conditions (pH, solvent, salts etc.) can easily alter the water molecule surroundings and hence the hydroxyl protonation site and the reaction mechanism and the following reaction.

2.1.2 New mechanisms to be considered

Some of the reported degradation mechanisms for xylose and glucose are analogous to each other (Rasmussen *et al* 2014) while other new mechanisms yet have to be modelled and tested. Two new reaction mechanisms for xylose degradation to furfural can be suggested:

Pyranose O protonation: Glucose follows an acyclic mechanism with isomerisation to fructose (Yang *et al* 2012). It can also be hypothesised that xylose could degrade *via* an analogous mechanism with a furan isomerisation intermediate (xylulose) which further dehydrates to furfural (Figure 7).

2-OH protonation: As for glucose (Kimura *et al* 2011, Qian 2012), xylose could possibly also proceed through a cyclic mechanism through the furan isomerisation intermediate (xylulose) or bypassing it to furfural (Figure 7).

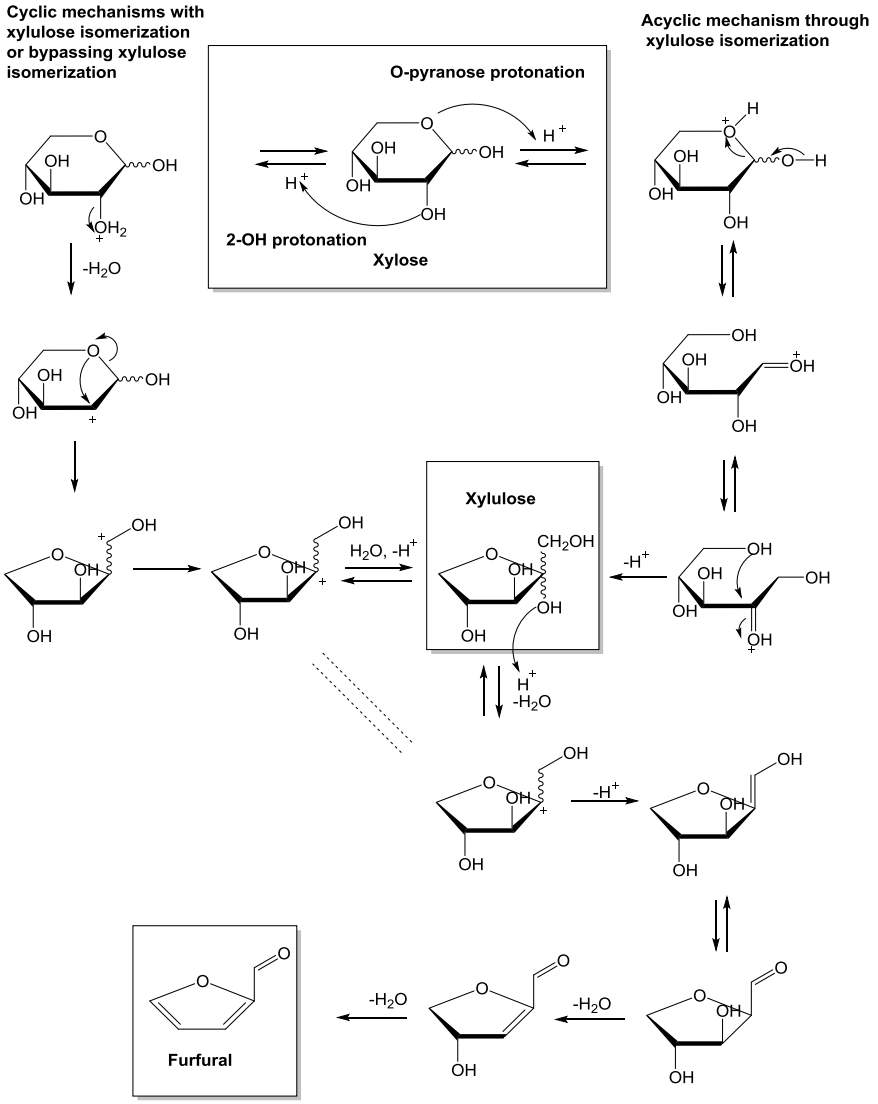


Figure 7. Hypothesised new mechanisms for xylose degradation to furfural.

2.2 Chapter 2 concluding remarks

Although the reaction mechanisms outlined in this chapter provide an important base for understanding how the degradation products are formed and offer some clues to predict which products that are likely to be formed, the results cannot be directly transferred to complex reaction mixtures resulting from biomass pretreatment, because of the significance of surrounding water molecule structure, which is effected by reaction conditions and other components in the reaction mixture. Several degradation routes and reaction mechanisms are possible for the same starting material, which is an important retrosynthesis consideration because one reactant can lead to different products depending on reaction mechanisms.

Furthermore reactions between compounds and intermediates in the reaction mixture influence the degradation product profile from biomass pretreatment.

The complexity of the theoretical base illustrate that it is necessary to perform experimental evaluations to gain full insight under the actual conditions.

3 New degradation compounds from wheat straw biomass pretreatment: Routes for formation of potent oligophenolic enzyme inhibitors

3.1 Introduction

Optimisation of enzyme performance during lignocellulosic saccharification and in turn yield is important for biorefinery processes. An important task towards this goal is a closer study of the large number of different degradation compounds, that are also inhibitory toward enzymes, generated or liberated during hydrothermal pretreatment of the biomass. Compound types that are reported to be especially inhibitory towards cellulases, include xylooligosaccharides (Kont *et al* 2013, Zhang and Viikari 2012, Baumann *et al* 2011, Qing *et al* 2010) and several phenolic compounds (Tejirian and Xu 2011, Mhlongo *et al* 2015, Kim *et al* 2011).
It complicates the picture that both the amounts and type of degradation compounds as well as the biomass structural elements that are liberated during pretreatment, can vary with pretreatment process parameters (Rasmussen *et al* 2014, Paper 4). Furthermore the different compounds most likely have different inhibitor potency i.e. some may be very inhibitory towards enzymatic catalysis while others are harmless. These complications urged the first task undertaken in this PhD study, which was development of a method to point out potent inhibitors in authentic pilot plant liquors from hydrothermal pretreated wheat straw and then subject these to analytical characterisation.
This chapter outlines the work with identification of potent cellulase inhibitors, consideration of their formation routes and prevention of their formation, which is all related to hypothesis 1 and 2.

3.2 Methodical considerations

3.2.1 Fractionation

In order to group compounds for further evaluation, fractionation *via* solvent extraction was carried out. Extraction solvents were chosen as polar as possible to compete with water and a protic and an aprotic solvent were chosen to obtain a specificity in solubility. Furthermore low boiling points were desired to enable efficient removal. 2-butanone and 2-butanol fulfilled the criteria and were selected for extraction of LfHP (Table 4).

Table 4. Solvent properties (Murov 2016).

Property	2-butanol	2-butanone
Polarity relative to water	0.506	0.327
Hydrogen bonding	protic	aprotic
Boiling point	99.5 °C	79.6 °C

3.2.2 Enzyme assay

The choice of evaluation method for enzyme inhibition was based on two considerations. i) The purpose was to evaluate groups of compounds from fractionation (section 3.2.1) (i.e. not single compounds) from

pretreated biomass and thus several enzyme activities in the catalytic cascade were expected to be affected. Thus an evaluation of overall inhibition of enzyme activities was most appropriate and a commercial enzyme preparation was well suited for this purpose. ii) A commercial enzyme preparation was also relevant for evaluation of inhibition due to the industrial aspect of the work with pilot scale pretreatment of biomass and subsequent enzymatic hydrolysis.

Accordingly, Cellic CTec3 obtained from Novozymes A/S (Bagsværd, Denmark) was utilised. It is a commercially available cellulase preparation based on the *Trichoderma reesei* complex. Apart from the cellulolytic enzyme base from *T. reesei* it contains at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, and a β-xylosidase (Rosgaard *et al* 2007) and also other proprietary hydrolysis-boosting proteins.

Initial attempts were made to determine initial reaction rates of cellulose conversion to glucose in order to investigate if inhibition was competitive on non-competitive *via* Lineweaver-Burk plots (Stryer 1995). However, liquid from hydrothermal pretreatment (LfHP) itself contains glucooligosaccharides that during enzymatic hydrolysis were hydrolysed to a final glucose content of 0.5 % (w/w) in the enzymatic hydrolysis mixture. This is a relative high amount and necessitated high substrate (avicel) concentrations in the assay to overshadow the released glucose from LfHP itself in order to evaluate inhibition. Thus further investigation of inhibition mechanisms was ceased. An enzymatic hydrolysis assay with 12 % (w/w) avicel and a run time of 23 hours, where glucose release in the tested fractions was stabilised, was found appropriate (Figure 13) for evaluation of overall enzyme inhibition. In all figures throughout the thesis, glucose originating from glucooligosaccharides in the LfHP is subtracted ie. blanks with no addition of avicel are subtracted. Glucose monosaccharide was present in concentrations < 0.05 % prior to enzymatic hydrolysis.

Table 5. Glucose release from LfHP 1 and its fractions from the extractions. WF: Water Fraction.

Fraction	Glucose content from oligosaccharides (% w/w)
LfHP 1	0.5 ± 0.01
WF from 2-butanol extraction	0.5 ± 0.01
WF from 2 butanone extraction	0.5 ± 0.04
2-butanol fraction	0.2 ± 0.01
2-butanone fraction	< 0.05

3.2.3 Evaluation of volatile compounds

No difference in inhibition between freeze dried and non-freeze dried LfHP was observed (Figure 8) indicating that potentially inhibitory, volatile compounds, such as furfural, acetic and formic acid, had not escaped evaluation Thus, in agreement with previous data (Kim *et al* 2011) it was affirmed, that volatile compounds, in the present amounts, where furfural was measured to 0.04 g/L, acetic acid to 5.5 g/L and formic acid to 2.6 g/L do not contribute to the inhibition from LfHP. It should be noted in this context that the LfHP is actually obtained from pilot plant reduced pressure evaporation of authentic pilot plant LfHP, which was concentrated to a similar concentration of that produced in a demonstration plant (16.6 % (w/w) dry

matter). It could be argued that volatile compounds had already been removed during the evaporation - thus authentic LfHP (10.9 % (w/w) dry matter) was also tested before and after freeze drying to ensure that no volatile compounds had escaped inhibitor evaluation, which was not the case (Figure 8). Furfural, acetic and formic acid levels in authentic LfHP were 0.2 g/L 3.9 g/L and 1.6 g/L respectively.

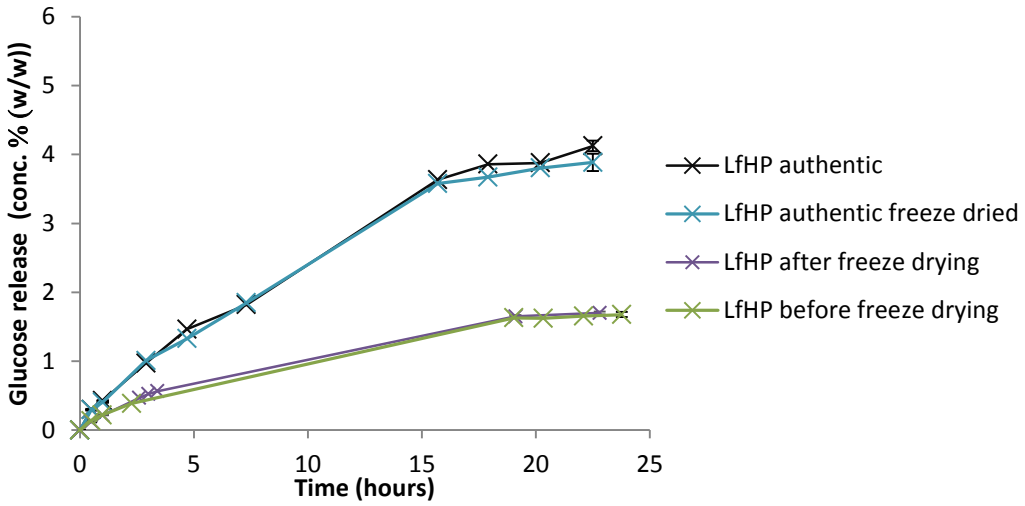


Figure 8. LfHP (16.6 % (w/w) dry matter) before and after freeze drying and authentic LfHP (10.9 % (w/w) dry matter) before and after freeze drying. LfHP authentic before and after freeze drying are similar (two-tailed T-Test, 95 % confidence interval).

3.2.4 Evaluation of ionisation in MS

Samples of all fractions were run at 2 different concentrations: 10 mg/mL and 1 mg/mL in order to evaluate if ionisation was a true function of concentration or just due to the ionisation ability of the compounds seen. The data showed good correlation between concentration and ion intensity of the investigated compounds (data not shown).

3.2.5 Evaluation of anomeric protection of xylose

The Fischer type glycosylation that was chosen as protection strategy (section 3.3.5) is well known for lower boiling alcohols (Bornaghi and Poulsen 2005, Mowery 1961). According to this, an initial test with ethanol was carried out to evaluate the reaction products in HPLC-RI analysis (Paper 2). Xylose (4 g) and Dowex 50WX2 (SigmaAldrich) (4 g) was suspended in 50 mL ethanol (abs 99.9 %) and heated to reflux. After 8 hours a sample was taken and centrifuged (15 minutes, 15.000 rcf), diluted 1:1 with MilliQ water and analysed on HPLC with RI detection (Figure 9).

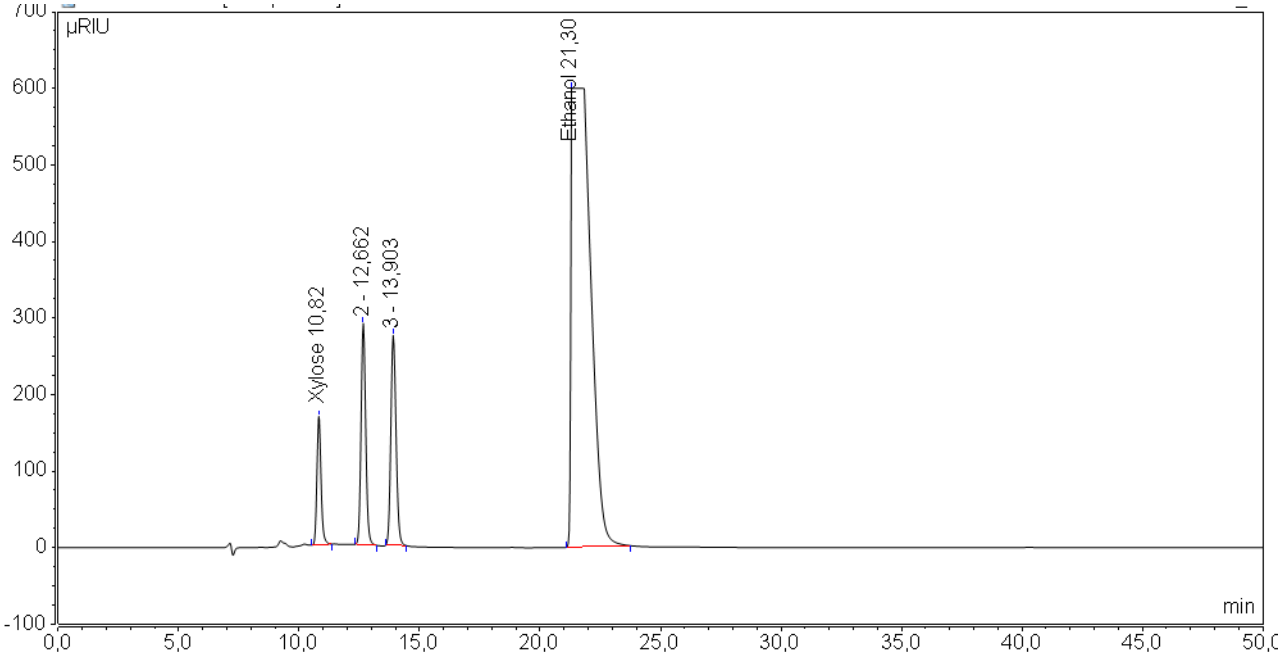


Figure 9. In process analysis of anomeric protection of xylose with ethanol.

The α and β anomers of the ethanol protected xylose eluted at 12.7-13.9 minutes. With ethylene glycol monobutyl ether as protection group the retention time shifted to 19.3-20.7 minutes (Paper 2).

3.2.6 Evaluation of inhibitor level

Because the new compounds identified with LC-MS were aromatic compounds (section 3.3.2), UHPLC-UV (260 nm) total UV count (Paper 2) was chosen to evaluate their total relative level in the experiments with anomeric protection (section 3.3.5, Figure 19). The retention time region for total UV count was determined as specified below.

Analysis of the 2-butanone fraction (10 g/L) (Figure 10 blue) showed that the oligophenolic compounds eluted mainly from 3.0 minutes to 6.0 minutes, which was in line with the very demarcated retention time region in the LC-MS analysis (Figure 15). It was also clear that the oligophenolic compounds had been removed from the water fraction (10 g/L) (Figure 10 pink), which was also in agreement the data from LC-MS (Figure 15). The liquids from hydrothermal pretreatment in the pilot plant (Figure 10 black) and Parr reactor (Figure 10 brown) conducted at the same temperature and time (183 °C, 18 min) were also analysed. The LfHP from the Parr reactor pretreatment does not have the same concentration as the LfHP from the pilot plant (84 g/L in the analysis), because the Parr reactor experiment could only be conducted at 8 % (w/w) dry matter (to ensure agitation) opposed to 40 % (w/w) dry matter in the pilot plant. Furthermore the LfHP from the pilot plant was concentrated by evaporation as described above (section 3.2.3).

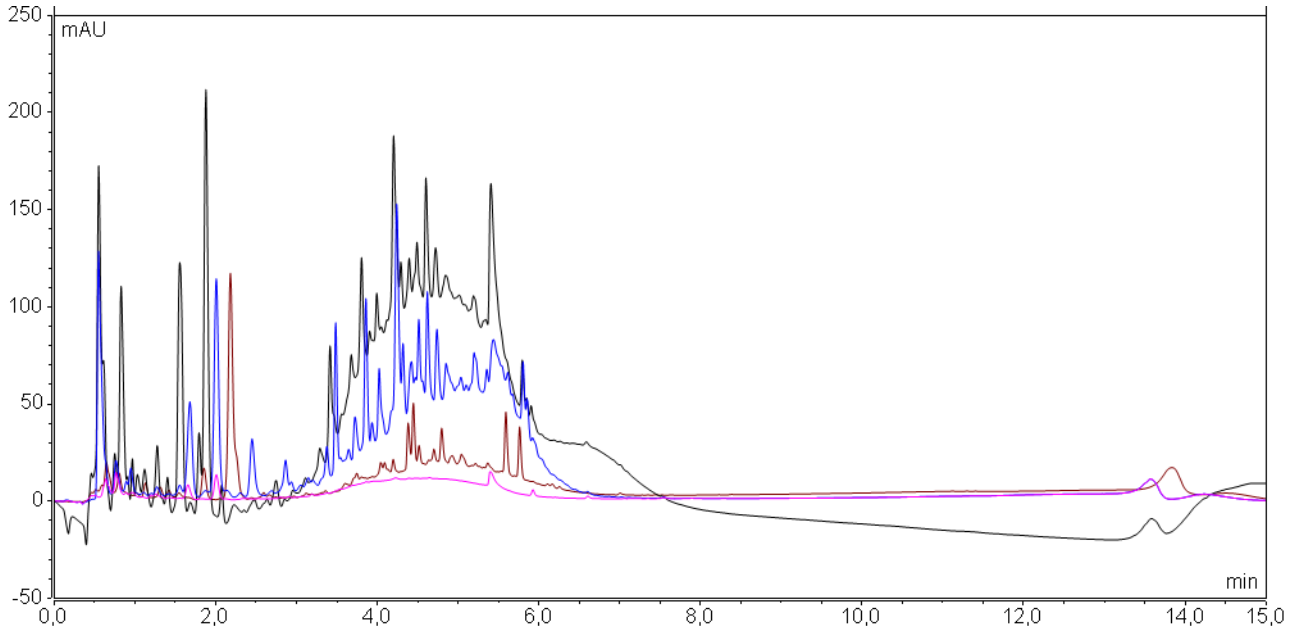


Figure 10. UHPLC UV (260 nm) analysis of LfHP from the pilot plant (black), the 2-butanone fraction (blue), LfHP from the Parr reactor (brown) and the water fraction form the 2-butanone extraction (pink).

An obstacle occurred during the analysis of the pretreatment with ethylene glycol monobutyl ether, because it dissolved a small part of the lignin, that interfered in the analysis. The lignin content decreased from 21 % (w/w) in the raw wheat straw to 17 % (w/w) in the pretreated wheat straw from the pretreatment with ethylene glycol monobutyl ether (Figure 11). When it was compared to the original pretreatment from the pilot plant (Figure 11), that does not dissolve lignin, it was clear that the pretreatment with ethylene glycol monobutyl ether had dissolved a small part of the lignin, because there was a difference of 10 % (w/w) in the lignin content.

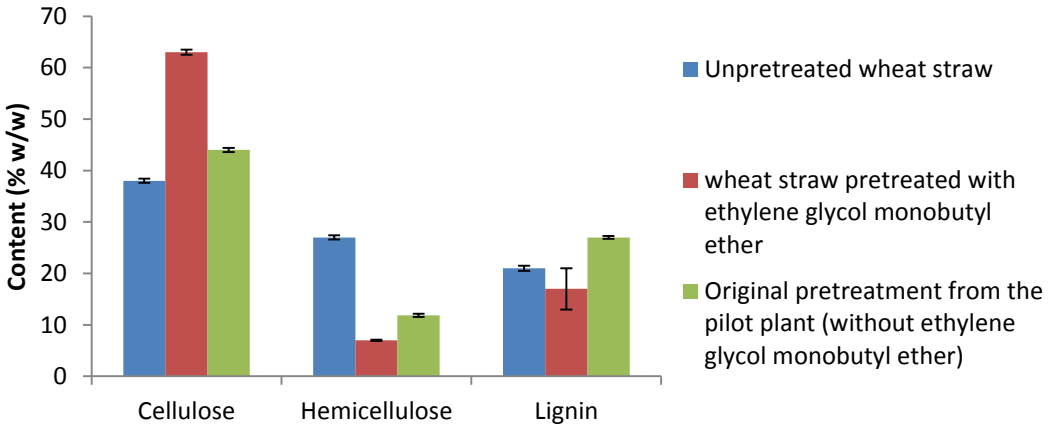


Figure 11. Composition of wheat straw and pretreated wheat straw. Analysed according to Sluiter *et al* 2008.

Along with the lignin solubilisation, a group of late eluting compounds beyond 6 minutes retention time showed up in the analysis of the liquid from pretreatment with ethylene glycol monobutyl ether (Figure 12, blue), strongly suggesting that the late eluting compounds were due to the dissolution of lignin. The late eluting compounds beyond 6 minutes retention time had an overlap with the late eluting part of the oligophenolic compounds.

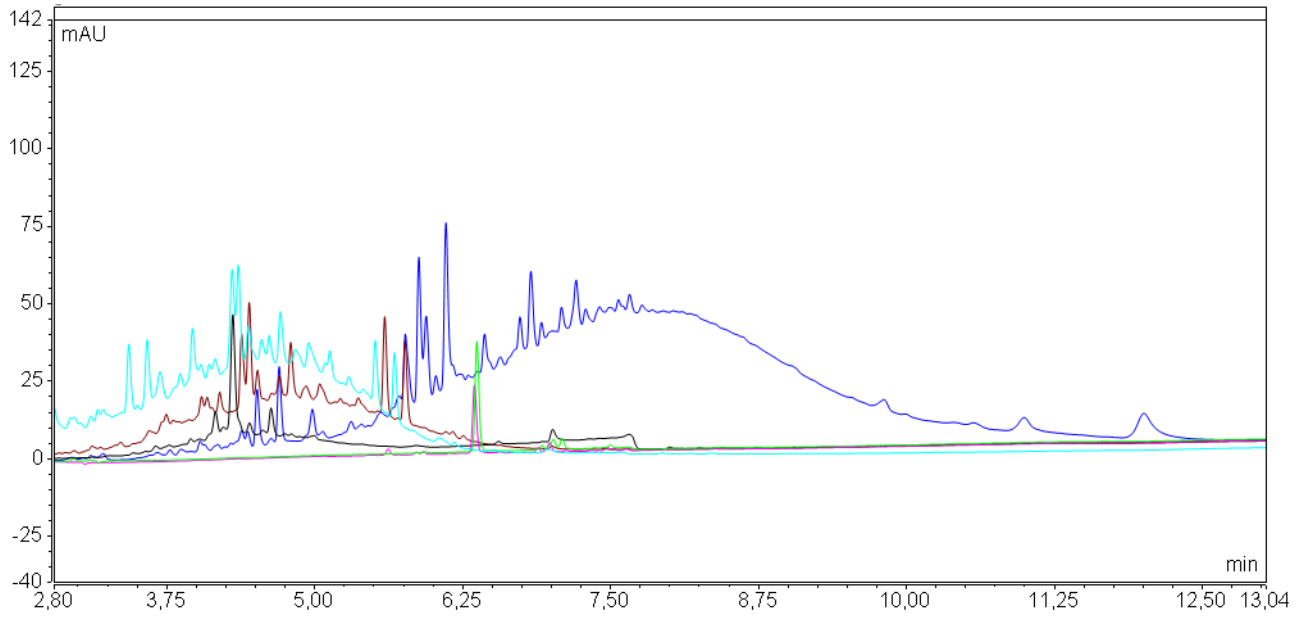


Figure 12. UHPLC UV (260 nm) analysis of liquids from pretreatments in the Parr reactor. Zoom from retention time 2.8 minutes to 13.0 minutes. Pretreatment of wheat straw, 183 °C, 18 min (brown). Pretreatment of wheat straw with ethylene glycol monobutyl ether, 18 eq. water, 4.5 % H_2SO_4 (w/w of solution), 100 °C, 30 min (blue). Pretreatment of wheat straw with water, 4.5 % H_2SO_4 (w/w of solution), 100 °C, 30 min (black). Pretreatment of xylose with ethylene glycol monobutyl ether, 18 eq. water, 4.5 % H_2SO_4 (w/w of solution), 100 °C, 30 min (pink). Ethylene glycol monobutyl ether (green). Pretreatment of wheat straw, 183 °C, 18 min with xylose spike (4.64 g) (turquoise).

To ensure that ethylene glycol monobutyl ether did not somehow induce polymerisations reactions of xylose, that could lead to the late eluting compounds beyond 6 minutes retention time, a control experiment was set up. Pure xylose (4.64 g which corresponds to the xylose content in the used amount of wheat straw in the experiments in the Parr reactor) was pretreated with ethylene glycol monobutyl ether. No late eluting compounds were formed (Figure 12 pink) - the late eluting compounds that were observed were from the ethylene glycol monobutyl ether itself (Figure 12 green).

From the above investigations, it was concluded that the late eluting compounds are solubilised lignin components. To avoid interference from the solubilised lignin components in the analysis, the retention time region for evaluation of the relative amounts oligophenolic compounds in the experiments with anomeric protection (section 3.3.5) was thus set to be from 3.0 minutes to 4.9 minutes instead of 3.0-6.0 minutes. 4.9 minutes were chosen, because it was the intercept of the ethylene glycol monobutyl ether pretreatment and its control (without ethylene glycol monobutyl ether) (Figure 12 blue and black), that do not solubilise lignin.

3.3 Results and discussion

3.3.1 Designation of potent enzyme inhibitors

The method developed for pointing out potent cellulase inhibitors consisted of a fractionation *via* organic solvent extraction of liquid from hydrothermal pretreated wheat straw (LfHP) where 2-butanone was found to be excellent in separation of compounds (Paper 2). Both the organic and the water fraction was dried completely and dissolved to the same dry matter content as the total dry matter content of the unextracted

LfHP in the enzymatic hydrolysis. In this way an inhibitor potency as per dry matter unit could be obtained (Paper 2). The most potent enzyme inhibitors were found in the 2-butane fraction from the extraction of LfHP. These compounds inhibited the enzymatic glucose release from Avicel (Figure 13) compared to unextracted LfHP, the compounds in the water fraction and buffer.

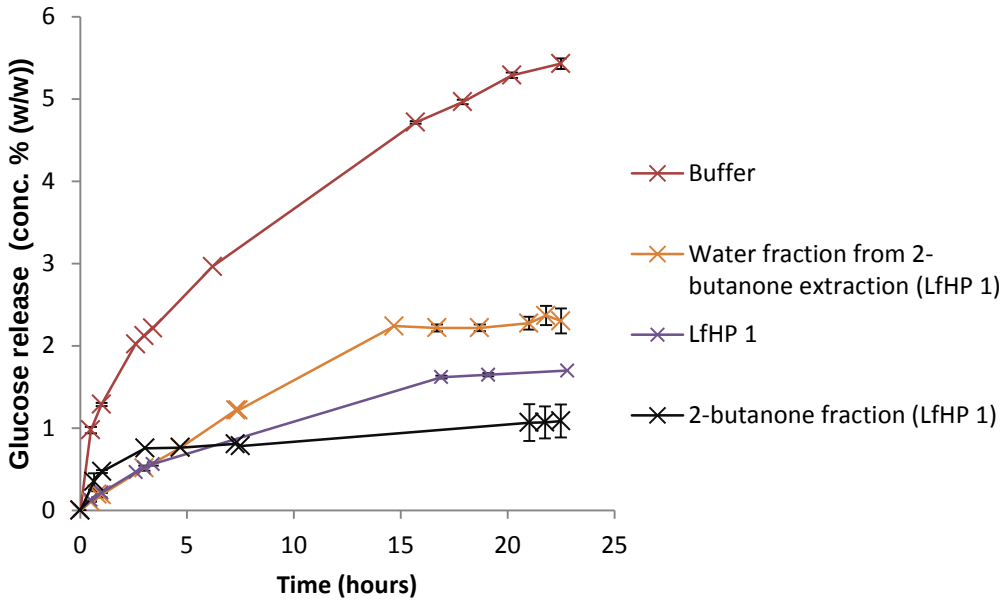


Figure 13. Glucose release during enzymatic hydrolysis of Avicel, with compounds added from solvent extractions of liquid from hydrothermal pretreatment (LfHP) of wheat straw. All fractions i.e. compounds from the organic fraction, water fraction or freeze dried LfHP were added to equal concentrations (w/w).

When the compounds in the 2-butanone fraction were tested in the enzymatic hydrolysis of authentic pilot plant pretreated wheat straw fiber they were also found to be very inhibitory towards glucose release compared to buffer and unextracted LfHP (Figure 14).

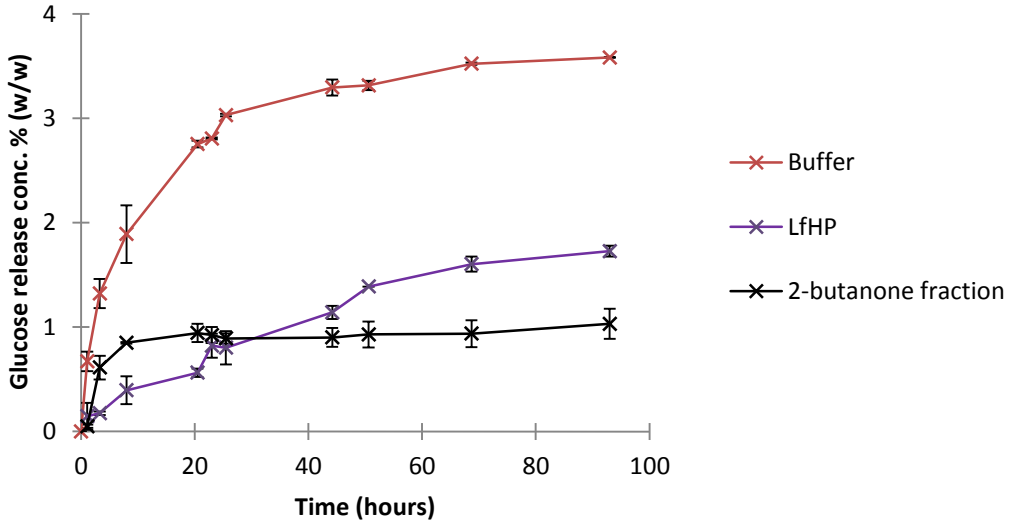


Figure 14. Glucose release during enzymatic hydrolysis of pretreated wheat straw, with addition of compounds from 2-butanone extraction of liquid from hydrothermal pretreatment (LfHP) of wheat straw. Both fractions i.e. compounds from the organic fraction and freeze dried LfHP were added to equal concentrations (w/w).

The base peak chromatograms from LC-MS revealed that a distinctive group of compounds with retention time 5.5-8 minutes had been extracted into the 2-butanone fraction (Figure 15). Because these compounds were almost completely removed from the water fraction they were most likely decisive in inhibition.

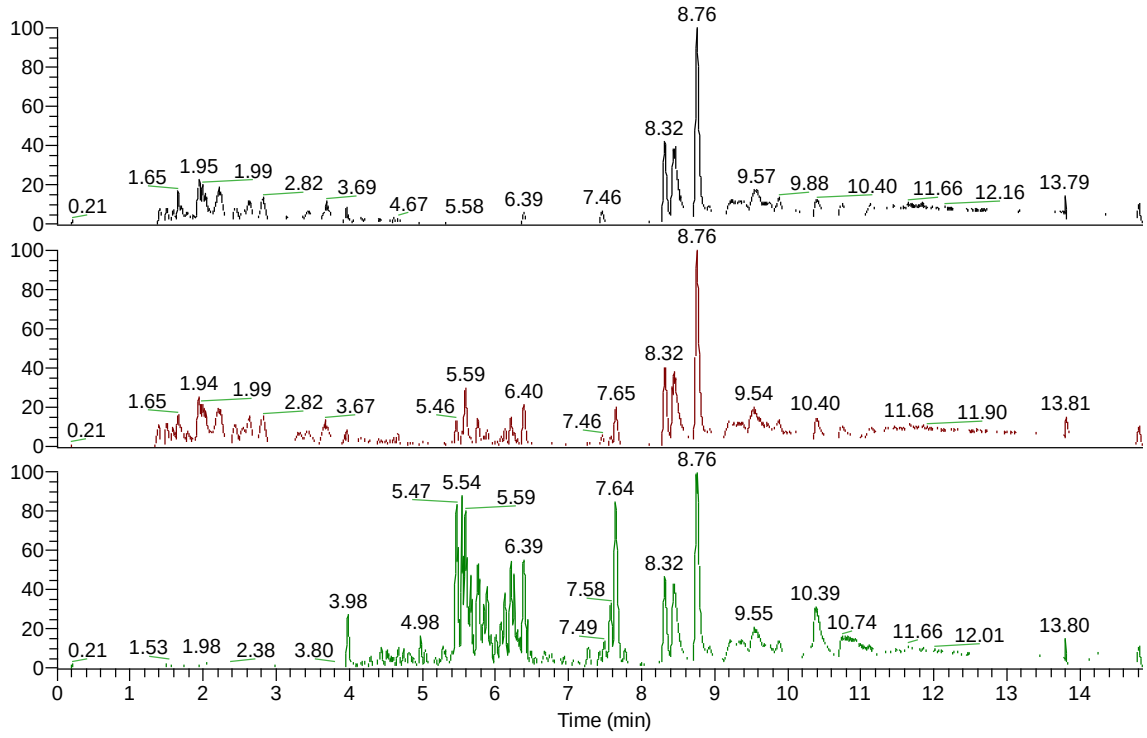


Figure 15. Base peak chromatograms (negative ionisation) of fractions from solvent extractions. Top: Water fraction from 2-butanone extraction. Middle: Non extracted LfHP. Bottom: 2-butanone fraction. Peaks beyond 8 minutes were also observed in blank control samples and thus interpreted as extractables.

3.3.2 Identification of potent enzyme inhibitors with LC-MS/MS

More than 100 different compounds were eluting in the region 5.5-8 minutes in the 2-butanone fraction (Figure 15 bottom). From earlier unsuccessful attempts to purify a compound for NMR analysis from a similar mixture, it was clear that it would not be possible to identify the compounds with NMR analysis in this case. Thus a different approach was taken by elucidating a number, as large as possible, of compounds to gain information of general structures and compound types even though the exact structures cannot be determined with LC-MS/MS. 30 compounds had sufficient intensity and quality of fragmentation.

The compounds had a high degree of double bond equivalents (DBE) compared to their masses (Paper 2, Supplementary material) i.e. they were quite condensed structures Common mass losses that gave rise to fragments were 18, 44 and 15 (Paper 2, Supplementary material), which corresponded to H_2O from elimination of hydroxyl, CO_2 from carboxylic acids and CH3• radical from cleavage of phenol methoxy groups (Sánchez-Rabaneda *et al* 2003) respectively. Lactones with hydroxyl substituents were strongly suggested by i) multiple sequential neutral mass losses involving $[M\text{-}H^+\text{-}44]^-$, $[M\text{-}H^+\text{-}18]^-$ and in some cases also $[M\text{-}H^+\text{-}28]^-$ CO loss, as well as by ii) the structures being so condensed, that they cannot contain carboxylic acids and hydroxyl groups to explain the multiple fragmentation pattern. Compounds with fused ring systems were strongly suggested by minor fragmentation, again consistent with a condensed structure. Additional fragmentation arose from straightforward heterobond cleavage and cleavage of bonds between non-fused

ring systems (Paper 2, Supplementary material). The structures (Figure 16) are thus mainly oligophenolic compounds, including lactone, flavonoid like structures and flavonoids. From a structure similarity search down to 70 % (based on molecular formula) in Chemical Abstract Service *via* Scifinder, it could be concluded that out of the 30 proposed structures, 26 are new. Three have been reported earlier (flavonoid **15** (Lan *et al* 2015, Río *et al* 2012), cumaroyl substituted pentose (**8**) (Carrasco *et al* 2012, Rusjan *et al* 2012) and feruloyl substituted pentose (**12**) (Carrasco *et al* 2012)) and one flavonoid regioisomer **9** of a previously described flavonoid structure (Xie *et al* 2014, Finger *et al* 2013, Ludwiczuk *et al* 2011) was also found. However, **9** has not been reported previously from wheat straw. Apart from the structures shown in Figure 15, 10 already known monophenolic compounds, including methoxylated phenols, ferulic and coumaric acids were also found (data not shown).

In relation to cellulase inhibition, the data strongly indicate that the higher inhibition of the 2-butanone fraction is contributed by the new oligophenolic compounds that we propose are a group of "super inhibitory" compounds in LfHP. Many of the proposed structures in Figure 15 are Michael acceptors and they are thus readily susceptible for nucleophilic attack from nucleophiles in amino acids in proteins. This may explain why they are potent enzyme inhibitors.

The super inhibitory action of oligophenols agree with earlier studies (Tejirian and Xu 2011), reporting that oligophenols, such as tannic acid and oligomeric proanthocyanidin isolated from a grape seed extract, are more potent cellulase inhibitors than monophenolic compounds.

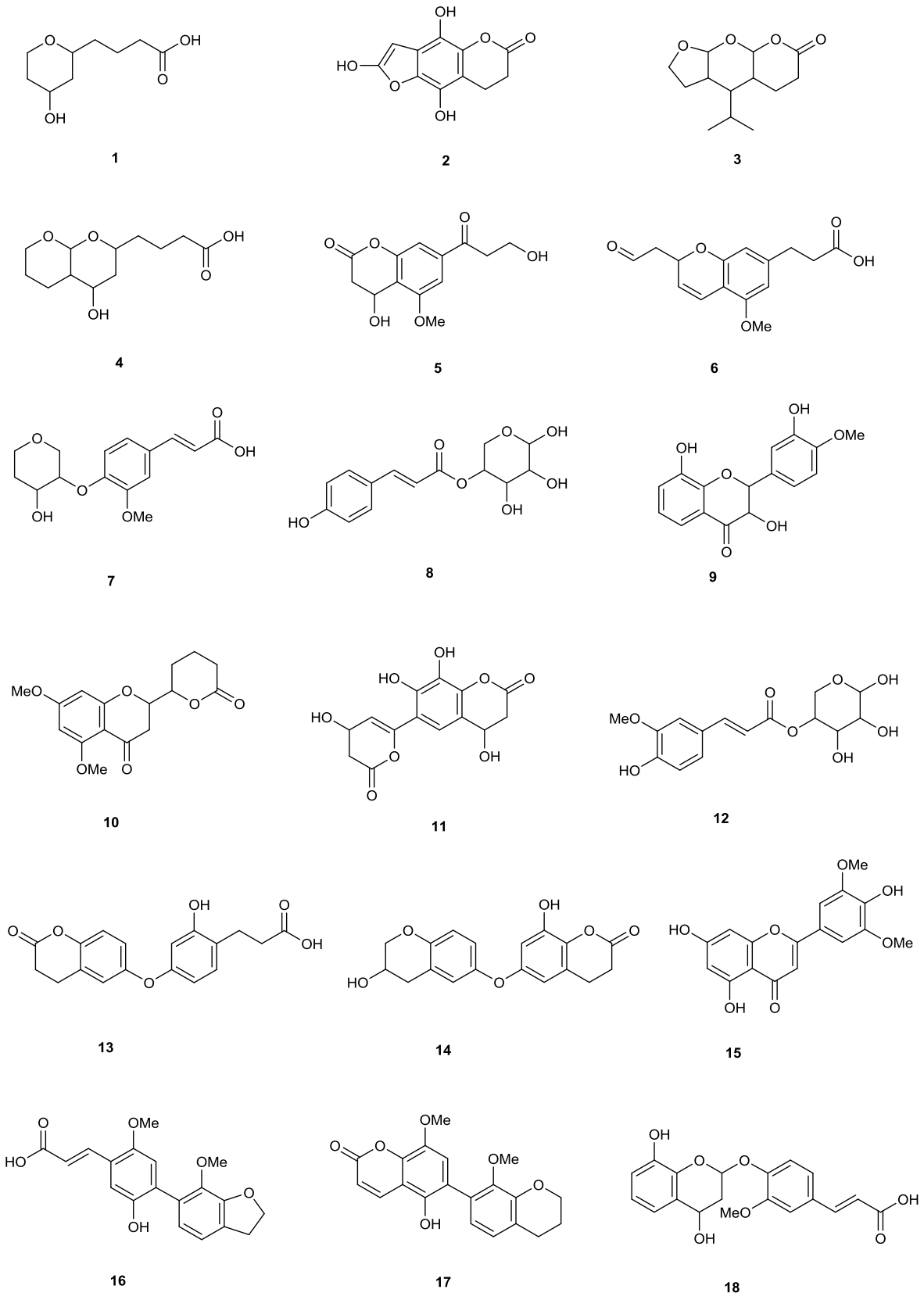


Figure 16. Proposed structures from LC-MS/MS. Note that ring substitution positions are given for clarity, but regioisomerism was not determined *(Figure continues).*

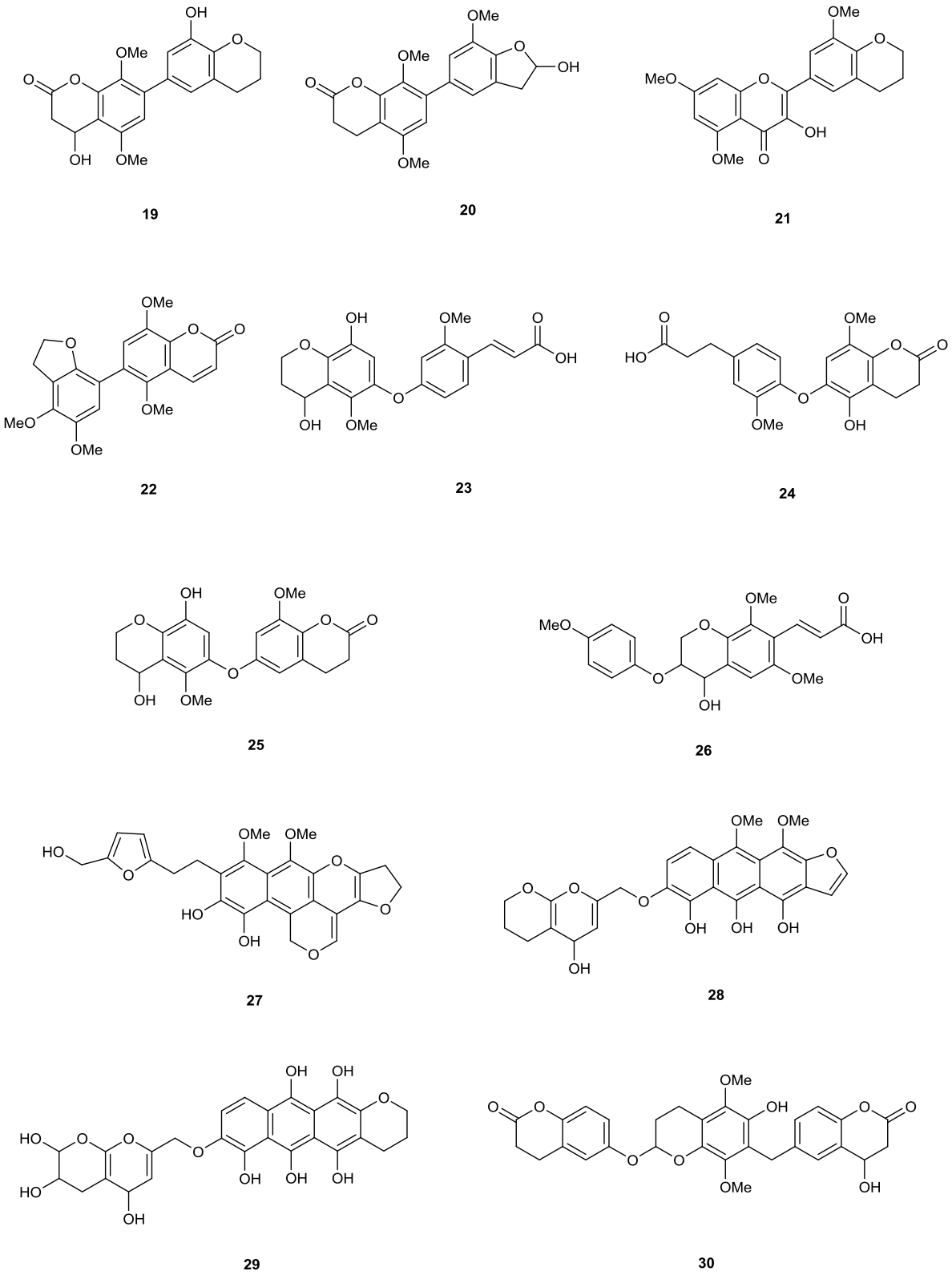


Figure 16 *(continued)*. Proposed structures from LC-MS/MS. Note that ring substitution positions are given for clarity, but regioisomerism was not determined.

3.3.3 Compounds in the water fraction and distributed across the 2-butanone and water phase fractions

The compounds eluting in the region 1-4 minutes in the water fraction (Figure 15 top) were identified as xylooligosaccharides (Paper 2), some possibly with arabinose substitution as xylose and arabinose substitution cannot be distinguished with the method applied here. A closer assessment of the compounds with retention time from 3.7-5.5 minutes, where the 2-butanone fraction and its water fraction had an overlap in the base peak chromatograms (Figure 15), indicated that the majority was feruloyl and/or acetyl substituted pentose oligomers (Paper 2).

The MS analysis also revealed the presence of one possible Maillard product as proposed in Chapter 1. One compound with accurate mass corresponding to acetyl azepinone was observed. This compound was reported previously (Wang *et al* 2011) to be a Maillard reaction product formed in acidic glucose/glycine rich solutions.

3.3.4 Origin of the identified compounds - reaction mechanisms

With the finding that the 26 new compounds were enzyme inhibitors, hypothesis 1 could be investigated:

Hypothesis 1) Liquors from biomass pretreatment contain an array of hitherto unidentified cellulase inhibitors that are believed to be reaction products from carbohydrate degradation.

To examine if the new compounds were actually reaction products that were formed during pretreatment, the reaction mechanisms were considered. The compounds arise from a wide array of reactions - some of the compounds appear to arise from pentose self-condensation reactions alone (Figure 17, Figure 18A), while other compounds are most likely reaction products from pentose reactions with lignin degradation compounds (Figure 18B and Figure 18C).

Reactions involving only pentoses

The reaction mechanism for formation of **11**, as an example (Figure 17), is proposed to be initiated with an acid catalysed aldol condensation between two pentoses resulting in compound **31**. Xylose was abundant in the LfHP (data not shown) and is therefore likely to be involved in the reaction (arabinose was present in less amounts, but may also be involved). In the suggested reaction, the aldol condensation is followed by further dehydration and cyclisation, resulting in the aldehyde substituted oxane **32**. The aldehyde is oxidised to the carboxylic acid by molecular oxygen, present during the reactions, presumably catalysed by trace metals present. The oxane hemiacetal is in equilibrium with its open chain form **33**, where the enol function is proposed to tautomerise to its keto form, resulting in the 3-keto acid **34**, that readily undergoes decarboxylation under acidic conditions. A dehydration gives a terminal carbonyl group, that is subject to nucleophilic attack from the enol, that acts as a carbon nucleophile, resulting in the ketone substituted cyclic compound **35**. The ketone is proposed to tautomerise to its enol form, resulting in the possibility of a new ring closure to the bicyclic hemiacetal **36**. Compound **36** is suggested to undergo further dehydration and protonation and subsequently undertake nucleophilic attack on a third pentose molecule. A cyclisation of the pentose moiety leads to a tricyclic compound, that further dehydrates and undergoes an electrocyclic ring

opening. Nucleophilic attack from water facilitates ring closure to compound **37**, which undergoes oxidation to the final product **11** due to atmospheric oxygen present during pretreatment. Previously reported data (Devlin and Harris 1984), show that phenols readily undergo oxidation under similar reaction conditions. Many variations of the reaction mechanism described for **11,** as an example, are possible. For example, the first cyclisation of compound **31**, will lead to a six membered ring with a different substituent pattern, if the nucleophilic attack takes place as a 1,4 addition to an α,β unsaturated carbonyl compound (Figure 18A). In this case the molecule will lack oxygen in the 2-position. Furthermore, a five membered ring i.e. an oxolane can be formed by nucleophile attack from the appropriate hydroxyl group (Figure 18A). Variations of the mechanisms in the proposed reaction schemes and reaction with other nucleophiles as for example lignin degradation compounds (Figure 18B and Figure 18C), explain the many different reaction products formed.

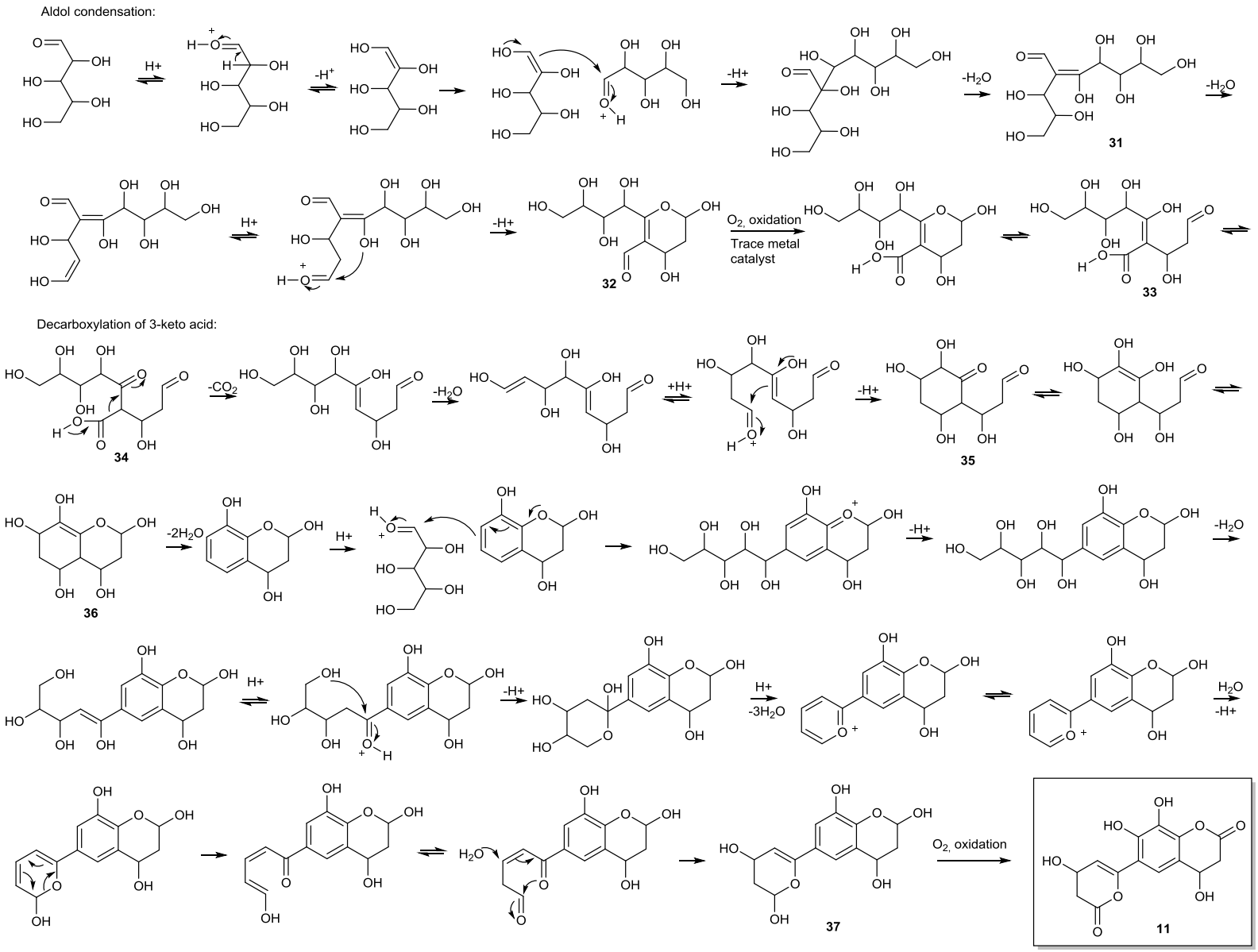


Figure 17. Proposed reaction mechanisms involving only pentose self-condensation reactions.

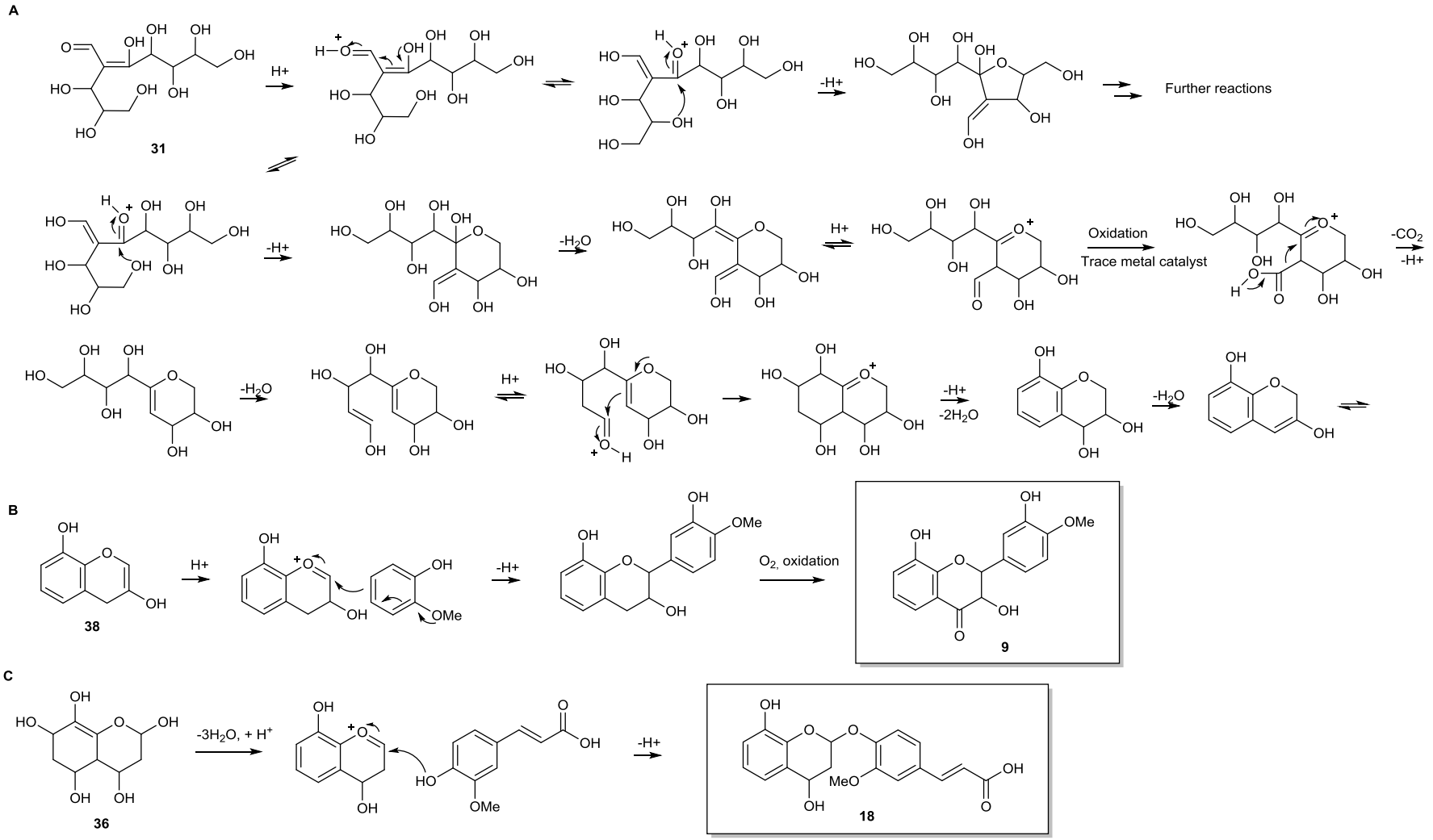


Figure 18. Examples of reactions leading to other of the discovered compounds. A) Variations in pentose self-condensation reactions. B) Reaction with methoxylated phenol resulting in methoxylated compounds and C) Reaction with ferulic acid resulting in the common (methoxyphenyl)prop-2-enoic acid motif.

Reactions involving pentoses and lignin degradation compounds

In addition to pentose self-condensation reactions, reactions involving other compounds in the reaction mixture such as methoxylated phenols, ferulic and coumaric acids arising from lignin degradation may take place (Figure 18B and Figure 18C). Methoxylated phenols explain the common methoxylations as exemplified with **9** (Figure 18B). It is worth noting that flavonoids can actually be formed as a reaction product during pretreatment.
Another common structural repeater was (methoxyphenyl)prop-2-enoic acid, which can be explained by reaction of ferulic acid with pentoses (Figure 18C) as exemplified with **18**. The ferulic acid moiety can in turn give rise to the corresponding bicyclic lactone by intramolecular esterification when a hydroxyl substituent is in *ortho* position. The lactone in the bicyclic moiety can be further dehydrated and/or react with other components in the reaction mixture.
Furthermore non methoxylated (phenyl)prop-2-enoic acid moieties are also proposed to be present and can, apart from reactions involving coumaric acid from lignin degradation, be explained by acid catalysed ring opening of lactones origination from pentose reactions alone (Figure 17).

The above findings and made it very clear that pentoses were heavily involved in formation of the identified inhibitors. This was confirmed with xylose spiking in pretreatment of wheat straw (Section 3.2.6) which increased the amount of oligophenolic compounds with 70 % with a xylose spike amount corresponding to the potential xylose from xylan in wheat straw. Hypothesis 1 was confirmed with the above findings and results.
Many of the new oligophenol compounds are possibly also precursors to pseudo lignin. This proposition is supported by the findings from Ma *et al* 2015 where pretreated holocellulose, isolated from bamboo, was found to generate pseudolignin which was more rich in alicyclic and hydroxyl substituted structures, than reference lignin. In addition, the pseudolignin went from being rich in aliphatic structures, to being rich in aromatic structures, with increasing pretreatment time. Furthermore Kumar *et al* 2013 have shown that pseudo lignin is formed from carbohydrates and that especially xylan and xylose are prone to undergo degradation, even at low severities, and precipitate as spheres of pseudo lignin on cellulose surfaces.
The degradation of pentoses is a double negative, because pentoses are now also becoming desired products in biorefineries. The pentoses are thus both lost, and at the same time unwanted products as inhibitors and/or pseudolignin are formed.

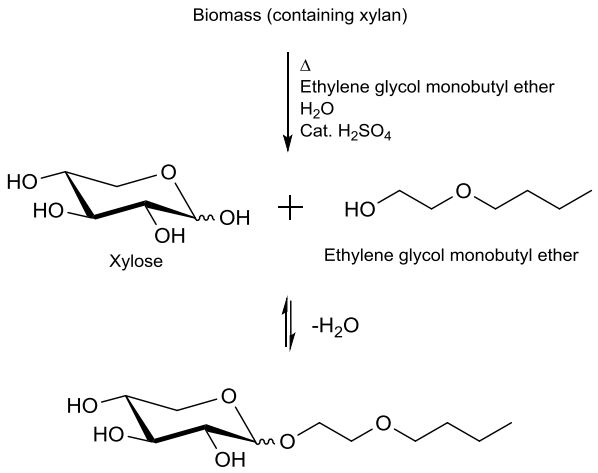
3.3.5 Protection of pentoses at the anomeric position

The revelation of the role of xylose in formation of oligophenolic enzyme inhibitors enabled the test of hypothesis 2:
Hypothesis 2). Formation of these inhibitor compounds can be prevented by protection of reactive chemical functionalities as revealed from their mechanisms for formation.

It was speculated that protection of the reactive anomeric center in xylose (Figure 19) could reduce the level of pentose degradation, even with no protection of the other hydroxyl groups. Anomeric protection could be possible in an industrial process of biomass pretreatment with a Fischer-type glycosylation with glycol ethers as protection groups. Glycol ethers have the desired high boiling points for biomass pretreatment and are water miscible, which is also important, because some water has to be present in order to hydrolyse the pentose oligosaccharides in the biomass to monomers, but water may also obstruct the protection reaction. An acid catalyst must be present in order for the protection reaction to take place, but the presence of acid would only additionally facilitate structural breakage of the biomass.

Ethylene glycol monobutyl ether (Figure 19) was chosen for further investigation, because it has an inverse solubility with water and in principle should be separable from water at 50 °C. The separation was needed for two reasons: 1) The presence of ethylene glycol monobutyl ether in the amounts used for the protection reaction in the current experiments inhibited the enzymatic hydrolysis (data not shown). 2) The recycling of ethylene glycol monobutyl ether is desirable for an environmental friendly process as well as for economic reasons.

A statistical experimental design was conducted in order to identify the optimal parameters for anomeric protection with ethylene glycol monobutyl ether (Paper 2). At optimal conditions (Figure 19) protection of the anomeric position in xylose reduced the level of degradation compounds by 73 % compared to the original pretreatment (Figure 19). The control experiment to the ethylene glycol monobutyl ether pretreatment, with no glycol ether present, only reduced the level of degradation compounds by 54 % (Figure 19). Thus hypothesis 2 was found to be relevant, because protection of the reactive anomeric position hindered formation of oligophenolic compounds to a large extent.



Pretreatment	Degradation compound/inhibitor reduction compared to original pretreatment (%)
Original: 183 °C, 18 minutes, 100 % water	0
Ethylene glycol monobutyl ether: 100 °C, 30 minutes, 18 equivalents of water (to xylose), 4.5 % H_2SO_4 (w/w of solution)	73
Control: 100 °C, 30 minutes, 95.5 % water, 4.5 % H_2SO_4 (w/w of solution)	54

Figure 19. Protection of the anomeric center in *in situ* generated xylose from biomass with ethylene glycol monobutyl ether (top) and reduction in the level of degradation compounds/inhibitors during a pretreatment of wheat straw (bottom).

Two further advantages of the pretreatment including anomeric protection was that: 1) xylooligosaccharides in the pretreatment liquor were hydrolysed completely (LC-MS data not shown) due to the severe conditions with sulphuric acid. 2) Xylose can be deprotected after the pretreatment i.e. the xylose can be recovered, simply by lowering the temperature after pretreatment and adding water to shift the equilibrium toward the reactants (Figure 19, top).

In relation to deprotection, the strategy was to do a combined deprotection of xylose with addition of water (1:1, water:ethylene glycol monobutyl ether) and separation of ethylene glycol monobutyl ether and subsequently test the combined water phase and pretreated fiber in the enzymatic hydrolysis for a full comparison with the current process (Figure 4). Initial 6 mL small scale tests showed promising results with both deprotection (~45 % from HPLC) and separation of the water phase and ethylene glycol monobutyl ether after 3 hours at 80 °C. However, when the experiments were run in 100 mL scale in order to obtain enough material for test in the enzymatic hydrolysis, separation of the water phase and ethylene glycol monobutyl ether was less efficient. It can be speculated that optimization of e.g. mechanical solutions such as continuous centrifugation or diafiltration or other approaches could improve the separation. However, this was not attempted in the course of this PhD.

Even though an experimental evaluation of the downstream effect on enzymatic hydrolysis could not be carried out, other measures could be undertaken to evaluate cellulose digestibility for the enzymes:

The current pilot plant pretreatment (183 °C, 18 minutes, 100 % water) is optimised between the trade-off of minimum inhibitor formation, minimum pentose degradation and maximum biomass digestibility for the enzymes. The pretreatment with anomeric protection does not contain this trade-off, because pretreatment severity and the resulting cellulose digestibility can be increased without the negative effect of pentose degradation to oligophenolic inhibitor compounds due to the protected pentose. Residual xylan content in pretreated wheat straw has previously been demonstrated to be a measure of cellulose digestibility with xylan content and cellulose digestibility conversely correlated (Kabel *et al* 2007). In the present work residual xylan was measured to 11 % (w/w) and 7 % (w/w) in the pretreated wheat straw from the original pretreatment and the pretreatment with anomeric protection respectively (section 3.2.6). These results illustrated, that the cellulose digestibility was increased in the pretreatment with anomeric protection, and at the same time the level of oligophenolic enzyme inhibitors were reduced with 73 % compared with the original pretreatment with lower cellulose digestibility.

Pretreatment parameters with anomeric protection, can likely be optimised even further towards biomass digestibility for the enzymes.

3.4 Chapter 3 conclusive remarks

This chapter confirmed hypothesis 1 and 2:

Hypothesis 1) Liquors from biomass pretreatment contain an array of hitherto unidentified cellulase inhibitors that are believed to be reaction products from carbohydrate degradation.

Hypothesis 2). Formation of these inhibitor compounds can be prevented by protection of reactive chemical functionalities as revealed from their mechanisms for formation.

The most potent enzyme inhibitors were pointed out and among them were 26 new compounds, that were found to be oligophenolic compounds. The compounds were formed during hydrothermal pretreatment from reactions with heavily involvement of xylose in aldol condensations, 1,4 additions to α,β unsaturated carbonyl compounds, 3-keto acid decarboxylations and oxidations. By protection of the reactive anomeric position in xylose with the glycol ether ethylene glycol monobutyl ether, during a hydrothermal pretreatment of wheat straw, the level of oligophenolic inhibitors were reduced with 73 % compared to the original pretreatment and 41 % compared to the control. Contemporary with the reduction in the level of inhibitors, the cellulose digestibility for the enzymes was increased.

These results illustrated that pretreatment with anomeric protection opens up new possibilities for pretreatment of lignocellulose biomasses towards high cellulose digestibility for the enzymes without the negative effect of monosaccharide degradation and formation of enzyme inhibitors.

4 Influence from process parameters on degradation compound profile

4.1 Introduction

The work presented in the last chapter revealed the presence of potent oligophenolic cellulase inhibitors in liquid from hydrothermal pretreated wheat straw (LfHP). When studying liquors from hydrothermal pretreatment processes another factor to consider is the actual amounts of oligophenolic and other compounds that are present in the liquors. Both the inhibitor potency, but also the actual amount of a compound determines its contribution to overall inhibition from the pretreatment liquors and this may in turn change when process parameters during pretreatment are changed. To shed light on these relations an "inhibition mass balance" method was applied to account for inhibition contributions from different compounds in LfHP and in turn account for all inhibition exerted by LfHP.

The LC-MS/MS analyses had, apart from the presence of oligophenolic compounds in the organic fraction, also clarified that xylooligosaccharides were crucial components in the water fraction. This chapter describes the work which aimed to investigate the consequences of increased pretreatment severity. In particular, i) if the amount of oligophenols and xylooligosaccharides would change. ii) if other degradation compounds are formed. These tasks are related to hypothesis 3.

4.2 Methodical considerations

4.2.1 Enzyme assay

Analogous to LfHP 1 (183 °C, 18 minutes) (section 3.2.2) LfHP 2 (191 °C, 18 minutes) also contains glucooligosaccharides that during enzymatic hydrolysis were hydrolysed glucose (Table 6).

Table 6. Glucose release from LfHP 2 and its fractions from the extractions. WF: Water fraction.

Fraction	Initial glucose content (% w/w)	Glucose content from mono and oligosaccharides (% w/w)
LfHP 2	0.2 ± 0.00	0.7 ± 0.02
WF from 2 butanone extraction	0.2 ± 0.01	0.8 ± 0.02
2-butanone fraction	< 0.05	< 0.05

4.2.2 Evaluation of volatile compounds

Volatile compounds in LfHP 2 were evaluated analogous to volatile compounds in LfHP 1 (section 3.2.3). No difference in inhibition before and after freeze drying of LfHP 2 was observed (Figure 20) even though the concentration of furfural and acetic and formic acid was higher in LfHP 2 than LfHP 1. Thus furfural and acetic and formic acid do not contribute to the inhibition from LfHP in the measured concentrations 1.1 g/L for furfural, 6.1 g/L for acetic acid and 3.4 g/L for formic acid.

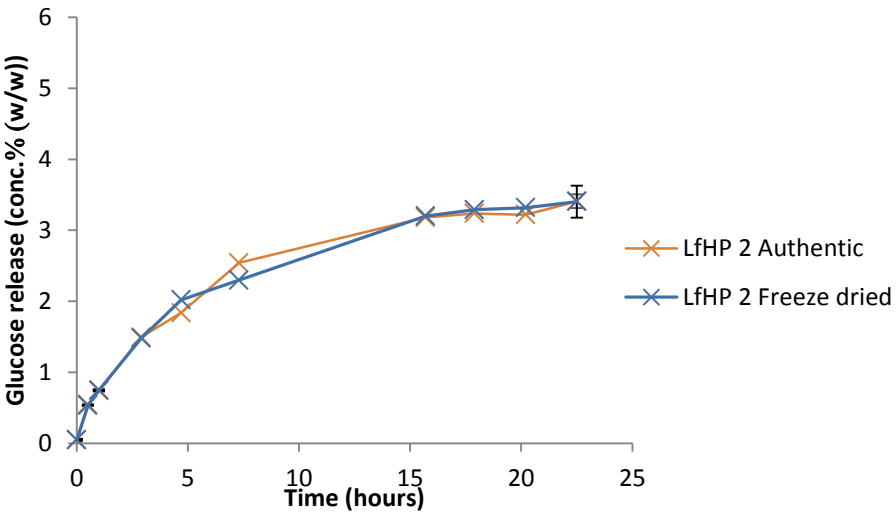


Figure 20. LfHP 2 authentic (9.8 % (w/w) dry matter) before and after freeze drying.

4.2.3 Chromatographic separation LC-MS

Due to abrasion of the column there was shift towards earlier retention time in the LC-MS and LC-MS/MS runs described in this chapter and section 5.3.1 (which is from the same LC-MS run) compared to the retention time in the analyses described in the rest of the thesis. It had no negative consequences because the samples that were planned to be compared were all analysed in the same run.

4.3 Results and Discussion

4.3.1 Contributions to overall cellulase inhibition from LfHP - inhibition mass balances

The inhibition mass balance method was an extension of the method developed to point out potent inhibitors in liquors from hydrothermal pretreatment described in Chapter 3. Based on the inhibitor potency per unit dry matter from the fractions and the actual amount of dry matter extracted into the fractions, the inhibition contribution from the fractions to overall inhibition from unextracted LfHP could be determined.

At present pilot plant pretreatment conditions optimised towards minimum inhibitor formation and minimum xylose degradation, the potent oligophenolic compounds in the 2-butanone fraction contributed less to total inhibition than the xylooligosaccharides in the water fraction, because the amount of dry matter extracted into to organic phase was much less than the amount in the water phase (Paper 3) (Figure 21, LfHP 1).

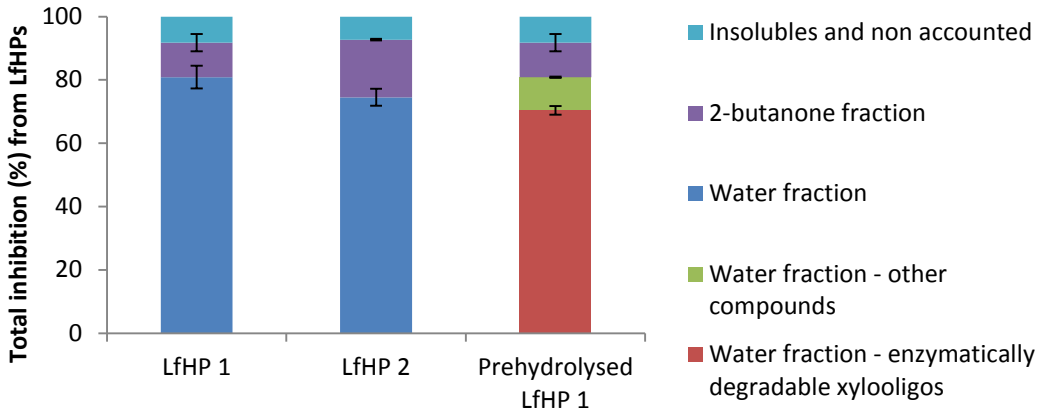
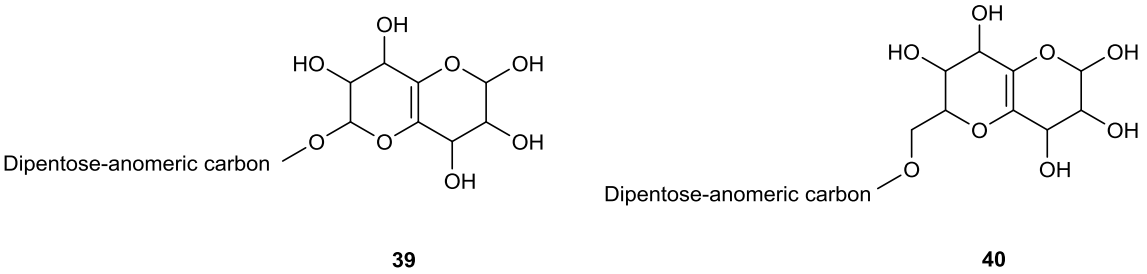


Figure 21. Inhibition of cellulases exerted by the compounds in LfHP 1 (pretreated 183 °C, 12 minutes), LfHP 2 (pretreated 191 °C, 12 minutes) and LfHP 1 with enzymatic removal of xylooligosaccharides (Prehydrolysed LfHP 1).

When pretreatment temperature was increased from 183 °C (LfHP 1) to 191 °C (LfHP 2) at 18 minutes pretreatment the overall inhibition from LfHP 2 increased slightly from 69 to 73 % (Paper 3). The amount by weight of compounds extracted into the 2-butanone fraction increased by 14 % and the amount decreased correspondingly in the water fraction, whereas the content of xylooligosaccharides in the water fraction decreased with 41 % (Paper 3). The consequence was an increased contribution to inhibition from the 2-butanone fraction containing the oligophenolic compounds - however, the water fraction containing xylooligosaccharides also contributed most to overall inhibition from LfHP 2 (Figure 21, LfHP 2).
The inhibition mass balance approach was applied to consider compounds contributing to overall inhibition. It was established that volatile small aliphatic acids remaining in the water fraction did not contribute to inhibition at the present concentrations (section 3.2.3 and 4.2.2), but there could possibly be other components left in the water fraction, apart from xylooligosaccharide, that contributed to inhibition from the water fraction. In order to investigate this, the xylooligosaccharides in the water fraction from LfHP1, that contained the most xylooligosaccharides, were enzymatically removed with Cellic CTec3 prior to test in the cellulase avicel hydrolysis assay. The major contributor to inhibition from the water fraction was actually due to enzymatically degradable xylooligosaccharides (Figure 21, prehydrolysed LfHP 1), but a small fraction (10 % out of 80 %) was due to other components in the water fraction. These other components could very likely be the residual compounds after the Cellic CTec3 prehydrolysis, that were identified to be feruloylated tripentoses and to new dipentose compounds with bicyclic moieties **39** and **40** (Figure 22) (Paper 3).



39 **40**

Figure 22. Proposed structures for two new residual compounds after enzymatic hydrolysis. Note that substitution position is given for clarity, but regioisomerism was not determined.

The two bicyclic residues **42** and **43** (Figure 23) are proposed to be formed from the isomerisation products xylulose and fructose, from xylose and glucose respectively (Ershova *et al* 2015, Kimura *et al* 2011), and their reaction with glyceraldehyde **41** (Figure 23). This links back to the reaction mechanisms for formation of xylulose and fructose that were depicted in Chapter 2 and is a new example of reactions between degradation products in the reaction mixture. The bicyclic residues are most likely been linked to the pentose oligomer at the anomeric position because it is able to form an oxocarbenium ion that is readily available for nucleophilic attack from the bicyclic compounds.

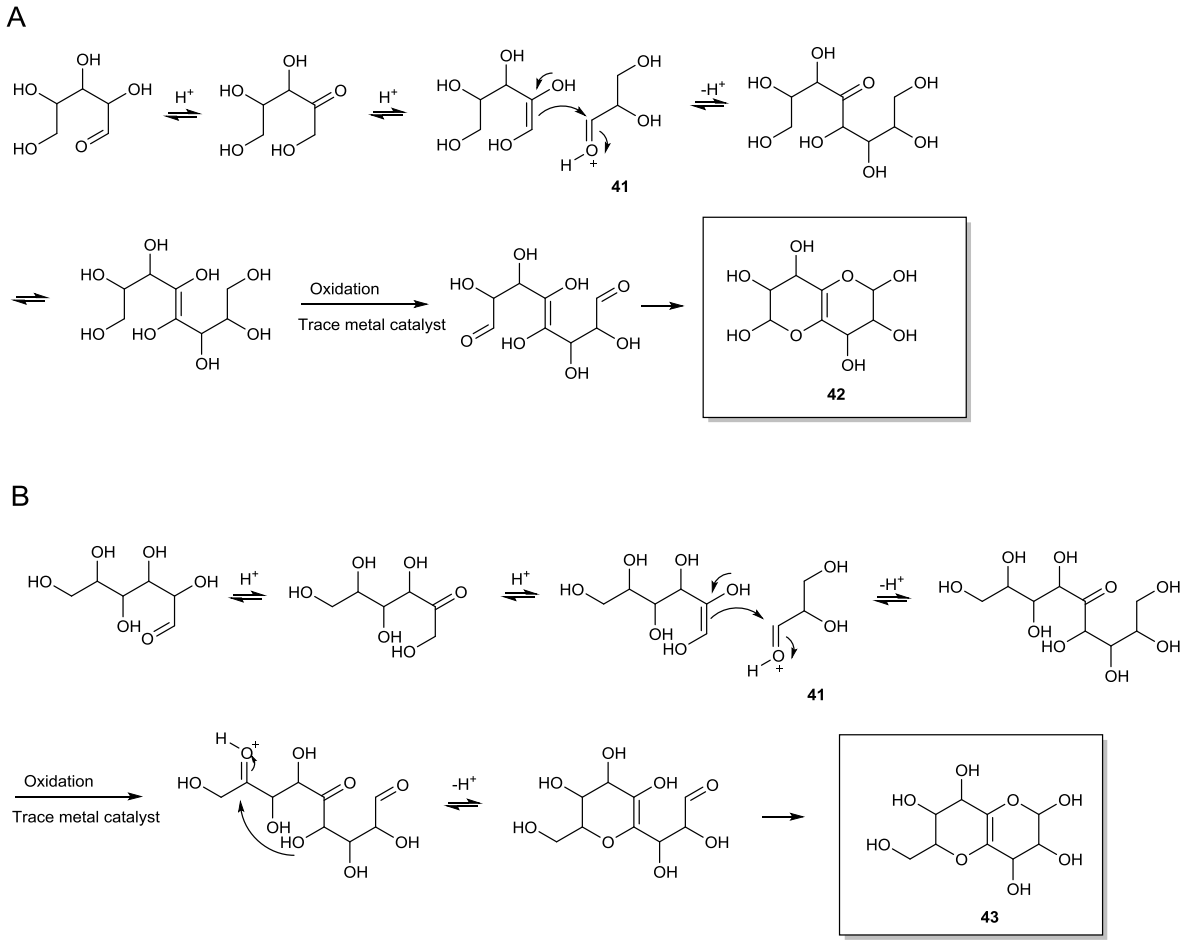


Figure 23. Proposed mechanisms for formation of the bicyclic residues in A) dipentose 39 and B) dipentose 40 (Figure 22).

4.3.2 Change in compound distribution with increased pretreatment severity

As established in the last section, the amount by mass of xylooligosaccharides decreased with increased pretreatment severity whereas the amount extracted into the 2-butanone phase containing the oligophenolic compounds increased. This is in line with that more xylooligosaccharides were degraded to xylose and in turn resulted in oligophenolic compounds *via* the mechanisms depicted in Chapter 3. However, the 2-butanone fraction from LfHP 2 was profoundly more inhibiting than the corresponding LfHP 1 2-butanone fraction when compared at the same dry matter basis (Figure 24). These data suggested that the more severe pretreatment had induced the formation of either new more potent inhibitory compounds or changed

the profile of oligophenolic compounds extracted into the 2-butanone fraction. In contrast the inhibitory effect exerted by the water fractions from the 2-butanone extraction were similar for the two pretreatments (Figure 24).

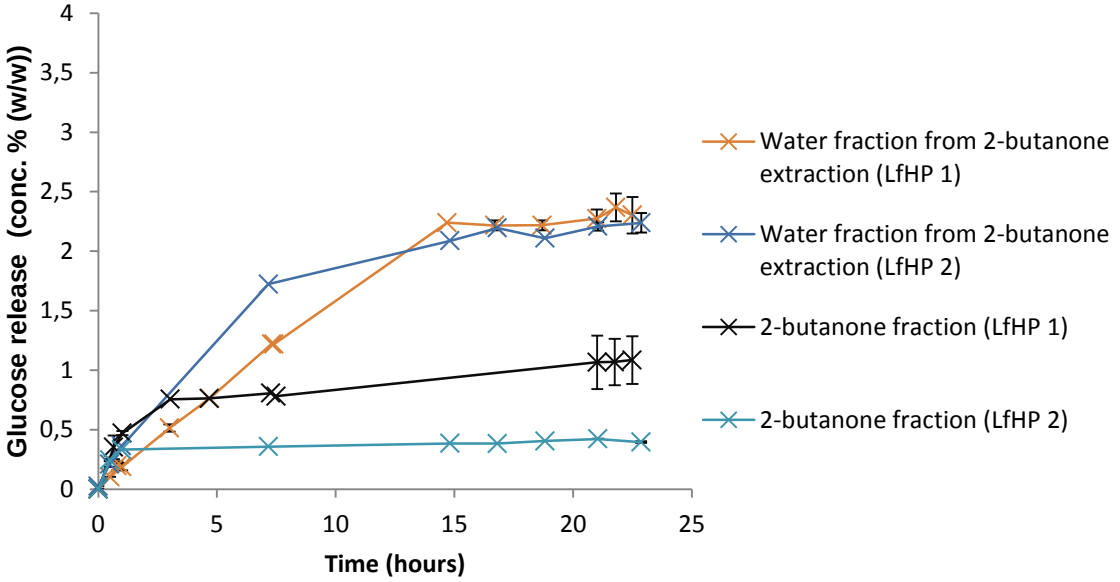


Figure 24. Enzymatic cellulose (avicel) hydrolysis assay with LfHP from pretreatment in 18 minutes at 183 ºC (LfHP 1) and 191 ºC (LfHP 2) and corresponding fractions from extractions.

In order to identify the differences introduced with more severe pretreatment the LC-MS (Figure 25) and LC-MS/MS spectra were analysed in detail (Paper 3).

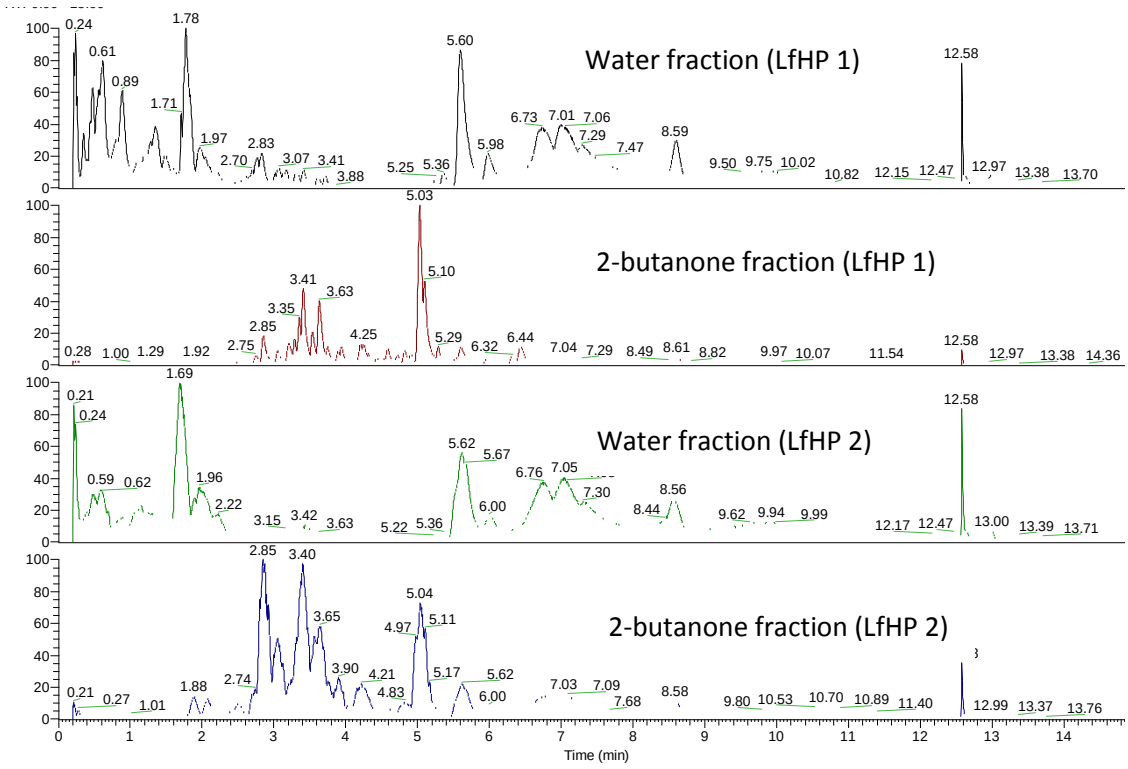
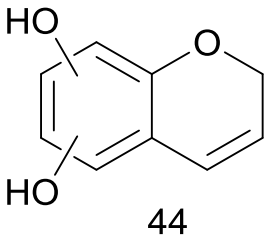


Figure 25. Base peak chromatograms (negative ionisation) of water fractions and 2-butanone fractions from LfHP 1 and LfHP 2.

The two pretreatments had a different distribution of the compounds abundance in the water fractions and 2-butanone fractions from LfHP 1 and LfHP 2 (Figure 25), but the compounds were found to be similar in LfHP 1 and LfHP 2 i.e. no formation of new structures was observed:

In the water fractions the amount of xylooligosaccharides without ester substitution (rt 0.21-1.69, Figure 25) were less abundant with more severe pretreatment. Isomers of tetrapentoses and pentapentoses with 1 acetyl substituent (rt 1.78 Figure 24 and rt 1.69 Figure 25) were present at similar abundance in the water fractions from LfHP and LfHP 2. In the 2-butanone fraction of LfHP 2, the shift in distribution as compared to LfHP 1 was towards earlier retention times and especially the compound at rt 2.85 had an increased relative abundance compared to its abundance in the 2-butanone fraction from LfHP 1 (Figure 25, pane 2 and 4). This ion, rt 2.85, *m/z* 163.03938 ($[M- H^+]^-$) was subjected to fragmentation with Higher-energy Collisional Dissociation (HCD) energies 25 % and 50 %, but did not result in any fragmentation, suggesting a condensed compound with no functionalities readily available for fragmentation. The accurate mass corresponds to the molecular formula $C_9H_8O_3$ with 6 double bond equivalents. On this basis compound **44** (Figure 26) was suggested. It is likely a xylose derived degradation compound with a synthesis route slightly modified from that leading to compound **36** (Chapter 3) and subsequently involving dehydrations. Compound **44** and other compounds eluting at the earlier retention time in the 2-butanone fraction from LfHP 2 may be responsible for the increased inhibition towards cellulases of this fraction (both in relation to the corresponding 2-butanone fraction of LfHP 1 and the water fraction of LfHP 2) (Figure 24).



44

Figure 26. Proposed structure of the compound (*m/z* 163.03938, $[M-H^+]^-$), that is present at elevated relative amounts, when the pretreatment temperature of wheat straw is increased from 183 ºC to 191 ºC.

Anomeric protection would potentially hinder the formation of compound **44** as well as the bicyclic moieties in compound **39** and **40**, because isomerisation cannot take place.

4.4 Chapter 4 conclusive remarks

The results from this chapter answer hypothesis 3:

Hypothesis 3) Process parameters influence the amount and type of reaction products (from hypothesis 1) that are formed and in turn change inhibition.

Process parameters as increased pretreatment severity do influence the amount of the different reaction products that are formed, but no new reaction products are formed. When pretreatment severity increases the amount of xylooligos decrease whereas the amount of oligophenolic compounds increase. At the same time the overall inhibition from liquid from hydrothermal pretreatment towards cellulytic enzymes increases. These results emphasise that pretreatment is a trade-off between degradation of inhibitory

xylooligosaccharides and xylose to even more potent oligophenolic inhibitors. This trade-off can be circumvented by applying a pretreatment with anomeric protection as described in Chapter 3, where xylooligosaccharides are hydrolysed to xylose, but xylose degradation is averted.

5 Influence from biomass on degradation products and released compounds during pretreatment

5.1 Introduction

In the previous chapters the investigations have been conducted on pilot plant liquid from hydrothermal pretreated wheat straw. In this chapter pilot plant liquid from hydrothermal pretreated sugar cane bagasse (SCB) and oil palm empty fruit bunches (EFB) are also considered to a certain extent to consider variations from biomass with respect to degradation compounds and xylooligosaccharides and lignin structural elements liberated during pretreatment.

In the pilot plant SCB and wheat straw are pretreated at similar conditions (184 ºC, 18 minutes versus 183 ºC, 18 minutes respectively) and can thus be compared, whereas EFB is pretreated at more severe conditions (200 ºC, 19 minutes) due to its recalcitrant nature. Consequently, EFB cannot be taken directly into the comparison study, but is included here as an example of how compounds liberated from lignin contribute to variations from biomass.

5.2 Methodical considerations

5.2.1 Chromatographic separation LC-MS

As mentioned in section 4.2.3 the retention times of the LC-MS analyses in section 5.3.1, section 5.3.2 as well as section 4.3.2 were shifted toward earlier retentions times compared to the retention time in the analyses described in the rest of the thesis, due to abrasion of the column. In order to investigate if the most abundant compound in LfHP from EFB (section 5.3.2) (rt 5.37, *m/z* 163.03935 (corresponds to $C_9H_8O_3$), Figure 27, A1 and B1) was the same as compound **44** (Chapter 4, section 4.3.2) it was compared to the sample of the 2-butanone fraction from LfHP 1, that was run in both the run with earlier retention times (section 4.3.2 and Figure 27, A3 and B3) and a run with normal retention time (Figure 27, A2 and B2). In the run with earlier retention time compound **44** eluted at 2.85 minutes (section 4.3.2 and Figure 27 A3 and B3) and when the same sample was analysed in a run with normal retention time compound **44** was located with retention time 5.44 minutes (Figure 27, A2 and B2) (it did also not undergo fragmentation in MS/MS). Because the molecular formula of the most abundant compound from EFB was $C_9H_8O_3$, which is the same as compound **44** and the retention time was similar to compound **44** in a different run with normal retention time, the most abundant compound from EFB was concluded to be compound **44**.

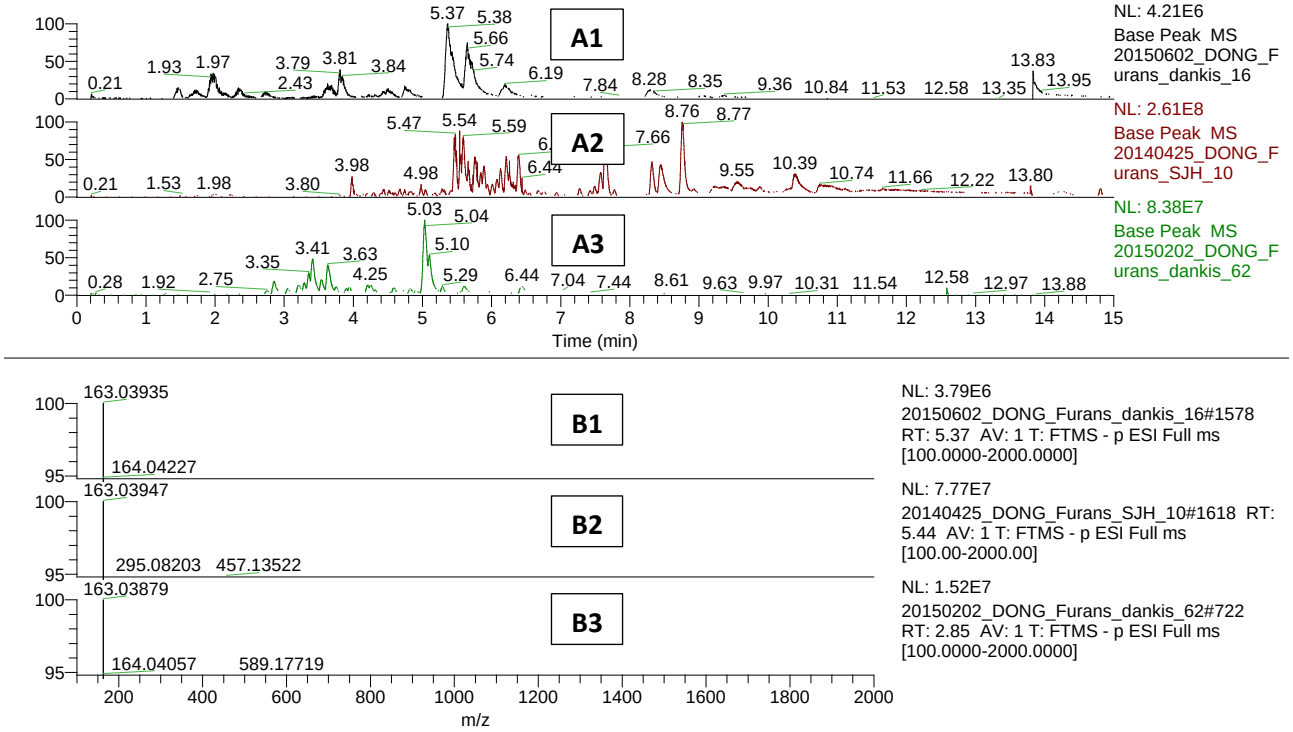


Figure 27. A panes: Base peak chromatograms (BPC), negative ionisation. LfHP from EFB (A1). 2-butanone fraction from extraction of LfHP 1 from wheat straw (A2). 2-butanone fraction from extraction of LfHP 1 from wheat straw at HPLC column shifted toward earlier retention time (A3). B panes: Mass spectra of the corresponding BPCs. B1: Mass spectrum of A1 peak with rt 5.37 minutes. B2: Mass spectrum of A2 peak with rt 5.44 minutes. B3: Mass spectrum of A3 peak with rt 2.85 minutes.

5.3 Results and discussion

5.3.1 Wheat straw versus sugar cane bagasse

Liquid from hydrothermal pretreatment (LfHP) of sugar cane bagasse was analysed with LC-MS/MS for profile comparison with wheat straw (Figure 28, Table 7). The pentoseoligosaccharides in LfHP of SCB has a more acetylated pentoseoligosaccharide profile than wheat straw (Table 7). The most abundant peak in the base peak chromatograms of LfHP from both SCB and wheat straw is the peak with rt 1.78. It consists of pentoses with a degree of polymerisation (DP) of 3-8 with one acetyl substitution. For wheat straw pentoses DP 9-10 with one acetyl substituent is also a constituent of the same peak. The reason why the latter is absent from SCB is that pentoses with DP 9-10 are simply not present with only one acetyl substituent. They have at least two acetyl groups, and up to 6 acetyl substituents. In a similar manner pentoses with DP 2-6 and no acetyl substitution are much more abundant in wheat straw than in SCB (Table 7).

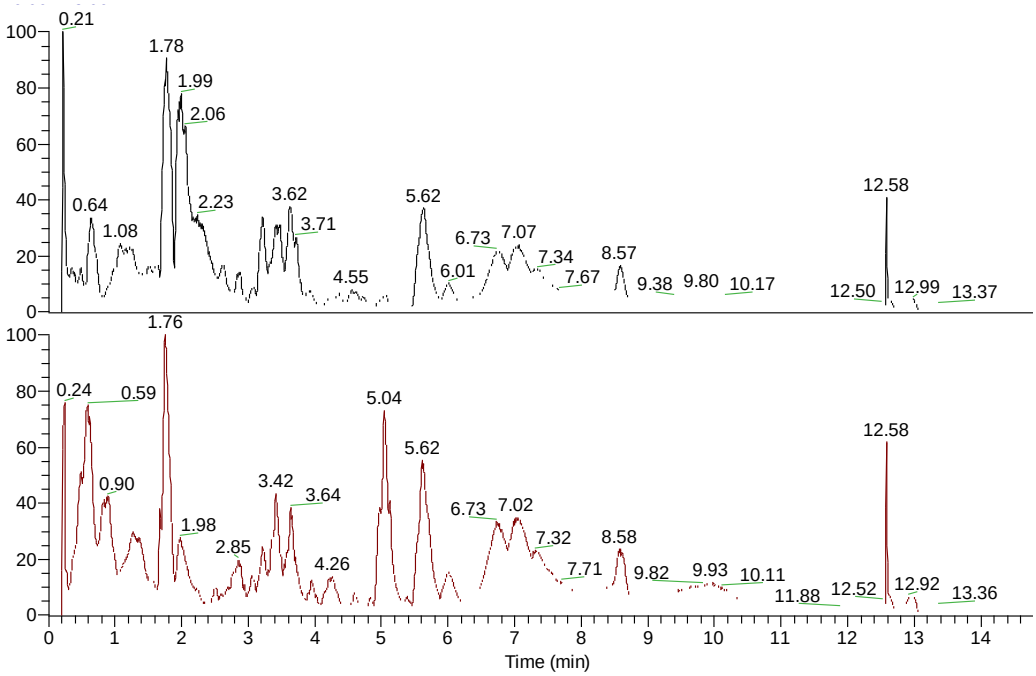


Figure 28. Base peak chromatograms, negative ionisation. LfHP from sugar cane bagasse (top) and wheat straw (bottom). Compounds beyond 5.4 minutes are extractables.

The 30 new oligophenolic compounds from wheat straw (Chapter 3), that have their earliest eluting representatives from retention time 2.85 minutes in the present MS run (Figure 25 pane 2 and Figure 28 bottom), were compared in detail with SCB, regarding retention time (Figure 28) and accurate masses of base peaks constituents. The same compounds were found and with comparable intensities (data not shown) except for the compound with retention time 5.04 (Figure 28) minutes that was more abundant in wheat straw. It is Tricin (compound **15**, Chapter 3) and thus a flavonoid, that is abundant in wheat straw, which is in line with earlier reports (Lan *et al* 2015, Río *et al* 2012). For the other compounds it could be concluded, that the investigated oligophenolic compounds generated from pretreatment of wheat straw and SCB were in general very similar. This is in accordance with, that xylose is heavily involved in the formation of the oligophenolic compounds and consequently many of the same compounds may be formed in biomasses containing xylose.

Table 7. Compounds in LfHP of sugar cane bagasse and wheat straw.

Retention time (min)	Ions, *m/z* [M- H^+]$^-$ (relative intensity)	Biomass	Compounds
0.49	281.08652 (23), 413.12857 (27), 545.17053 (100), 677.21271 (45)	Sugar cane bagasse	Pentoses DP2, DP 3, DP 4, DP 5.
0.49	281.08643 (18), 413.12851 (24), 545.17053 (100), 677.21259 (72)	Wheat straw	Pentoses DP2, DP 3, DP 4, DP 5.
(0.59-)0.64	191.01823 (100), 413.12866 (5), 545.17017 (13), 677.21240 (28), 809.25458 (14)	Sugar cane bagasse	$C_6H_8O_7$ (possibly oxidised glucuronic acid); pentoses DP2, DP 3, DP 4, DP 5, DP6.
0.59	191.01816 (8), 281.08636 (5), 413.12842 (11), 545.17004 (40), 677.21301 (100), 809.25494 (59)	Wheat straw	$C_6H_8O_7$ (possibly oxidised glucuronic acid); pentoses DP2, DP 3, DP 4, DP 5, DP6.
0.90	545.16992 (10), 677.21228 (32), 809.25494 (100), 941.29669 (66)	Wheat straw	Pentoses DP 3, DP 4, DP 5, DP 6, DP 7.
(1.76-)1.78	455.13931 (8), 587.18109 (86), 719.22327 (100), 851.26520 (49), 983.30707 (20), 1115.34937 (7)	Sugar cane bagasse	Pentoses + 1 acetyl, DP 3, DP 4, DP 5, DP 6, DP 7, DP 8.
1.76	455.13931 (8), 587.18066 (61), 719.22296 (100), 851.26520 (78), 983.30713 (47), 1115.34912 (26), 1247.39099 (12), 1379.43164 (6)	Wheat straw	Pentoses + 1 acetyl, DP 3, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9, DP 10.
(1.98-)1.99	629.19202 (99), 761.23413 (100), 893.27637 (58), 1025.31848 (29), 1157.35986 (12), 1289.40186 (4), 1421.44360 (1)	Sugar cane bagasse	Pentoses + 2 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9, DP 10.
1.98	629.19135 (50), 761.23340 (90), 893.27570 (100), 1025.31787 (90), 1157.35950 (66), 1289.40186 (43), 1421.44141 (22), 1553.48328 (10), 1685.52649 (3)	Wheat straw	Pentoses + 2 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9, DP 10, DP 11, DP 12.
2.23	629.19153 (90), 761.23346 (44), 803.24451 (82), 935.28693 (100), 1067.32874 (64), 1199.37048 (35), 1331.41248 (12), 1463.45276 (4)	Sugar cane bagasse	Pentoses + 2 acetyl, DP 4, DP 5. Pentoses + 3 acetyl, DP 5, DP 6, DP 7, DP 8, DP 9, DP 10.

Retention time (min)	Ions, *m/z* [M- H^+]$^-$ (relative intensity)	Biomass	Compounds
2.61	671.20233 (100), 803.24445 (50), 935.28644 (18). 845.25500 (38), 977.29706 (62), 1109.33923 (47), 1241.38110 (22), 1415.43250 (20), 1547.47.400 (10), 1679.51758 (3)	Sugar cane bagasse	Pentoses + 3 acetyl, DP 4, DP 5, DP 6. Pentoses + 4 acetyl, DP 5, DP 6, DP 7, DP 8. Pentoses + 5 acetyl, DP 9, DP 10, DP 11.
2.85	163.03867 (100), 325.09149 (20), 589.17566 (24), 721.21802 (34), 845.25525 (38), 977.29706 (30), 1151.34985 (26), 1283.39160 (21), 1457.44299 (10), 1589.48523 (8), 1721.52771 (4), 1895.57202 (1)	Sugar cane bagasse	Compound **44** Feruloyl substituted pentoses, DP 1, DP 3, DP 4. Pentoses + 4 acetyl DP 5, DP 6. Pentoses + 5 acetyl DP 7. Pentoses + 6 acetyl, DP 9, DP 10, DP 11. Pentoses + 7 acetyl, DP 12.
2.85	163.03860 (100), 325.09131 (24), 589.17554 (34), 721.21808 (87), 853. 26007 (35), 1027.31262 (12), 1159.35449 (13), 1291.39587 (8), 1423.43799 (4)	Wheat straw	Compound **44** Feruloyl substituted pentoses, DP 1, DP 3, DP 4, DP 5. Feruloyl + 1 acetyl substituted pentoses, DP 6, DP 7, DP 8, DP 9.
3.05	295.08096 (100), 427.12372 (29), 559.16571 (24), 325.09134 (24), 721.21832 (41), 763.22864 (22), 895.27051 (36), 1159.35535 (17), 865.26031 (26), 1019.30731 (24), 1325.40234 (15), 1457.44312 (8), 1631.49426 (4)	Sugar cane bagasse	Cumaroyl substituted pentose DP 1, DP 2, DP 3. Feruloyl substituted pentose DP 1, DP 4. Feruloyl + 1 acetyl substituted pentose DP 4, DP 5, DP 7. Cumaroyl + 1 acetyl substituted pentose DP 5. Pentoses + 5 acetyl DP 6. Pentoses + 6 acetyl DP 8, DP 9. Pentoses + 7 acetyl DP 10.
3.05	295.08087 (29) , 559.16516 (30), 325.09091 (16), 721.21771 (66), 853.25946 (43), 763.22858 (12), 895.27039 (30), 1027.31189 (12), 1159.35588 (14), 1291.39551 (12), 1423.43909 (5)	Wheat straw	Cumaroyl substituted pentose DP 1, DP 3. Feruloyl substituted pentose DP 1, DP 4, DP 5. Feruloyl + 1 acetyl substituted pentose DP 4, DP 5, DP 6, DP 7, DP 8, DP 9.
3.21	325.09171 (100), 457.13400 (25), 589.17566 (14), 733.21808 (14), 865.26001 (14), 763.22852 (15), 895.27087 (13), 1027.31238 (15), 1201.36475 (6), 1333.40649 (5)	Sugar cane bagasse	Feruloyl substituted pentose DP 1, DP 2, DP 3. Cumaroyl + 1 acetyl substituted pentose DP 3, DP 4. Feruloyl + 1 acetyl substituted pentose DP 4, DP 5, DP 6. Feruloyl + 2 acetyl substituted pentose DP 7, DP 8.

Retention time (min)	Ions, *m/z* $[M- H^+]^-$ (relative intensity)	Biomass	Compounds
3.21	325.09143 (100), 457.13428 (45), 589.17542 (54), 721.21906 (8), 733.21796 (6), 865.26044 (9), 997.30121 (7), 763.22827 (21), 895.27051 (18), 1027.31213 (24), 1159.35376 (10), 1333.40637 (4)	Wheat straw	Feruloyl substituted pentose DP 1, DP 2, DP 3, DP 4. Cumaroyl + 1 acetyl substituted pentose DP 4, DP 5, DP 6. Feruloyl + 1 acetyl substituted pentose DP 4, DP 5, DP 6, DP 7. Feruloyl + 2 acetyl substituted pentose DP 8.
3.42	601.17578 (22), 733.21832 (24), 631.18628 (79), 763.22852 (100), 895.27075 (22), 937.27997 (26), 1069.32239 (22), 1201.36499 (6), 1375.41736 (4)	Sugar cane bagasse	Cumaroyl + 1 acetyl substituted pentose DP 3, DP 4. Feruloyl + 1 acetyl substituted pentose DP 3, DP 4, DP 5. Feruloyl + 2 acetyl substituted pentose DP 5, DP 6, DP 7. Feruloyl + 3 acetyl substituted pentose DP 8.
3.42	601.17584 (6), 733.21686 (5), 631.18628 (9), 763.22821 (20), 895.27008 (9), 937.27856 (2), 1069.32251 (2), 1201.36462 (2)	Wheat straw	Cumaroyl + 1 acetyl substituted pentose DP 3, DP 4. Feruloyl + 1 acetyl substituted pentose DP 3, DP 4, DP 5. Feruloyl + 2 acetyl substituted pentose DP 5, DP 6, DP 7.
(3.62-)3.64	469.14453 (18), 601.17578 (10), 499.14453 (24), 631.18622 (100), 763.22852 (8), 805.23883 (20), 937.28101 (25), 1069.32227 (5), 1243.37646 (4)	Sugar cane bagasse	Cumaroyl + 1 acetyl substituted pentose DP 2, DP 3. Feruloyl + 1 acetyl substituted pentose DP 2, DP 3, DP 4. Feruloyl + 2 acetyl substituted pentose DP 4, DP 5, DP 6. Feruloyl + 3 acetyl substituted pentose DP 7.
3.64	601.17609 (5), 631.18646 (15), 763.22675 (3), 937.28082 (3), 1069.32251 (1)	Wheat straw	Cumaroyl + 1 acetyl substituted pentose DP 3. Feruloyl + 1 acetyl substituted pentose DP 3, DP 4. Feruloyl + 2 acetyl substituted pentose DP 5, DP 6.
3.71	367.10205 (52), 499.14432 (100), 631.18634 (48), 673.19702 (17), 805.23907 (38), 937.28119 (20), 979.29156 (5), 1111.33374 (7), 1243.37500 (2)	Sugar cane bagasse	Feruloyl + 1 acetyl substituted pentose DP 1, DP 2, DP 3. Feruloyl + 2 acetyl substituted pentose DP 3, DP 4, DP 5. Feruloyl + 3 acetyl substituted pentose DP5, DP 6, DP 7.
3.71	601.17908 (7), 631.18652 (14), 805.23749 (6), 937.28052 (5),	Wheat straw	Cumaroyl + 1 acetyl substituted pentose DP 3. Feruloyl + 1 acetyl substituted pentose DP 3. Feruloyl + 2 acetyl substituted pentose DP 4, DP 5. Feruloyl + 3 acetyl substituted pentose DP5, DP 6, DP 7.

5.3.2 Oil palm empty fruit bunches

Native structural substitution patterns as acetyl substitution of the hemicellulose part of SCB give variations to the compounds that are liberated during pretreatment of different biomasses as outlined above. During the hydrothermal pretreatment of EFB a variation was observed with the consistent appearance of an unknown compound that was formed as a distinct product (Paper 4). It was characteristic for EFB i.e. it was not observed after hydrothermal pretreatment of wheat straw or corn stover at similar severity. The compound was identified to be 4-hydroxybenzoic acid (4-HBA) (Paper 4) and a study was undertaken to examine if 4-HBA arose from a native structural element in the biomass or if it was a new reaction product.
Lu *et al* 2015 have recently demonstrated that 4-HBA is a structural lignin component characteristic for EFB and that it is readily accessible for cleavage due to its position on lignin unit side chains. The release of 4-HBA during pretreatment (Figure 29) in is in line with the findings of Lu and coworkers.

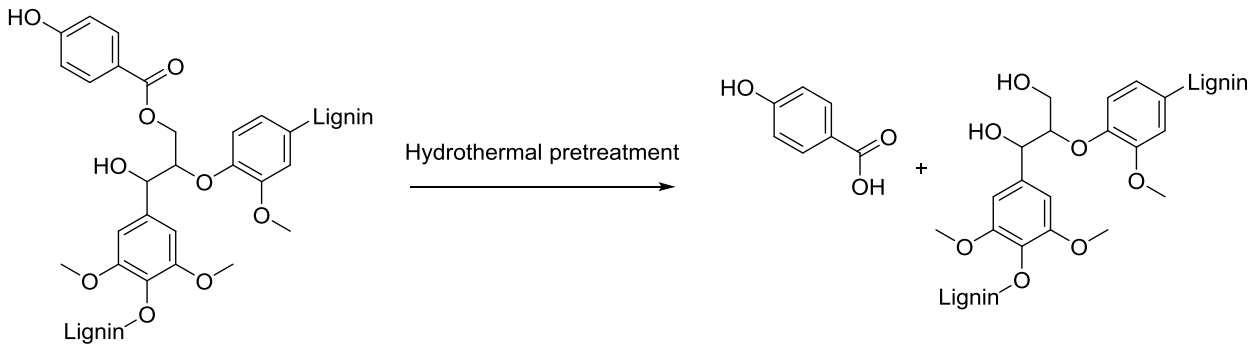


Figure 29. Release of 4-hydroxybenzoic acid during hydrothermal pretreatment.

It was hypothesised, that 4-HBA could be produced from an additional route arising from biomass degradation compounds reacting with each other. Slightly higher rhamnose levels have been reported in EFB compared to wheat straw (Holopainen-Mantila *et al* 2013, Sun *et al* 1999) and a route involving rhamnose degradation to 5-methylfuran-2-carbaldehyde by a degradation route analogues to HMF formation from glucose (Rasmussen *et al* 2014), followed by a carboxylation with formic acid can be suggested (Figure 30). In the proposed mechanism 5-methylfuran-2-carbaldehyde is cleaved to give an 1,4 dione as other furans and the aldehyde function facilitates intramolecular cyclisation to a six membered ring, which undergo dehydration to an activated dihydroxylated benzene. The carboxylation may take place as a Koch-Haaf type reaction with in situ formation of CO. The carboxylation may be speculated to take place, due to the high temperature and pressure during the pretreatment, even though no mineral acid is present.

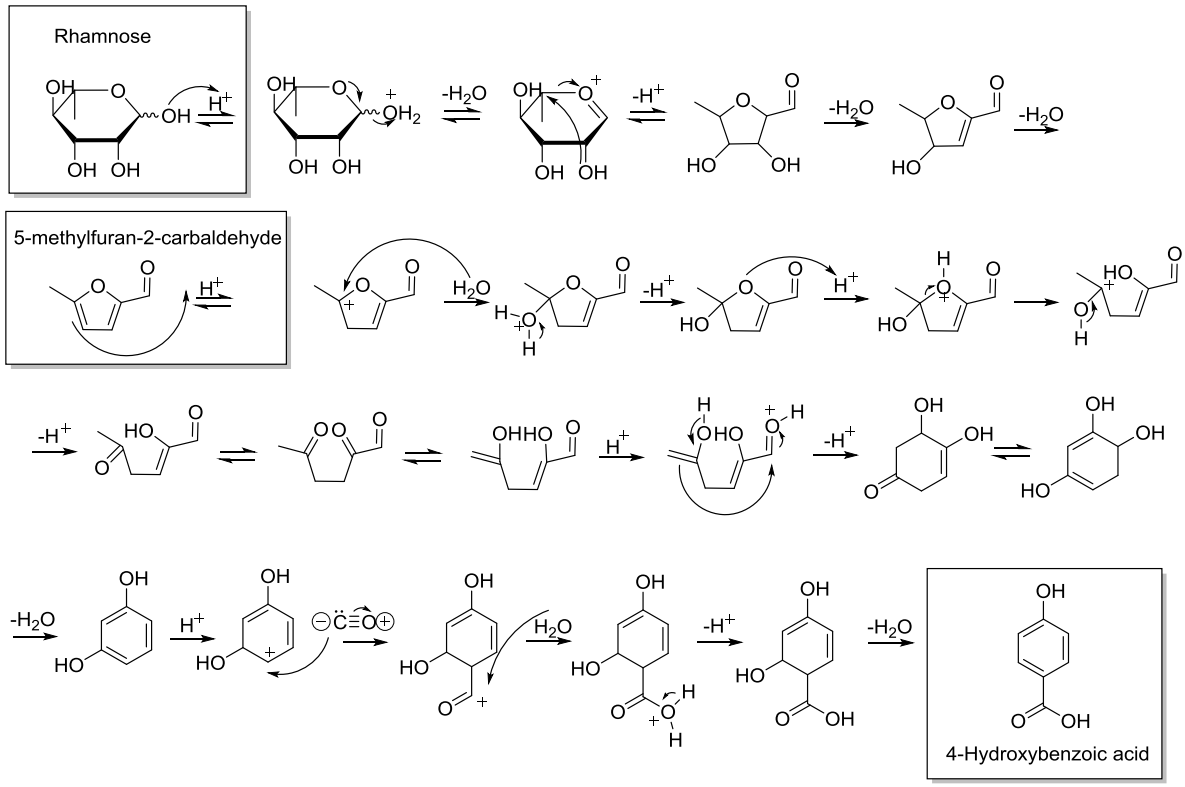


Figure 30. Hypothesised mechanism for formation of 4-hydroxybenzoic acid from rhamnose and formic acid.

When the hypothesis was tested, by reaction of rhamnose and formic acid at pretreatment conditions, it was found that 5-methylfuran-2-carbaldehyde was actually formed as a reaction product (Paper 4). No 4-HBA was formed, thus suggesting that 5-methylfuran-2-carbaldehyde does not react further with formic acid to form 4-HBA, which may be due to the lack of a mineral acid catalyst *vide supra*. From these results it could be concluded that 4-HBA acid is present after hydrothermal pretreatment of EFB, because 4-HBA is a structural element in EFB lignin from where the ester linkage is hydrolysed during hydrothermal pretreatment.

Even though the liquid from hydrothermal pretreatment of EFB was not taken into the comparison study with wheat straw, due to the more severe pretreatment of EFB, it was unavoidable to notice that the most abundant compound in the LC-MS chromatogram from EFB was compound **44** from Chapter 4 (data shown in section 5.2.1). It is in line with that increased pretreatment severity (183 °C versus 191 °C) of wheat straw lead to an increased amount of compound **44** and thus the severe pretreatment (200 °C) of EFB also resulted in compound **44**. In despite of the liberation of 4-HBA from EFB lignin this xylose derived degradation compound is important for the profile of compounds in liquid from hydrothermal pretreatment of EFB.

As a closing remark on EFB it should be mentioned that the inhibitory effect of 4-hydroxybenzoic acid was assessed in the enzymatic hydrolysis of pretreated oil palm empty fruit bunches as well as in the fermentation. It was found that 4-hydroxybenzoic acid is not inhibitory neither in the enzymatic hydrolysis or fermentation in the quantified range from 0.1 g/L to 1 g/L (Paper 4), which is in line with earlier reported results (Dumitrache *et al* 2016, Zha *et al* 2014, Ximenes *et al* 2011).

5.4 Chapter 5 conclusive remarks

From the work presented in this chapter it was concluded that the biomass type influences the type of compounds that are liberated during hydrothermal pretreatment, because different biomasses have different native structural elements or substitutions. Both lignin structural elements, as 4-HBA that is characteristic to EFB lignin and hemicellulose substitution, as acetyl substitution of pentoses that is characteristic for SCB hemicellulose, influence the profile of compounds that are liberated as native compounds.
In contrary the degradation compound profile is more decided by the presence of xylose and its reactions to oligophenolic compounds as depicted in chapter 3. Hence the degradation product profile for wheat straw and SCB was similar at similar pretreatment conditions and wheat straw and EFB pretreated at more severe condition had their most abundant compound in LC-MS in common.

6 Overall conclusions and future perspectives

6.1 Overall conclusions

The thesis answered the 3 outlined hypotheses:

Hypothesis 1) Liquors from biomass pretreatment contain an array of hitherto unidentified cellulase* inhibitors that are believed to be reaction products from carbohydrate degradation.

(*cellulases include endo-cellulases, cellobiohydrolases, LPMO, and beta-glucosidase enzyme activities)

Hypothesis 2). Formation of these inhibitor compounds can be prevented by protection of reactive chemical functionalities as revealed from their mechanisms for formation.

Hypothesis 3) Process parameters influence the amount and type of reaction products (from hypothesis 1) that are formed and in turn change inhibition.

The reactions taking place during pretreatment of biomass are complex. The structural elements of the biomass as monosaccharides and lignin components are released during pretreatment and undergo degradation reactions where the same starting material can result in different reaction products (Figure 31D). The structural elements can furthermore react with each other as exemplified with the reactions of xylose, that undergo self-condensation reactions (Figure 31A), but also reactions with lignin structural elements (Figure 31B). Variations in the reaction mechanism for these reactions lead to a plethora of compounds whereof 26 new oligophenolic compounds were identified. The compounds were potent cellulase inhibitors - they reduced glucose release from cellulose with up 90 %, depending on pretreatment severity, compared to the control.

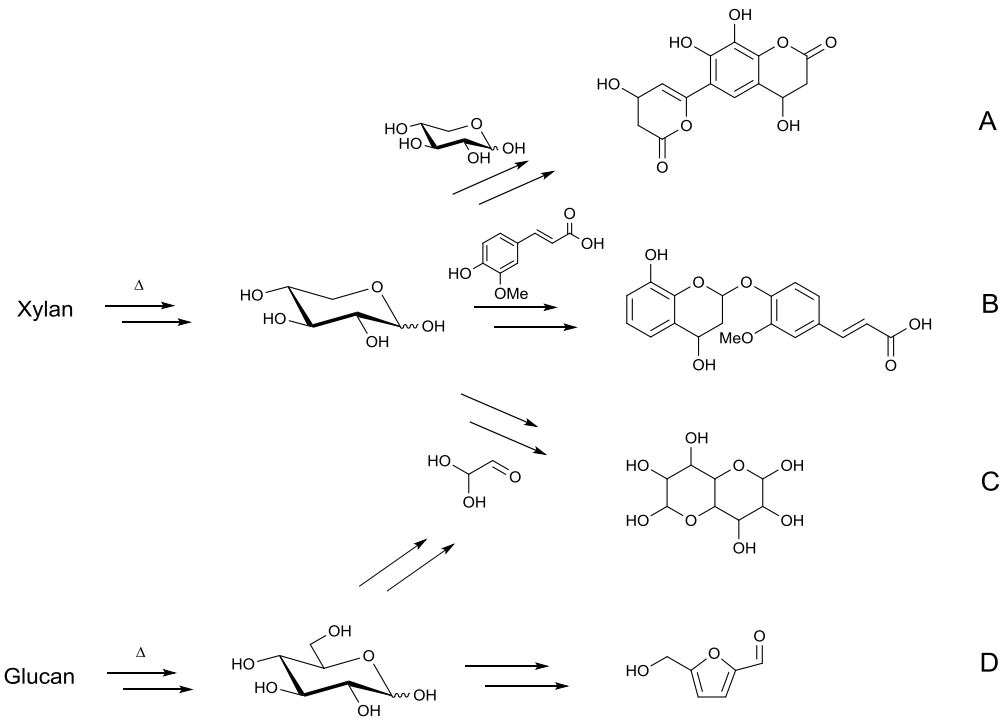


Figure 31. Reactions of biomass structural elements (stoichiometry not shown). A: Xylose self-condensation reactions. B: Reactions of xylose and lignin degradation compounds (here ferulic acid). C: Reaction of xylose and hexose degradation compounds (here glyceraldehyde). D: The same starting material (here glucose) can lead to different degradation compounds (here glyceraldehyde and HMF).

The new oligophenolic compounds thus represented a double negative in relation to biomass pretreatment: Xylose is lost because it is transformed into undesired potent cellulase inhibitors. The formation of oligophenolic reaction products was diminished by protection of the reactive anomeric position in xylose with ethylene glycol monobutyl ether. The pretreatment of wheat straw with anomeric protection resulted in a reduction of the oligophenolic compounds with 73 % compared to the original pretreatment and 41 % compared to the control.

Apart from reactions solely between biomass structural elements, reaction between biomass structural elements and degradation compounds also occurred (Figure 31C). The isomerisation products xylulose and fructose were found to undergo reaction with glyceraldehyde to form two new bicyclic moieties, that furthermore reacted with oligopentoses. Oligopentoses with bicyclic moieties were not enzymatically degraded further than dipentose level.

Biomass structural elements, that were liberated during pretreatment was found to vary with type of biomass, because different biomasses have different native structural elements or substitutions. Both lignin structural elements, as 4-HBA that is characteristic to EFB lignin and hemicellulose substitution, as acetyl substitution of pentoses that is characteristic for SCB hemicellulose, influence the profile of compounds that are liberated as native compounds. In contrary the degradation compound profile from different biomasses were decided by the presence of xylose and its reactions to oligophenolic compounds: The degradation product profile for wheat straw and SCB was similar at similar pretreatment conditions and wheat straw and EFB pretreated at more severe condition had their most abundant degradation compound in common.

Process parameters as increased pretreatment severity influenced the amount of the different reaction products that were formed, but no new reaction products were formed. When the pretreatment severity was increased the amount of xylooligos decreased whereas the amount of oligophenolic compounds increased. At the same time the overall inhibition from liquid from hydrothermal pretreatment towards cellulytic enzymes increased. These results emphasised that pretreatment is a trade-off between degradation of inhibitory xylooligosaccharides and xylose to even more potent oligophenolic inhibitors. This trade-off can be circumvented by applying a pretreatment with anomeric protection, where xylooligosaccharides are hydrolysed to xylose, but xylose degradation is averted.

At the present pilot plant pretreatment conditions optimised towards minimum inhibitor formation i.e. oligophenolic compounds and minimum xylose degradation, the potent oligophenolic compounds in the 2-butanone fraction actually contributed less to total inhibition than the xylooligosaccharides, because the mild pretreatment preserved high levels of xylooligosaccharides that are also inhibitors. A drawback of mild pretreatment is that the biomass digestibility for the enzymes is not optimal. Pretreatment with anomeric protection has integrated minimum inhibitor formation and minimum pentose degradation. Consequently, the pretreatment parameters with anomeric protection, can be optimised even further to include biomass digestibility for the enzymes.

Nevertheless, the present work has cast new light on the reactions taking place during lignocellulosic biomass pretreatment and from this a new type of pretreatment with anomeric protection has been proposed and tested. The results open up for new possibilities for pretreatment of lignocellulosic biomass without degradation of monosaccharides and formation of inhibitors.

6.2 Future perspectives

In the current pilot plant pretreatment process approximately 27 % of the xylose is lost due to degradation to inhibitor compounds during pretreatment. A theoretical calculation based on the reduced level of inhibitor compounds in pretreatment with anomeric protection, implies a xylose recovery of 93 %. Thus 20 % more xylose can be deployed in a mixed glucose-xylose fermentation to ethanol and thus increases the theoretical ethanol yield from xylose with 20 %. Hereto comes advantages with improved cellulose digestibility and less inhibition for the enzymes. If these considerations are taken into account, theoretical calculations imply up to 30 % ethanol yield improvement from mixed glucose-xylose fermentation and further potential with optimisation towards cellulose digestibility.

To evaluate further consequences in the downstream process towards ethanol production, it would also be relevant to evaluate the inhibitory effect from the new compounds on the fermentation by yeast, because mainly small aliphatic acids, furans and monophenolic compounds are considered in the literature (Adeboye *et al* 2015, Gu *et al* 2015, Huang *et al* 2011, Klinke *et al* 2004, Klinke *et al* 2003).

Another continuation of the work reported here would be to study other protection groups than glycol ethers. Any nucleophilic compound that forms a stable xyloside can in principle be applied for protection of the anomeric position. However, both environmental and economic issues have to be considered. It could be interesting to test compounds with more than one xylosidation/glycosidation site as for example compounds with more hydroxyl groups, to study if it affects reactivity and/or optimal reaction conditions.

Glycerol and other alcohols are already applied in pretreatment today (Sun *et al* 2016, Sun et al 2015, Martín *et al* 2013, Zhang and Pei *et al* 2016, Zhang and Harrison *et al* 2016), but mainly in organosolv processes with the purpose of delignification although a few have reported ethyl/methyl glycoside formation (Bouxin *et al* 2014, Dora *et al* 2012, Deng *et al* 2011).

There are other ways to improve biorefinery processes than by considering the pretreatment. Although the work reported here are in the pretreatment category, identification of the 26 new compounds opens up new possibilities towards studies on which enzyme activities that are inhibited by potent oligophenolic inhibitors. This could be relevant for optimisation of endo-cellulase, cellobiohydrolase, and beta-glucosidase enzyme activities in enzyme preparations. With the recent discovery of the importance of LPMOs in cellulytic degradation (Vaaje-Kolstad *et al* 2010, Eibinger *et al* 2014, Forsberg *et al* 2011,Quinlan *et al* 2011, Agger *et al* 2014, Kracher *et al* 2016), LPMOs could also be relevant to study in this context.

7 References

1. Adeboye, P. T.; Bettiga, M.; Aldaeus, F.; Larsson, P. T.; Olsson, L. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. Microb Cell Fact, 2015, 14, 149.
2. Agger, J. W.; Isaksen, T.; Várnai, A.; Vidal-Melgos, S.; Willats, W. G. T.; Ludwig, R.; Horn, S.J.; Eijsink, V. G. E.; Westereng, B. Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation. Proc. Natl. Acad. Sci. U.S.A. 2014, 111, 6287–6292.
3. Ahmad, T.; Kenne, L.; Olsson, K.; Theander, O. The formation of 2-furaldehyde and formic acid from pentoses in slightly acidic deuterium oxide studied by 1H NMR spectroscopy Carbohydr. Res., 1995, 276, 309-320.
4. Antal, M.; Leesomboon, T.; Mok, W.; Richards, G. Mechanism of formation of 2-furaldehyde from D-xylose. Carbohydr. Res., 1991, 217, 71-85.
5. Appeldoorn, M. M.; de Waard, P.; Kabel, M. A.; Gruppen, H.; Schols, H. A. Enzyme resistant feruloylated xylooligomer analogues from thermochemically treated corn fiber contain large side chains, ethyl glycosides and novel sites of acetylation Carbohydr. Res., 2013, 381, 33-42.
6. Baumann, M. J.; Borch, K.; Westh, P.. Xylan oligosaccharides and cellobiohydrolase I (TrCel7A) interaction and effect on activity. Biotechnol. Biofuels, 2011, 4, 45.
7. Behera, S.; Arora, R.; Nandhagopal, N.; Kumar, S. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renewable Sustainable Energy Rev. 2014, 36, 91–106.
8. Bornaghi, L. F.; Poulsen, S-A. Microwave-accelerated Fischer glycosylation. Tetrahedron Lett. 2005, 46, 3485–3488.
9. Bouxin, F. P.; Jackson, S. D.; Jarvis, M. C. Organosolv pretreatment of Sitka spruce wood: Conversion of hemicelluloses to ethyl glycosides. Bioresour. Technol. 151, 2014, 441–444.
10. Carrasco, C.; Solano, C.; Penarrieta, J. M.; Baudel, H. M.; Galbe, M.; Lidén, G. Arabinosylated phenolics obtained from SO2-steam-pretreated sugarcane bagasse. J. Chem. Technol. Biotechnol. 2012; 87, 1723–1726.
11. Chen, X.; Shekiro, J.; Franden, M. A.; Wang, W.; Zhang, M.; Kuhn, E.; Johnson, D. K.; Tucker, M. P. The impacts of deacetylation prior to dilute acid pretreatment on the bioethanol process. Biotechnol. Biofuels, 2012, 5, 8.
12. Chundawat, S. P. S.; Vismeh, R.; Sharma, L. N.; Humpula, J. F.; Sousa, L. d. C.; Chambliss, C. K.; Jones, A. D.; Balan, V.; Dale, B. E. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. Bioresour. Technol., 2010, 101, 8429–8438.
13. Climent, M. J.; Corma, A.; Iborra, S. Conversion of biomass platform molecules into fuel additives and liquid hydrocarbon fuels, Green Chem., 2014, 16, 516–547.

14. Dee, S. J.; Bell, A. T. A Study of the Acid-Catalyzed Hydrolysis of Cellulose Dissolved in Ionic Liquids and the Factors Influencing the Dehydration of Glucose and the Formation of Humins. ChemSusChem, 2011, 4, 1166-1173.
15. Deng, W.; Liu, M.; Zhang, Q.; Wang, Y. Direct transformation of cellulose into methyl and ethyl glucosides in methanol and ethanol media catalyzed by heteropolyacids. Catal. Today, 2011, 164, 461–466.
16. Devlin, H. R.; Harris, I. J. Mechanism of the Oxidation of Aqueous Phenol with Dissolved Oxygen. Ind. Eng. Chem. Fundam., 1984, 23, 387-392.
17. Dora, S.; Bhaskar, T.; Singh, R.; Naik, D. V.; Adhikari, D. K. Effective catalytic conversion of cellulose into high yields of methyl glucosides over sulfonated carbon based catalyst. Bioresour. Technol. 2012, 120, 318–321.
18. Du, B.; Sharma, L. N.; Becker, C.; Chen, S.; Mowery, R. A.; van Walsum, G. P.; Chambliss, C. K. Effect of Varying Feedstock–Pretreatment Chemistry Combinations on the Formation and Accumulation of Potentially Inhibitory Degradation Products in Biomass Hydrolysates. Biotechnol. Bioeng., 2010, 107, 430-440.
19. Dumitrache, A.; Akinosho, H.; Rodriguez, M.; Meng, X.; Yoo, C G.; Natzke, J.; Engle, N. L.; Sykes, R. W.; Tschaplinski, T. J.; Muchero, W.; Ragauskas, A J.; Davison, B. H.; Brown, S. D. Consolidated bioprocessing of Populus using *Clostridium* (*Ruminiclostridium*) *thermocellum*: a case study on the impact of lignin composition and structure. Biotechnol. Biofuels, 2016, 9, 31.
20. Echavarría, A. P.; Pagán, J.; Ibarz, A. Melanoidins Formed by Maillard Reaction in Food and Their Biological Activity. Food Eng. Rev. 2012, 4, 203–223.
21. Eibinger, M.; Ganner, T.; Bubner, P.; Rosker, S.; Kracher, D.; Haltrich, D.; Ludwig, R.; Plank, H.; Nidetzky, B. Cellulose Surface Degradation by a Lytic Polysaccharide Monooxygenase and Its Effect on Cellulase Hydrolytic Efficiency. J. Biol. Chem. 2014, 289, 35929–35938.
22. Ershova, O.; Kanervo, J.; Hellsten, S.; Sixta, H. The role of xylulose as an intermediate in xylose conversion to furfural: insights via experiments and kinetic modelling. RSC Adv. 2015, 5, 66727-66737.
23. Finger, D.; Machado, C. S.; Torres, Y. R.; Quinaia, S. P.; Thomaz, A. C. G.; Gobbo, A. R.; Monteiro, M. C.; Ferreira, A. G.; Saway, A. c. H. F.; Eberlin, M. N. Antifungal Bioassay-Guided fractionation of an Oil Extract of Propolis. J. Food Qual. 2013, 36, 291–301.
24. Flannelly, T.; Lopes, M.; Kupiainen, L.; Dooley, S.; Leahy, J. J. Non-stoichiometric formation of formic and levulinic acids from the hydrolysis of biomass derived hexose carbohydrates. RSC Adv., 2016, 6, 5797–5804.
25. Forsberg, Z.; Vaaje-Kolstad, G.; Westereng, B.; Bunæs, A. C.; Stenstrøm, Y.; MacKenzie, A.; Sørlie, M.; Horn, S. J.; Eijsink, V. G. H. Cleavage of cellulose by a CBM33 protein. Protein Sci. 2011, 20, 1479–1483.
26. Forsskåhl, I.; Popoff, T.; Theander, O. Reactions of D-Xylose and D-Glucose in alkaline, Aqueous Solutions. Carbohydr. Res., 1976, 48, 13-21.

27. Gu, H.; Zhang, J.; Bao, J. High Tolerance and Physiological Mechanism of Zymomonas Mobilis to Phenolic Inhibitors in Ethanol Fermentation of Corncob Residue. Biotechnol. Bioeng. 2015, 112, 1770-1782.
28. Han, J.; Sen, S. M.; Alonso, D. M.; Dumesic, J. A.; Maravelias, C. T. A strategy for the simultaneous catalytic conversion of hemicellulose and cellulose from lignocellulosic biomass to liquid transportation fuels. Green Chem., 2014, 16, 653–661.
29. Holopainen-Mantila, U.; Merali, K. M. Z.; Käsper, A.; Bot, P.; Jääskeläinen, A.; Waldron, K.; Kruus, K.; Tamminen, T. Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers, Bioresour. Technol. 138 (2013) 156–162.
30. Hu, F.; Jung, S.; Ragauskas, A. Pseudo-lignin formation and its impact on enzymatic hydrolysis Bioresour. Technol., 2012, 117, 7-12.
31. Huang, H.; Guo, X.; Li, D.; Liu, M.; Wu, J.; Ren, H. Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids. Bioresour. Technol, 2011, 102, 7486–7493.
32. Humpula, J. F.; Uppugundla, N.; Vismeh, R.; Sousa, L.; Chundawat, S. P. S.; Jones, A. D.; Balan, V.; Dale, B. E.; Cheh, A. M. Probing the nature of AFEX-pretreated corn stover derived decomposition products that inhibit cellulase activity. Bioresour. Technol. 2014, 152, 38–45.
33. Ibarz, A.; Garza, S.; Pagán, J. Inhibitory effect of melanoidins from glucose–asparagine on carboxypeptidases activity. Eur. Food Res. Technol. 2008, 226, 1277–1282.
34. Jönsson, L. J.; Martín, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 2016, 199, 103–112.
35. Kabel, M. A.; Bos, Zeevalking, J.; Voragen, A. G. J.; Schols, H. A. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour. Technol. 2007, 98, 2007, 2034–2042.
36. Kim, Y.; Ximenes, E.;, Mosier, N. S.; Ladisch, M. R. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme Microb. Technol., 2011, 48, 408–415.
37. Kimura, H.; Nakahara, M.; Matubayasi, N. In Situ Kinetic Study on Hydrothermal Transformation of D-Glucose into 5-Hydroxymethylfurfural through D-Fructose with 13C NMR. J. Phys. Chem. A, 2011, 115, 14013-14021.
38. Klinke, H. B.; Olsson, L.; Thomsen, A. B.; Ahring, B. K. Potential Inhibitors from Wet Oxidation of Wheat Straw and Their Effect on Ethanol Production of Saccharomyces cerevisiae: Wet Oxidation and Fermentation by Yeast. Biotechnol. Bioeng. 2003, 81, 738-747.
39. Klinke, H. B.; Thomsen, A. B.; Ahring, B. K. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 2004, 66, 10–26.
40. Kont, R.; Kurasin, M.; Teugjas, H.; Väljemäe, P. Strong cellulase inhibitors from the hydrothermal pretreatment of wheat straw. Biotechnol. Biofuels., 2013, 6, 135.

41. Kracher, D.; Scheiblbrandner, S.; Felice, A. K.; Breslmayr, E.; Preims, M.; Ludwicka, K.; Haltrich, D.; Eijsink, V. G. H.; Ludwig, R. Extracellular electron transfer systems fuel cellulose oxidative degradation. Science, 2016, 352, 1098-1101.
42. Kumar, R.; Hu, F.; Sannigrahi, P.; Jung, S.; Ragauskas, A. J.; Wyman, C. E. Carbohydrate Derived-Pseudo-Lignin Can Retard Cellulose Biological Conversion. Biotechnol. Bioeng., 2013, 110, 737-753.
43. Lan, W.; Lu, F.; Regner, M.; Zhu, Y.; Rencoret, J.; Ralph, S. A.; Zakai, U. I.; Morreel, K.; Boerjan, W.; Ralph, J. Tricin, a Flavonoid Monomer in Monocot Lignification. Plant Physiology, 2015, 167, 1284–1295.
44. Larsen, J.; Petersen, M. O.; Thirup, L.; Li, H. W.; Iversen, F. K. The IBUS process - Lignocellulosic Bioethanol Close to a Commercial Reality. Chem. Eng. Technol., 2008, 31, 765-772.
45. Larsson, S.; Palmqvist, E.; Hahn-Hagerdal, B.; Tengborg, C.; Stenberg, K.; Zacchi, G.; Nilvebrant, N. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb. Technol., 1999, 24, 151-159.
46. Lu, F.; Karlen, S. D.; Regner, M.; Kim, H.; Ralph, S. A.; Sun, R.; Kuroda, K.; Augustin, M. A.; Mawson, R.; Sabarez, H.; Singh, T.; Jimenez-Monteon, G.; Zakaria, S.; Hill, S.; Harris, P. J.; Boerjan, W.; Wilkerson, C. G.; Mansfield, S. D.; Ralph, J. Naturally p-Hydroxybenzoylated Lignins in Palms. Bioenerg. Res. 2015, 8, 934-952.
47. Ludwiczuk, A.; Saha, A.; Kuzuhara, T.; Asakawa, Y. Bioactivity guided isolation of anticancer constituents from leaves of *Alnus sieboldiana* (*Betulaceae*). Phytomedicine, 2011, 18, 491–498.
48. Luijkx, G.; Vanrantwijk, F.; Vanbekkum, H. Hydrothermal formation of 1,2,4-benzenetriol from 5-hydroxymethyl-2-furaldehyde and D-fructose. Carbohydr. Res., 1993, 242, 131-139.
49. Luterbacher, J. S.; Rand, J. M.; Alonso, D. M.; Han, J.; Youngquist, J. T.; Maravelias, C. T.; Pfleger, B. F.; Dumesic, J. A. Nonenzymatic Sugar Production from Biomass Using Biomass-Derived γ-Valerolactone. Science, 2014, 343, 277-280.
50. Ma, X.; Yang, X.; Zheng, X.; Chen, L.; Huang, L.; Cao, S.; Akinosho, H. Toward a further understanding of hydrothermally pretreated holocellulose and isolated pseudo lignin. Cellulose, 2015, 22, 1687–1696.
51. Martín, C.; Puls, J.; Schreiber, A.; Saake, B. Optimization of sulfuric acid-assisted glycerol pretreatment of sugarcane bagasse. Holzforschung, 2013; 67, 523–530.
52. Matson, T.D.; Barta, K.; Iretskii, A. V.; Ford, P. C. One-Pot Catalytic Conversion of Cellulose and of Woody Biomass Solids to Liquid Fuels. J. Am. Chem. Soc., 2011, 133, 14090–14097.
53. Mhlongo, S. I.; Haan, R. d.; Viljoen-Blooma, M.; van Zyla, W. H. Lignocellulosic hydrolysate inhibitors selectively inhibit/deactivatecellulase performance. Enzyme Microb. Technol., 2015, 81, 16–22.
54. Mitchell, V. D.; Taylor, C. M.; Bauer, S. Comprehensive Analysis of Monomeric Phenolics in Dilute Acid Plant Hydrolysates. Bioenerg. Res. 2014, 7, 654–669.
55. Möller, M.; Schröder, U. Hydrothermal production of furfural from xylose and xylan as model compounds for hemicelluloses. RSC Adv., 2013, 3, 22253-22260.

56. Mowery, D. F. Isomer Distributions during Methyl Mannoside and during Methyl Arabinoside Formations by the Fischer Method Using a Cation Exchange Resin as Catalyst. J. Org. Chem. 1961, 26, 3484-3486.
57. Murov. http://murov.info/orgsolvents.htm. 2016-06-13.
58. Nimlos, M. R.; Qian, X.; Davis, M.; Himmel, M. E.; Johnson, D. K. Energetics of Xylose Decomposition as Determined Using Quantum Mechanics Modeling. J. Phys. Chem. A, 2006, 110, 11824-11838.
59. Oefner, P.; Lanziner, A.; Bonn, G.; Bobleter, O. Quantitative Studies on Furfural and Organic Acid Formation during Hydrothermal, Acidic and Alkaline Degradation of D-Xylose. Monatsh. Chem., 1992, 123, 547-556.
60. Panagiotou, G.; Olsson L. Effect of Compounds Released During Pretreatment of Wheat Straw on Microbial Growth and Enzymatic Hydrolysis Rates. Biotechnol. Bioeng., 2007, 96, 250-258.
61. Patil, S. K. R.; Lund, C. R. F. Formation and Growth of Humins via Aldol Addition and Condensation during Acid-Catalyzed Conversion of 5-Hydroxymethylfurfural. Energy Fuels, 2011, 25, 4745-4755.
62. Pedersen, M.; Meyer, A. S. Lignocellulose pretreatment severity – relating pH to biomatrix opening New Biotechnol., 2010, 27, 739-750.
63. Popoff, T.; Theander, O. Formation of Aromatic Compounds from Carbohydrates. Part I. Reaction of D-Glucuronic acid, D-Galaturonic acid, D-Xylose and L-Arabinose in Slightly Acidic, Aqueous Solution Carbohydr. Res., 1972, 22, 135-149.
64. Popoff, T.; Theander, O. Formation of Aromatic Compounds from Carbohydrates. Part III. Reaction of D-Glucose and D-Fructose in Slightly Acidic, Aqueous Solution. Acta Chem. Scand., Ser. B, 1976, 30, 397-402.
65. Qian, X. Mechanisms and Energetics for Brønsted Acid-Catalyzed Glucose Condensation, Dehydration and Isomerization Reactions. Top. Catal., 2012, 55, 218-226.
66. Qian, X.; Nimlos, M.; Davis, M.; Johnson, D.; Himmel, M. Ab initio molecular dynamics simulations of β-D-glucose and β-D-xylose degradation mechanisms in acidic aqueous solution. Carbohydr. Res., 2005, 340, 2319-2327.
67. Qing, Q.; Yang, B.; Wyman, C. E. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresour. Technol. 2010, 101, 9624–9630.
68. Quinlana, R. J.; Sweeney, M. D.; Leggio, L. L.; Otten, H.; Poulsen, J-C. N.; Johansen, K. S.; Krogh, K. B. R. M.; Jørgensen, C. I.; Tovborg, M.; Anthonsen, A.; Tryfona, T.; Walter, C. P.; Dupree, P.; Xu, F.; Davies, G. J.; Walton, P. H. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc. Natl. Acad. Sci. U.S.A. 2011, 108, 15079–15084.
69. Rasmussen, H.; Sørensen, H. R.; Meyer, A. S. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms. Carbohydr. Res. 2014, 385, 45–57.
70. Rinaldi, R. Angew. Chem. Int. Ed., 2014, 53, 8559–8560.

71. Río, J. C. D.; Lino, A. G.; Colodette, J. L.; Lima, C. L.; Gutierrez, A.; Martínez, A. T.; Lu, F.; Ralph, J.; Rencoret, J. Differences in the chemical structure of the lignins from sugarcane bagasse and straw. Biomass Bioenergy, 2015, 81, 322-338.
72. Río, J.C. D.; Rencoret, J.; Prinsen, P.; Martínez, Á.; Ralph, J.; Gutiérrez, A. Structural Characterization of Wheat Straw Lignin as Revealed by Analytical Pyrolysis, 2D-NMR, and Reductive Cleavage Methods. J. Agric. Food Chem. 2012, 60, 5922−5935.
73. Rio, J. C. D.; Marques, G.; Rencoret, J.; Martinez, A. T.; Gutierrez, A. Occurrence of Naturally Acetylated Lignin Units. J. Agric. Food Chem., 2007, 55, 5461-5468.
74. Rosgaard, L.; Pedersen, S.; Langston, J.; Akerhielm, D.; Cherry,J. R.; Meyer, A. S. Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates. Biotechnol. Prog., 2007, 23, 1270–1276.
75. Rusjan, D.; Veberič, R.; Mikulič-Petkovšek, M. The response of phenolic compounds in grapes of the variety 'Chardonnay' (*Vitis vinifera L.*) to the infection by phytoplasma Bois noir. Eur. J. Plant Pathol. 2012, 133, 965–974.
76. Sánchez-Rabaneda, F.; Jáuregui, O.; Casals, I.; Andrés-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventós, R. M. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). J. Mass Spectrom., 2003, 38, 35-42.
77. Sannigrahi, P.; Kim, D. H.; Jung, S.; Ragauskas, A. Pseudo-lignin and pretreatment chemistry. Energy Environ. Sci., 2011, 4, 1306-1310.
78. Silveira, M. H. L.; Morais, A. R. C.; Lopes, A. M. d. C.; Olekszyszen, D. N.; Łukasik, R. B.; Andreaus, J.; Ramos, L. P. Current Pretreatment Technologies for the Development of Cellulosic Ethanol and Biorefineries. ChemSusChem, 2015, 8, 3366 – 3390.
79. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. Determination of structural carbohydrates and lignin in biomass. NREL Technical Report 2008, 510:42618.
80. Stryer, L. Biochemistry 4th edition, W. H. Freeman and Company, New York.
81. Sun, R.; Fang, J. M.; Mott, L.; Bolton, J. Fractional isolation and characterization of polysaccharides from oil palm trunk and empty fruit bunch fibres. Holzforschung, 1999, 53, 253–260.
82. Sun, F. F.; Wang, L.; Hong, J.; Ren, J.; Du, F.; Hu, J.; Zhang, Z.; Zhou, B. The impact of glycerol organosolv pretreatment on the chemistry and enzymatic hydrolyzability of wheat straw. Bioresour. Technol. 2015, 187, 354–361.
83. Sun, F. F.; Zhao, X.; Hong, J.; Tang, Y.; Wang, L.; Sun, H.; Li, X.; Hu, J. Industrially relevant hydrolyzability and fermentability of sugarcane bagasse improved effectively by glycerol organosolv pretreatment. Biotechnol. Biofuels, 2016, 9, 59.
84. Tejirian, A.; Xu, F. Inhibition of enzymatic cellulolysis by phenolic compounds. Enzyme Microb. Technol. 2011, 48, 239–247.
85. Vaaje-Kolstad, G.; Westereng, B.; Horn, S. J.; Liu, Z.; Zhai, H.; Sørlie, M.; Eijsink, V. G. H. An Oxidative Enzyme Boosting the Enzymatic Conversion of Recalcitrant Polysaccharides. Science, 2010, 330, 219–222.

86. Wang, H.; Qian, H.; Yao, W. Melanoidins produced by the Maillard reaction: Structure and biological activity. Food Chem., 2011, 128, 573-584.
87. Xie, G-Y.; Zhu, Y.; Shu, P.; Qin, X-Y.; Wu, G.; Wang, Q.; Qin, M-J. Phenolic metabolite profiles and antioxidants assay of three *Iridaceae* medicinal plants for traditional Chinese medicine “She-gan” by on-line HPLC–DAD coupled with chemiluminescence (CL) and ESI-Q-TOF-MS/MS. J. Pharm. Biomed. Anal. 2014, 98, 40–51.
88. Ximenes, E.; Kim, Y.; Mosier, N.; Dien, B.; Ladisch, M. Deactivation of cellulases by phenols. Enzyme Microb. Technol, 2011, 48, 54–60.
89. Yang, G.; Pidko, E. A.; Hensen, E. J. M. Mechanism of Brønsted acid-catalyzed conversion of carbohydrates J. Catal., 2012, 295, 122-132.
90. Yelle, D. J.; Kaparaju, P.; Hunt, C. G.; Hirth, K.; Kim, H.; Ralph, J.; Felby, C. Two-Dimensional NMR Evidence for Cleavage of Lignin and Xylan Substituents in Wheat Straw Through Hydrothermal Pretreatment and Enzymatic Hydrolysis. Bioenerg. Res. 2013, 6, 211–221.
91. Zha, Y.; Westerhuis, J. A.; Muilwijk, B.; Overkamp, K. M.; Nijmeijer, B. M.; Coulier, L.; Smilde, A. K.; Punt, P. J. Identifying inhibitory compounds in lignocellulosic biomass hydrolysates using an exometabolomics approach. BMC Biotechnol. 2014, 14, 14-22.
92. Zhang, J.; Viikari, L. Xylo-oligosaccharides are competitive inhibitors of cellobiohydrolases I from *Thermoascus aurantiacus*. Bioresour. Technol, 2012, 117, 286–291.
93. Zhang, Z.; Harrison, M. D.; Rackemann, D. W.; Doherty, W. O. S.; O’Hara, I. M. Organosolv pretreatment of plant biomass for enhanced enzymatic saccharification. Green Chem., 2016, 18, 360-381.
94. Zhang, K.; Pei, Z.; Wang, D. Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: A review. Bioresour. Technol. 2016, 199, 21–33.

8 Papers

Paper 1

Helena Rasmussen, Hanne R. Sørensen, Anne S. Meyer. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms. *Carbohydrate Research* 385 (2014) 45–57.

Paper 2

Helena Rasmussen, David Tanner, Hanne R. Sørensen, Anne S. Meyer. New degradation compounds from lignocellulosic biomass pretreatment: Routes for formation of potent oligophenolic enzyme inhibitors. Submitted after revision. *Green Chemistry*.

Paper 3

Helena Rasmussen, Hanne R. Sørensen, David Tanner, Anne S. Meyer. Pentose dimers with bicyclic moieties from pretreated biomass. Ready for submission (follow up to paper 2 and thus awaits publication acceptance of paper 2 before submission).

Paper 4

Helena Rasmussen, Kit H. Mogensen, Martin D. Jeppesen, Hanne R. Sørensen and Anne S. Meyer. 4-Hydroxybenzoic acid from hydrothermal pretreatment of oil palm empty fruit bunches - its origin and influence on biomass conversion. Submitted and invited for resubmission in revised form. *Biomass & Bioenergy.*

Patent application

Anomeric protection (filed not published).

Paper 1

Carbohydrate Research 385 (2014) 45–57

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Minireview

Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms

Helena Rasmussen [a], Hanne R. Sørensen [a], Anne S. Meyer [b,*]

[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark
[b] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Lyngby, Denmark

ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 27 August 2013
Accepted 31 August 2013
Available online 11 September 2013

Keywords:
Carbohydrate degradation
5-(Hydroxymethyl)-2-furaldehyde (HMF)
Furfural
Humins
Protonation site
Reaction mechanism

ABSTRACT

The degradation compounds formed during pretreatment when lignocellulosic biomass is processed to ethanol or other biorefinery products include furans, phenolics, organic acids, as well as mono- and oligomeric pentoses and hexoses. Depending on the reaction conditions glucose can be converted to 5-(hydroxymethyl)-2-furaldehyde (HMF) and/or levulinic acid, formic acid and different phenolics at elevated temperatures. Correspondingly, xylose can follow different reaction mechanisms resulting in the formation of furan-2-carbaldehyde (furfural) and/or various C-1 and C-4 compounds. At least four routes for the formation of HMF from glucose and three routes for furfural formation from xylose are possible. In addition, new findings show that biomass monosaccharides themselves can react further to form pseudo-lignin and humins as well as a wide array of other compounds when exposed to high temperatures. Hence, several aldehydes and ketones and many different organic acids and aromatic compounds may be generated during hydrothermal treatment of lignocellulosic biomass. The reaction mechanisms are of interest because the very same compounds that are possible inhibitors for biomass processing enzymes and microorganisms may be valuable biobased chemicals. Hence a new potential for industrial scale synthesis of chemicals has emerged. A better understanding of the reaction mechanisms and the impact of the reaction conditions on the product formation is thus a prerequisite for designing better biomass processing strategies and forms an important basis for the development of new biorefinery products from lignocellulosic biomass as well.

1. Introduction

When lignocellulosic biomass is processed into biofuels—and potentially other biorefinery products—the biomass is usually pretreated in order to make the cellulose and hemicellulose amenable to enzymatic depolymerisation. The pretreatment may be done according to a number of regimes,[1,2] but current large scale pretreatment processes for lignocellulosic biomass generally involve some kind of high temperature treatment. The biomass pretreatment, particularly pretreatment regimes involving acid and/or temperatures above 160–180 °C, induces the formation of degradation products that may inhibit the cellulolytic enzymes (Table 1) and/or the ethanol producing microorganisms (notably yeast, *Saccharomyces cerevisiae*) (Table 2) that are required for the subsequent sugar conversion.

5-(Hydroxymethyl)-2-furaldehyde (HMF) and furan-2-carbaldehyde (furfural) are considered the key primary degradation compounds from the carbohydrate fraction of the lignocellulosic biomass, but several other products, notably other aldehydes, aliphatic and aromatic acids may also form and these compounds have a different inhibitory impact on the enzymes and yeast (Tables 1 and 2).

Knowledge of the degradation routes is therefore obviously an important foundation for controlling the inhibitor reactivity and in turn for improving the current cellulosic ethanol processes.

The advances in the recent two years have also revealed that the chemistry of formation of the putative inhibitors during thermal biomass pretreatment for biofuel production have many features overlapping with the discipline of industrial scale synthesis of biobased platform chemicals from glucose and potentially from other biomass monosaccharides.[3,4] The deliberate production of these type of products from biomass carbohydrates for industrial uses currently only appears feasible via targeted catalytic or biocatalytic technologies.[5] Nevertheless, with regard to the formation of degradation or synthesis compounds two topics are particularly important to understand in detail for making the production of biofuels more cost-effective and for preparing for industrial scale synthesis of chemicals from monosaccharides:

* Corresponding author. Tel.: +45 45 25 2800.
E-mail address: am@kt.dtu.dk (A.S. Meyer).

0008-6215/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carres.2013.08.029

Table 1
Inhibition of cellulolytic enzyme activity (commercial cellulase preparations) by lignocellulosic biomass degradation products

Inhibitor	Concn (mM)	Commercial cellulases (% activity relative to reference)
Furfural and 5-(hydroxymethyl)-2-furaldehyde (HMF) total	19	~100[a]
Vanillic acid	12	~100[b]
Syringic acid	10	~100[b]
Acetosyringone	5	~100[b]
Syringaldehyde	5/10	~100[b]/~30[c]
Ferulic acid	10	~100[d]
Tannic acid	1	~40[d]
Vanillin	13	~100[d]
Gallic acid	12	~100[d]
Cinnamic acid	14	~100[d]
p-Coumaric acid	12	~100[d]
Sinapic acid	9	~100[d]
4-Hydroxybenzoic acid	14	~100[d]
Lignin derivatives	1	~20-30[c]
Acetic acid	218	95–100[a]
Butyric acid	567	42[e]
Formic acid	326	5–20[b]
Succinic acid	34	~100[b]
Itaconic acid	384	83[e]
Lactic acid	555	87[e]
Propionic acid	675	64[e]

Activity (%) compared to reference hydrolysis with no inhibitor present (data from a,[30] b,[31] c,[32] d,[33] e,[34]).

Table 2
Ethanol yields (g ethanol/g consumed glucose) relative to reference fermentation

Inhibitor compound	Concn (mM)	*S. cerevisiae* (Baker's yeast)
Furfural	21–65	82–50
5-(hydroxymethyl)-2-furaldehyde (HMF)	8–57	50
4-Hydroxybenzaldehyde	4–17	50
Vanillin	1–18	~50–100
Syringaldehyde	1–25	50
Levulinic, acetic and formic acids	Total <100	Increase in yield
	Total >100	Decrease in yield

Data adapted from Refs. 35,36.

1. The degradation products and their routes of formation.
2. The influence of process parameters and pretreatment on the degradation product profile.

In the literature, different authors promote different degradation routes for biomass monosaccharides, with the degradation routes for xylose to furfural and for glucose to HMF appearing to be particularly disputed.[6–10] In the present treatise we will critically discuss the recently proposed degradation mechanisms and routes and show that several degradation routes for glucose and xylose are in fact possible. We will also highlight how a combination of advanced modelling and systematic analytical approaches has recently cast new light on the details of the molecular reactions, and underline how this understanding can pave the way for the industrial production of new chemicals in the biobased economy.

2. Biomass degradation during hydrothermal treatment

2.1. Cellulose and hemicellulose

The D-glucose originating from cellulose can be thermally degraded directly during the biomass pretreatment. In this type of degradation, D-glucose is firstly dehydrated to HMF, which can then further degrade to formic acid and levulinic acid[8] (Fig. 1). As the monomeric substituents in hemicellulose include both hexoses and pentoses, hemicellulose may also give rise to HMF, formic acid and levulinic acid (Fig. 1). In contrast, furfural is formed exclusively from pentoses, that is, mainly from D-xylose and L-arabinose released from the hemicellulose (Fig. 1).

Hydrothermal biomass pretreatment can also lead to cleavage of the acetyl linkages in xylan and presumably also induce hydrolytic cleavage of the acetyl substitutions in, for example, acetylated galacto-glucomannans, leading to the formation of free acetic acid[11] (Fig. 1).

Although it is generally acknowledged that phenolics found in biomass liquors stem from lignin, early reports show that various phenolic compounds may also form as degradation products from D-glucose, D-xylose and L-arabinose[12,13] (Fig. 1). Recently this formation of phenolics has been confirmed by demonstration of pseudo-lignin generation from model substrates of cellulose (i.e., Avicel), xylan and D-xylose.[14] These findings mark a significant 'game-changer' in the field, since they show that phenolics can originate from carbohydrates.

2.2. Lignin

The aromatic residues in lignin can be degraded to different types of phenolic structures depending on the type of structural monomeric unit in the lignin.[15] Furthermore it cannot be excluded that acetylated lignin[16] liberates acetic acid when thermally treated (Fig. 1).

2.3. Degradation product inter-reactions

The degradation compounds shown in Figure 1 are moreover able to undergo various reactions with each other. Such intermolecular reactions can lead to several new products and polymerisation reactions.

2.3.1. Pseudo lignin

Pseudo lignin is an aromatic material containing hydroxyl and carbonyl functional groups. In this way pseudo lignin resembles native lignin but is not derived thereof. Sannigrahi et al.[17] proposes the broad definition of pseudo-lignin to be an aromatic material that yields a positive Klason lignin value and is not derived from native lignin. The existence of pseudo lignin has been recognised because the amount of Klason lignin in pretreated

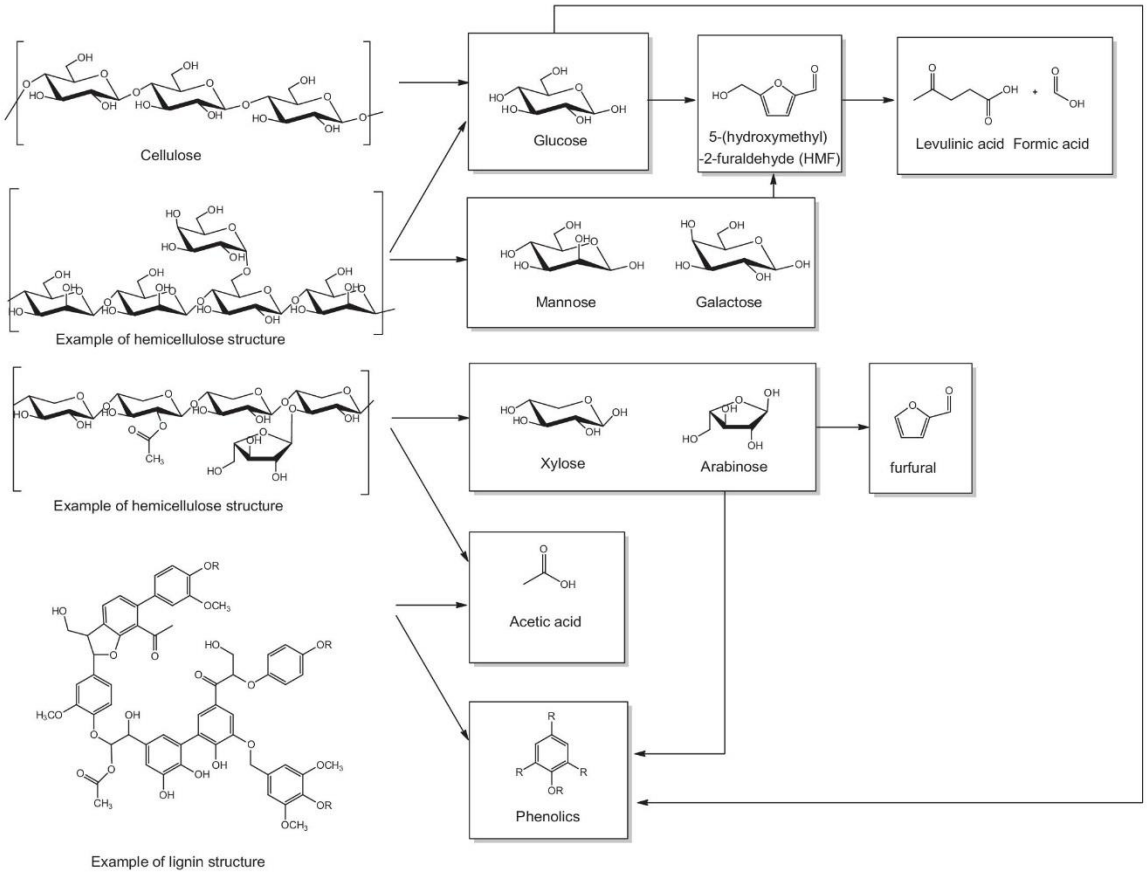


Figure 1. Suggested products and summary reaction routes for the degradation of biomass saccharides (cellulose and hemicelluloses structures) plus lignin during hydrothermal treatment.

biomass has often been found to be higher than in the untreated biomass and has furthermore been reported to increase with pretreatment severity.[17] As already mentioned above recent progress has confirmed that pseudo-lignin can form solely via carbohydrate degradation.[14] It has furthermore been proposed[18] that pseudo-lignin arises from polymerisation and/or condensation reactions from the key intermediates 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol derived from furfural and HMF respectively. However, these reactions need experimental support regarding at least three aspects: (1) it is uncertain whether 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol are in fact 'key intermediates' in the sense that it is unclear whether the pseudo lignin formation also involves other compounds. (2) The formation of 3,8-dihydroxy-2-methylchromone from furfural has not been explicitly demonstrated. (3) It has not been unequivocally proven that 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol give rise to pseudo lignin formation.

Popoff and Theander[19] suggested that degradation of different monosaccharides at low pH (pH 3.5 and pH 4.5) at 96 °C produced 3,8-dihydroxy-2-methylchromone as a major degradation product, but they did not show that its formation took place through furfural. As well, the formation of 1,2,4-benzenetriol through HMF has been found to occur at 290–400 °C and high pressure (27.5 MPa),[20] but this reaction remains to be demonstrated to occur at more typical lignocellulosic biomass pretreatment conditions.

Pseudo lignin has in several reports[14,17,18] been demonstrated to deposit as droplets on the surface of pretreated lignin free biomass and carbohydrate model substrates and is in this way thought to retard the enzymatic hydrolysis by enzyme inhibition and/or reduced substrate accessibility.[14]

2.3.2. Humins

Humins are dark coloured substances that are formed during the thermal hydrolysis of glucose. Formation of humins has been shown to arise from reaction of HMF with glucose[21] and/or via reaction of HMF with 2,5-dioxo-6-hydroxy-hexanal (hydrated HMF)[22] and subsequent polymerisation (Fig. 2). The formation of humins via HMF reactions has been reported relatively recently,[21,22] and whether, and to which extent, humins impact cellulolytic enzymes and/or yeast during cellulosic ethanol production are at present uncertain.

3. Mechanistic considerations

3.1. Furfural and xylose

Both a ring opening of xylose, that is, an acyclic mechanism and two different types of direct cyclic mechanisms (Fig. 3) have been

suggested for the degradation of xylose to furfural. There is experimental support of both mechanisms[6,7] indicating that the degradation of xylose is complex and may not occur according to one mechanism only. Moreover, several different organic acids, aldehydes and ketones have been reported as products from xylose degradation[7,23] and some of these are chemicals of potential commercial interest.

Via ab initio molecular dynamic simulations it has been shown that the rate limiting step in the reaction of xylose is the protonation of hydroxyl groups on the xylose or direct protonation of the pyranose oxygen. Hence, the subsequent degradation product formed depends on the hydroxyl site of protonation.[24] The significance of the initial hydroxyl group protonation for the subsequent reactions is supported by calculation of activation energies by using quantum mechanics modelling in combination with NMR studies.[25] For ab initio molecular dynamic simulations and quantum mechanics modelling combined with NMR with no solvent effects (in vacuum) the reaction schemes are as outlined in Figure 4.

It is worth noting that in the models and simulations (summarised in Fig. 4), the furfural formation from the ring opened xylose, that is, the acyclic mechanisms followed by *O*-pyranose protonation, shown in Figure 3, does not occur. Protonation of the pyranose oxygen, which leads to ring opening, only leads to the equilibrium between pyranose and aldose and not to further reaction to furfural according to the models. Hence the models contradict with the experimental data which support furfural formation via the acyclic mechanism (Fig. 3). Furthermore the ring closed mechanism resulting from protonation at 1-OH (as suggested in Fig. 3) does not give any observable reaction when modelled by ab initio molecular dynamics simulations and quantum mechanics. Hence via this modelling the furfural formation appears to arise exclusively from the 2-OH protonation of xylose (Fig. 3).

However, the solvent water structure is crucial for the protonation site since water molecules compete for protons and hydrogen bond to the hydroxyl groups. In addition, reaction conditions (pH, solvent, salts etc.) can easily alter the water molecule surroundings and hence the hydroxyl protonation site and the reaction mechanism and the following reaction. Although these reaction mechanisms provide an important base for understanding how the degradation products are formed and offer some clues to predict which products that are likely to be formed, the results cannot be directly transferred to complex reaction mixtures resulting from biomass pretreatment because of the significance of the surrounding water molecule structure. It is tempting to infer that in genuine biomass processes operated at low liquid-to-solid ratios water will be present on a mole basis to promote the reactions. However, other effects such as solubility might affect the course of the reactions. In addition, as for any chemical reaction, mixing challenges resulting in hot spots will most likely be an issue, in particular considering the high viscosities of pretreated lignocellulosic biomass slurries.The complexity of the theoretical base as well as the divergent analytical observations clearly illustrate that it is necessary to perform experimental evaluations to gain full insight under the actual conditions.

3.2. HMF, glucose and fructose

HMF formation from glucose is quite complex and several reaction routes and mechanisms have been suggested. Both cyclic and acyclic mechanisms have been proposed and each of these can furthermore take place through fructose isomerisation or via mechanisms without isomerisation (Fig. 5). The acyclic mechanism results when glucose is protonated at the pyranose oxygen which leads to ring opening. Ring opening is followed by either the aldose acyclic mechanism[7] or rearrangement and ring reclosure

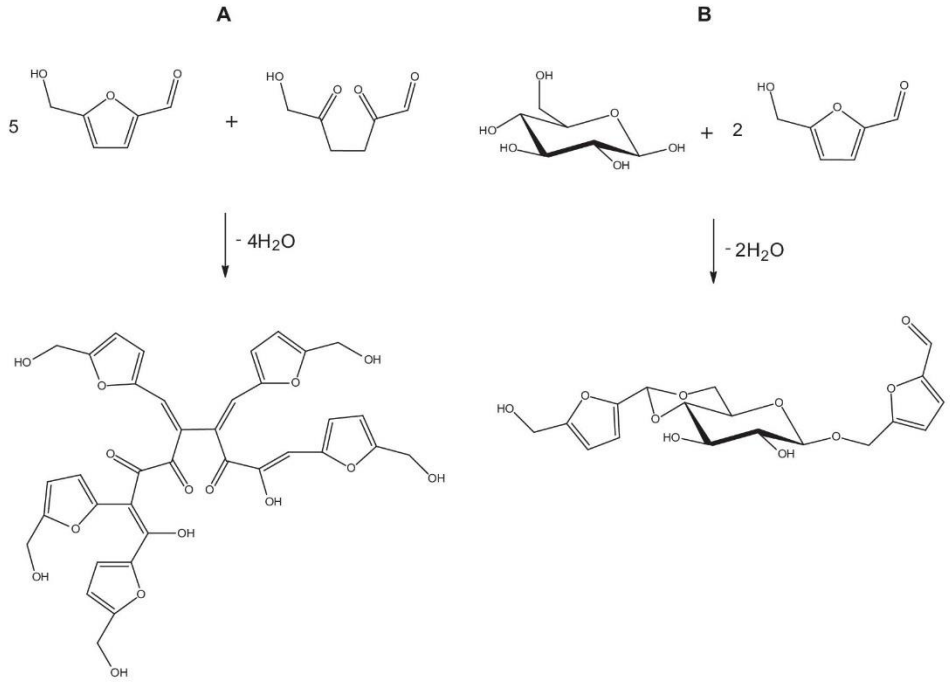


Figure 2. Examples of humins formation. (A) From HMF reaction with hydrated HMF (2,5-dioxo-6-hydroxy-hexanal. (B) From HMF and glucose[22] and Ref. 21.

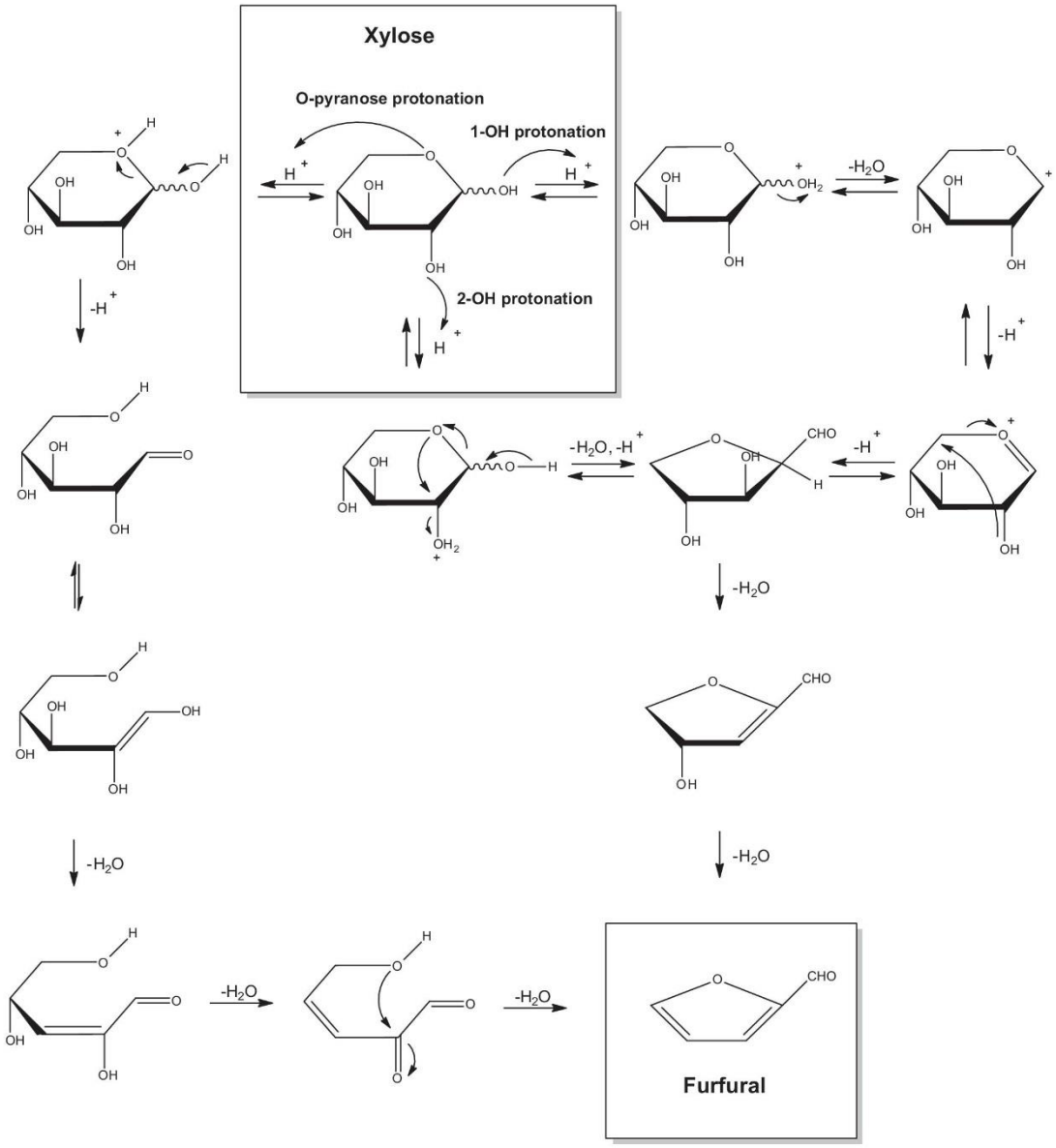


Figure 3. Suggested acyclic and cyclic mechanisms for furfural formation from xylose resulting from protonation at either *O*-pyranose, 1-OH or 2-OH. Reaction scheme adapted and summarised from schemes proposed by Refs. 6,7.

to fructose[8] (Fig. 5). In either case the compounds dehydrate further through a series of steps to produce HMF (Fig. 5). Experimental support for the direct acyclic aldose mechanism has been reported; however it should be noted that the reaction conditions involved the presence of LiCl and dimethylacetamide (DMA) as solvent.[26]

The cyclic mechanism is initiated by 2-OH protonation of glucose followed by fructose formation via a furanose tertiary carbocation or, as recently proposed, HMF formation via a furanose secondary carbocation with no isomerisation to fructose[10] (Fig. 5). In situ NMR support for isomerisation to fructose, with no revealing of an acyclic or cyclic mechanism prior to isomerisation, has been given for hydrothermal conditions.[9] Glucose isomerisation to fructose with preservation of the closed ring structure is supported by Car–Parrinello molecular dynamics metadynamic simulations (CPMD-MTD) in the gas phase.[10] However gas phase simulation can drastically change when water solvent molecules are incorporated in the model.[24] Glucose acyclic isomerisation to fructose is supported by quantum chemical calculations with density functional theory in water.[8] The recently proposed direct cyclic mechanism for the formation of HMF from glucose with no fructose intermediate is supported by CPMD-MTD simulations in water,[10] but experimental evidence is still lacking.

Another cyclic mechanism bypassing fructose as indicated by the dotted lines in Figure 5 may also be hypothesised. This possibility emerges because the same tertiary fructose carbocation is

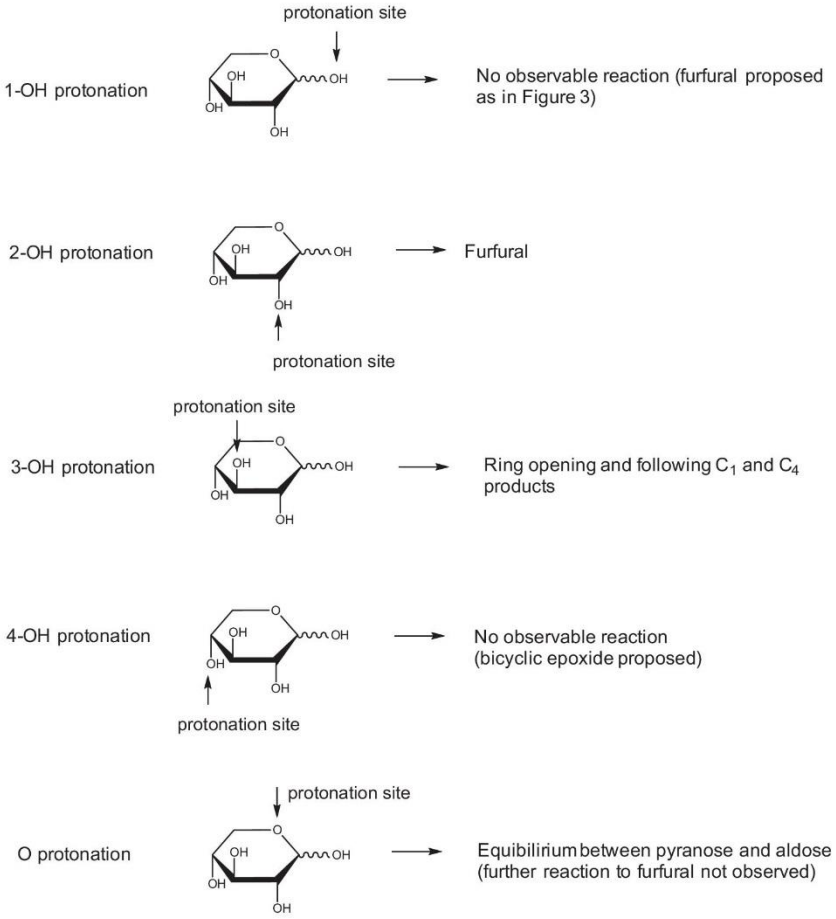


Figure 4. Xylose: Site of protonation and subsequent degradation products determined by ab initio molecular dynamic simulations in the gas phase and quantum mechanics modelling with no solvent water. Summarised from Refs. 24,25. Note that site of protonation and the subsequent reaction mechanism can be altered by changing the reaction conditions.

formed both prior to and after fructose formation. As outlined for xylose, the protonation site for glucose also determines subsequent degradation products and is dependent on reaction conditions[24] (Fig. 6).

There are some discrepancies in the literature regarding the degradation products subsequent to protonation. For example Yang et al.[8] did not observe any HMF as a result of 2-OH protonation but only levulinic acid suggesting that levulinic acid formation has an alternative path of formation than degradation of HMF. On the other hand Qian[10] suggested HMF as the only product formed from 2-OH protonation, with HMF being formed via the direct cyclic mechanism or via fructose isomerisation as depicted in Figure 6.

3.3. Comparison of xylose and glucose degradation mechanisms: new mechanisms to be considered

Some of the reported degradation mechanisms for xylose and glucose are analogous to each other while other new mechanisms still have to be modelled and tested.

Pyranose O protonation: The acyclic direct mechanisms to respectively furfural (Fig. 3) and HMF (Fig. 5) are analogous. However, glucose additionally follows an acyclic mechanism with isomerisation to fructose (Fig. 5). It can also be hypothesised that xylose could degrade via an analogous mechanism with a furan isomerisation intermediate (xylulose) which further dehydrates to furfural (Fig. 7).

1-OH protonation: xylose degradation to furfural follows a proposed cyclic mechanism resulting from 1-OH protonation (Fig. 3). An analogous 1-OH protonation mechanism for glucose to HMF could be speculated to take place (Fig. 8). An 1-OH protonation of glucose leading to HMF via an alternative mechanism involving an epoxide structure has been considered by,[8] but this was found to be energetically unfavourable.

2-OH protonation: Xylose and glucose follow analogous mechanisms leading to furfural and HMF respectively from the cyclic direct mechanisms (Figs. 3 and 5). As for glucose, xylose could possibly also proceed through a cyclic mechanism through the furan isomerisation intermediate (xylulose) or bypassing it to furfural (Fig. 7).

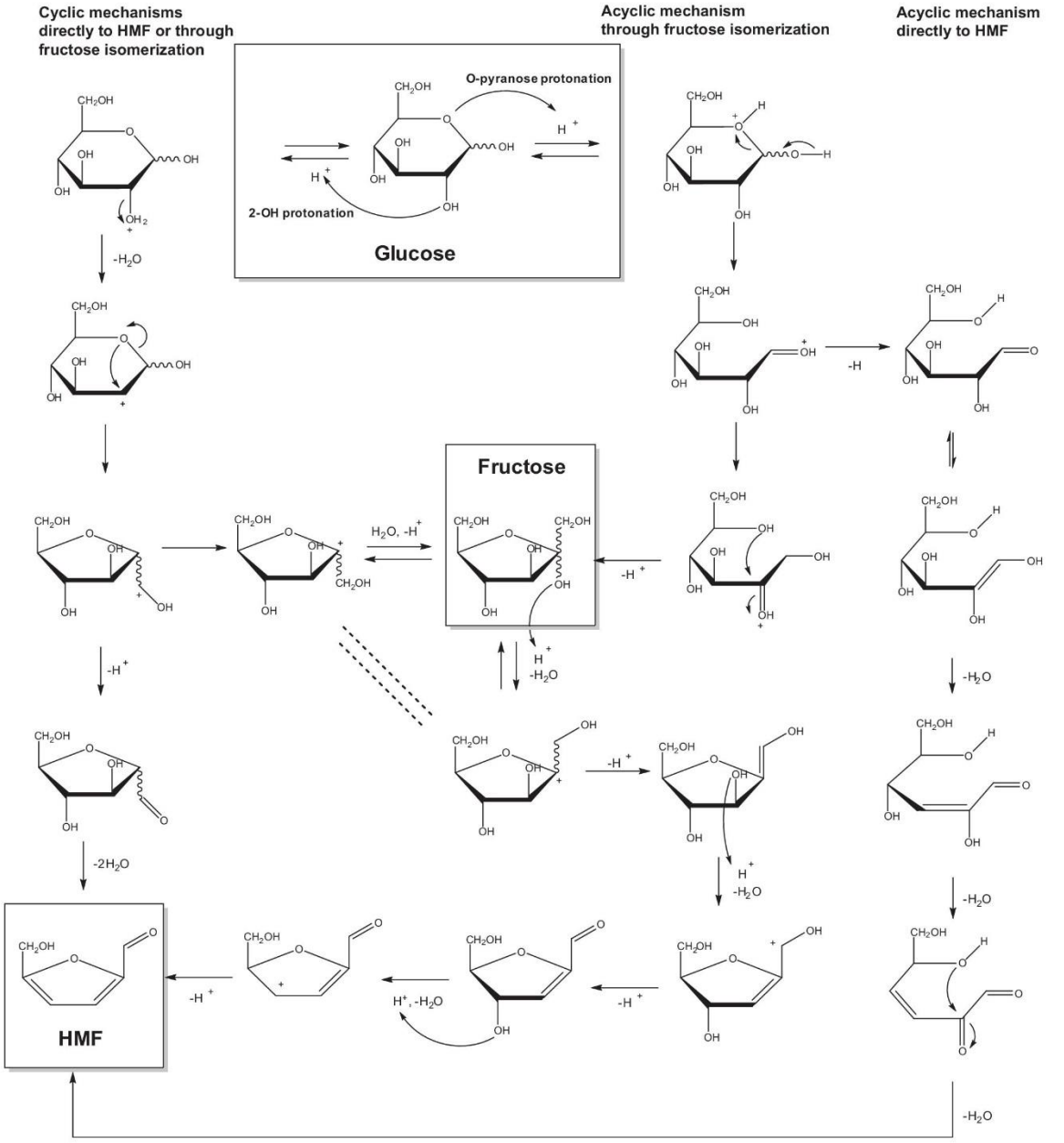


Figure 5. HMF formation from glucose through fructose (acyclic and cyclic isomerisation) and direct cyclic and acyclic non fructose isomerised mechanism from glucose. These are resulting from *O*-pyranose and 2-OH protonation which leads to ring opening and ring closed mechanisms respectively.

In addition to the above considerations regarding the formation of furfural and HMF it is also important to realise that further reactions most likely take place when processing genuine biomass. Detailed analyses of various pretreated biomass liquors have thus revealed a plethora of different compounds, including various carboxylic, aliphatic and aromatic acids, as well as a series of aldehydes and ketones in addition to HMF, furfural, levulinic and formic acid. The exact reaction routes for the formation of these compounds are still not clear.

4. Influence of pretreatment method on degradation products

Based on the knowledge about degradation routes and mechanisms it is relevant to consider any possible relations between pretreatment methodology and reported degradation products for genuine biomass.

There exist multiple studies reporting the profile of degradation products formed from a particular pretreatment regime. Currently, the most studied types of pretreatments include dilute acid

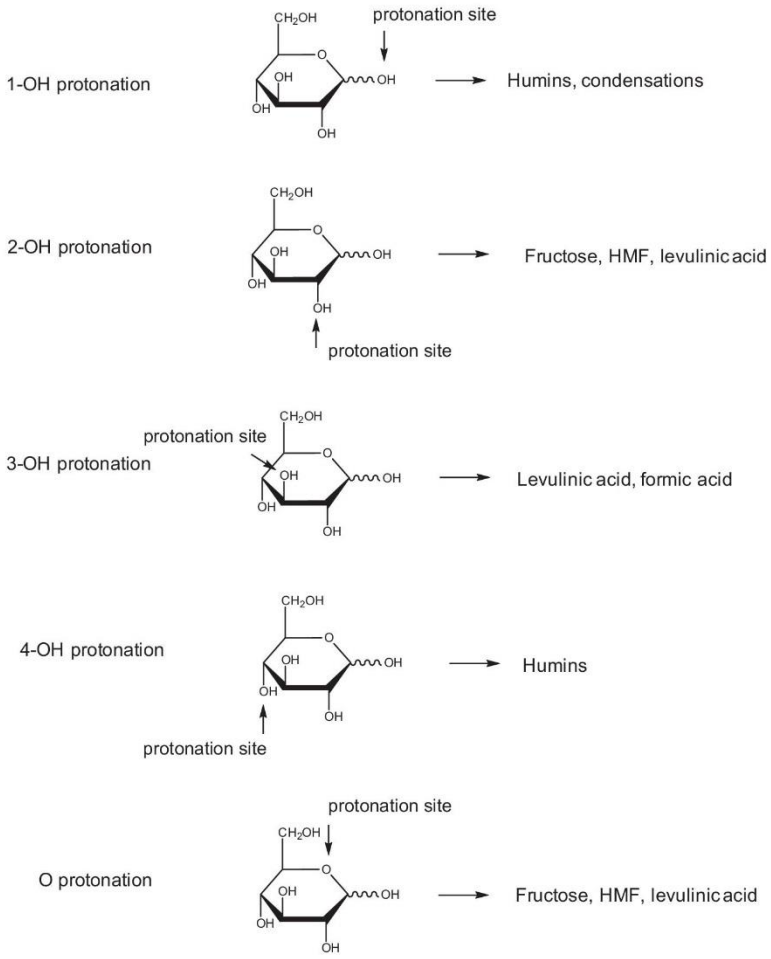


Figure 6. Glucose: Site of protonation and subsequent degradation products determined by ab initio molecular dynamics in water and quantum mechanics modelling with solvent water. Summarised from Refs. 8,10 Note that the site of protonation and the subsequent reaction mechanism can be altered by changing the reaction conditions.

hydrothermal pretreatment (with or without steam explosion), steam or liquid hot water treatments, ammonia fibre expansion (AFEX) and alkaline wet oxidation.[27,28] Unfortunately, even within the same type of pretreatment several different process conditions such as time and temperature are applied, which makes it difficult to unravel correlations between pretreatment conditions and degradation product formation.

It should be noted that in most studies only the liquid fractions are analysed. Hence the possible polymerisation products, such as pseudo lignin and humin compounds, may not have been identified as they may have escaped analysis if they precipitate.

In Table 3 the dilute acid and hydrothermal pretreatment data from corn stover are obtained from the same study, that is, based on the same biomass. In general the formation of degradation products were, not surprisingly, higher in the dilute acid pretreatment. When comparing dilute acid and hydrothermal pretreatments the most distinct difference is the increase in the levels of levulinic, acetic and formic acids together with HMF and furfural with dilute acid pretreatment. The increase in levulinic and formic acids corresponds well with the increase in HMF as described in Figure 6. Similarly the increase in furfural, formic and acetic acid after dilute acid pretreatment is in accord with Figure 4 as well as more pronounced degradation of pentoses and acetylated xylan, that is, the degradation of pentose rich hemicellulose and the products as outlined in Figure 1.

The data from pretreatment of corn stover (Table 3) clearly show that reaction conditions highly influence the degradation product profile.

It is still to be revealed how reaction conditions can be coupled to degradation mechanisms and routes in order to control the degradation product profile. Detailed knowledge about the impact of process conditions is important with respect to cost reduction of biofuel processes, and is also highly relevant for industrial scale synthesis of biomass derived chemicals where the aim is to increase the yield of one or more particular compounds of interest. Many of the biomass derived chemicals are platform chemicals or building blocks for further synthesis and are thus chemicals with annual large production volumes (Table 4). Whereas neither HMF nor furfural was included in the US Department of Energy's original Top 10 list for 'top value added chemicals from biomass',[29] both of these chemicals have recently been included in an updated evaluation of the original Top 10 list.[5] In addition to a number of

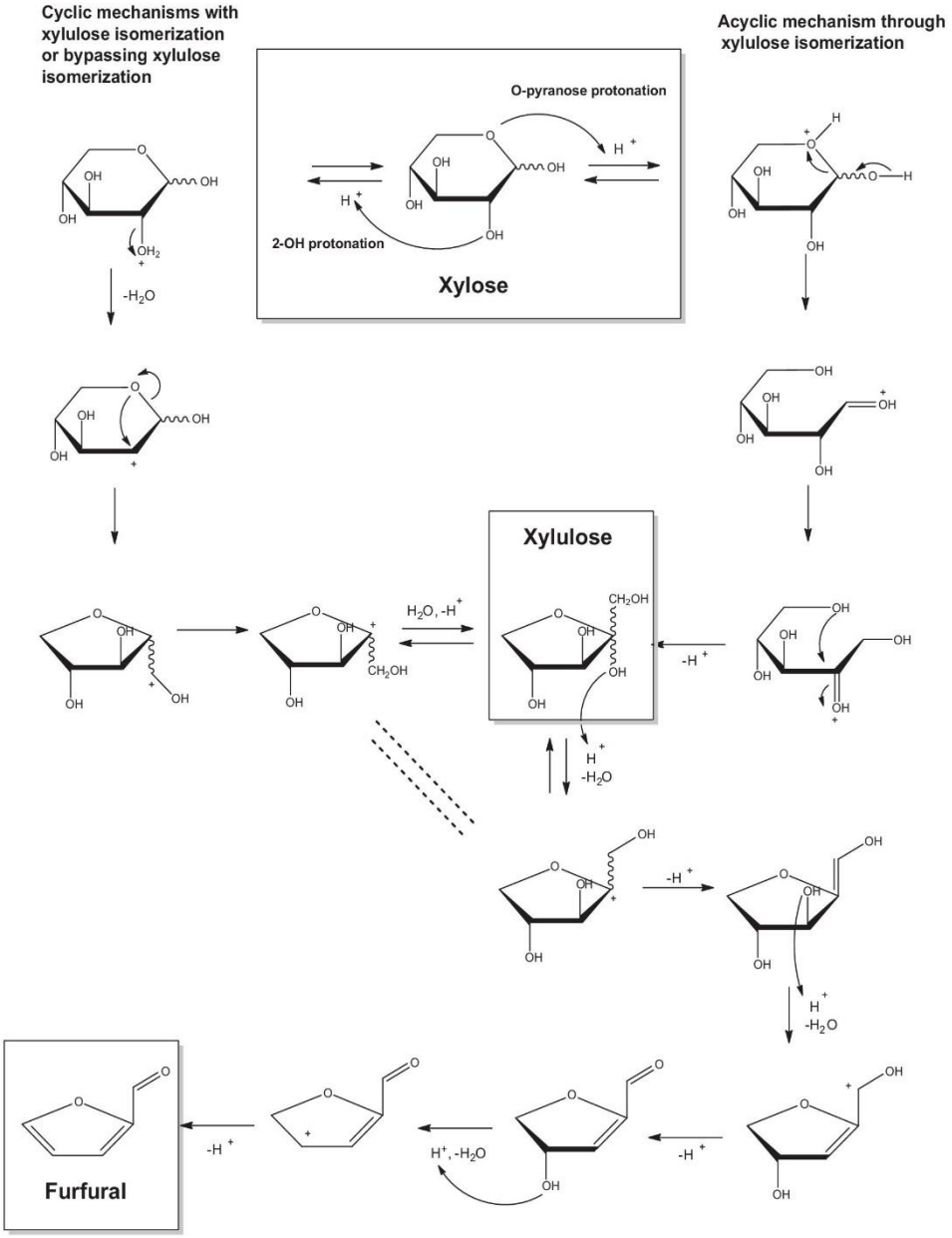


Figure 7. Hypothesised new mechanisms of xylose degradation to furfural.

technology criteria, yield improvement is a particularly important key factor for platform chemicals for the realisation of cost effective processes leading to biomass derived chemicals.

5. Conclusions

Whether the goal is to reduce or increase degradation/synthesis compounds from lignocellulosic biomass, it is important to understand the chemistry of the degradation or synthesis of compounds. Understanding of the chemistry is both important for avoiding the inhibition of any subsequent enzymatic and fermentation processes, and for the potential commercial biorefinery development of as many of the inhibitor compounds that are of commercial interest for industrial scale synthesis of biobased chemicals.

It has long been discussed which are the exact reaction routes and mechanisms that lie behind glucose and xylose degradation during hydrothermal pretreatment of lignocellulosic biomass. In fact the different reaction routes proposed in the literature may all be correct and may even occur side by side. The site of protonation initiating the monosaccharide degradation determines the

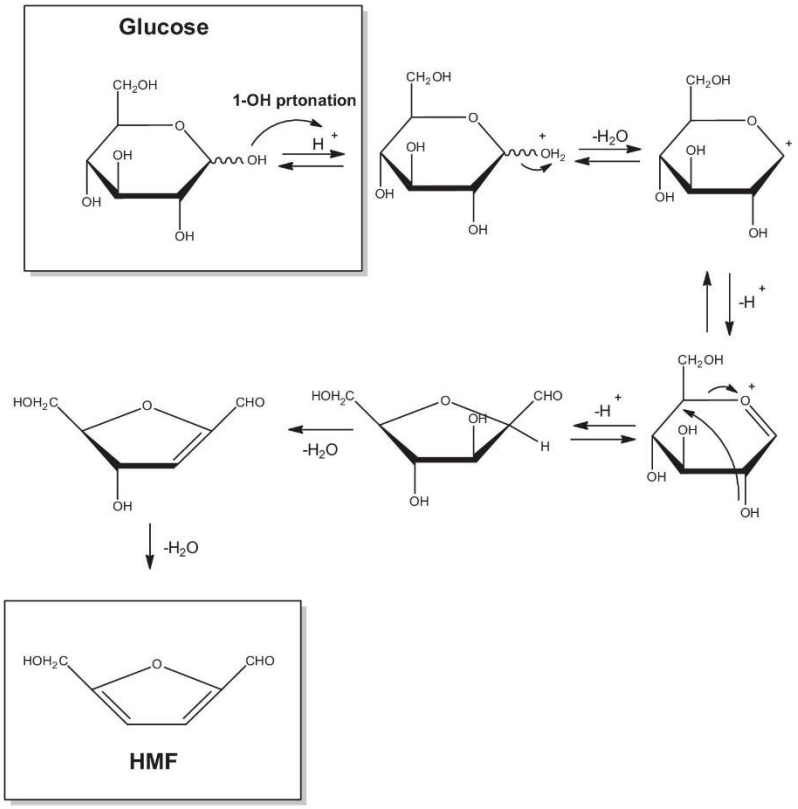


Figure 8. Hypothesised new mechanism of glucose degradation to HMF.

Table 3

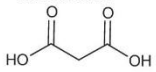
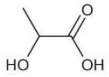
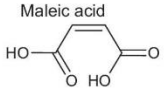
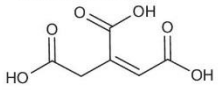
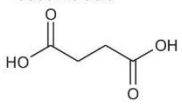
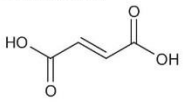
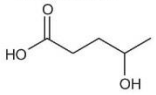
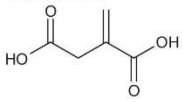
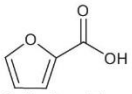
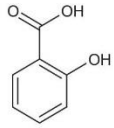
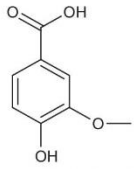
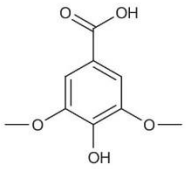
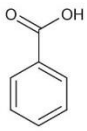
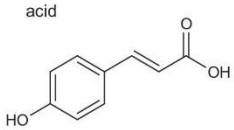
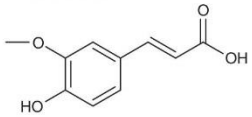
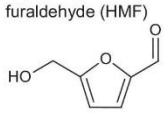
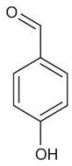
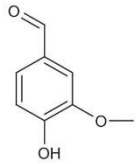
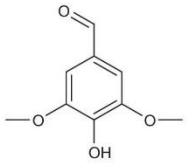
Degradation product	Corn stover	Corn stover
	Dilute acid pretreatment[a] (μM)	Hydrothermal pretreatment[b] (μM)
Aliphatic acids		
Malonic acid 	10	0
Lactic acid 	220	60
Maleic acid 	10	20
Cis-Aconitic acid 	10	0
Succinic acid 	20	20

Table 3 (*continued*)

Degradation product	Corn stover	Corn stover
	Dilute acid pretreatment[a] (μM)	Hydrothermal pretreatment[b] (μM)
Fumaric acid 	30	30
Levulinic acid 	350	0
Itaconic acid 	60	10
Acetic acid 	2830	570
Formic acid 	2610	1190
Aromatic acids		
2-Furoic acid 	20	10
Salisylic acid 	10	10
Vanillic acid 	20	20
Syringic acid 	10	10
Benzoic acid 	10	0

(*continued on next page*)

Table 3 (*continued*)

Degradation product	Corn stover	Corn stover
	Dilute acid pretreatment[a] (μM)	Hydrothermal pretreatment[b] (μM)
4-Hydroxycoumaric acid 	30	70
Ferulic acid 	30	10
Aldehydes and ketones		
5-(hydroxymethyl)-2-furaldehyde (HMF) 	350	20
Furfural 	2290	80
4-Hydroxybenzaldehyde 	30	20
Vanillin 	30	20
Syringaldehyde 	10	10
Total phenolics	210	170

[a] 0.7% (w/w) H_2SO_4, 180 °C, 8 min solids concd 10 g/L.[37]
[b] 180 °C, 8 min, solids concd 10 g/L.[37]

mechanism and degradation route and hence the biomass degradation products. It has been shown by ab initio molecular dynamic simulations and quantum mechanics modelling that the protonation site is depending on solvent water structure surroundings, which in turn depend on the reaction conditions. The key point is thus that degradation product profile depends on reaction conditions and may potentially be controlled by controlling the process parameters. Experimental evidence for different mechanisms for monosaccharide degradation to furans supports that degradation product profiles can be controlled via the tuning of process parameters.

Glucose can be converted to HMF, levulinic acid, formic acid and/or different phenolics. Correspondingly, xylose can follow different reaction mechanisms resulting in the formation of furfural

Table 4
Annual production volumes of potential biomass derived chemicals a,[38] b,[39] c,[40] d[41]

Compound	Annual production (tons)
Acetic acid	6.500.000[b]
Benzoic acid	700.000[d]
Formic acid	415.000[c]
Lactic acid	300.000–400.000[a]
Furfural	225.000[c]
Succinic acid	30.000–50.000[a]
Vanillin	10.000[d]

or C-1 and C-4 compounds. At least four routes for the formation of HMF from glucose and three routes for furfural formation from xylose are possible. By comparing xylose and glucose degradation mechanisms reported in the literature one new mechanism for the degradation of glucose to HMF and two new mechanisms for xylose degradation to furfural have been hypothesised.

It is not only the degradation products themselves that should be taken into account when considering degradation product formation. The reactions between degradation compounds, including polymerisation reactions, may result in new compounds as for example humins and pseudo lignin.

The determination of reaction mechanisms for the formation of degradation products is highly complex. Ab initio molecular dynamic simulations and quantum mechanics modelling are excellent tools in predicting molecular behaviour in the understanding of degradation compound formation and reaction mechanisms. However it is important that the predictions are experimentally evaluated to gain full insight under the actual conditions. Experimental evaluation is thus a crucial prerequisite to obtain the final goal of improved process control with respect to controlling the desired degradation product profile.

When the process outcome can be completely controlled it has wide perspectives. A better understanding of the influence of reaction conditions on mechanisms and routes for the degradation of biomass components during processing forms an important foundation for realising the biobased economy.

Acknowledgement

This work was supported by the Danish National Advanced Technology Foundation via the Technology Platform 'Biomass for the 21st century—B21st'.

References

1. Pedersen, M.; Meyer, A. S. *New Biotechnol.* **2010**, *27*, 739–750.
2. Larsen, J.; Petersen, M. O.; Thirup, L.; Li, H. W.; Iversen, F. K. *Chem. Eng. Technol.* **2008**, *31*, 765–772.
3. Alonso, D. M.; Wettstein, S. G.; Dumesic, J. A. *Green Chem.* **2013**, *15*, 584–595.
4. Gallezot, P. *Chem. Soc. Rev.* **2012**, *41*, 1538–1558.
5. Bozell, J. J.; Petersen, G. R. *Green Chem.* **2010**, *12*, 539–554.
6. Ahmad, T.; Kenne, L.; Olsson, K.; Theander, O. *Carbohydr. Res.* **1995**, *276*, 309–320.
7. Antal, M.; Leesomboon, T.; Mok, W.; Richards, G. *Carbohydr. Res.* **1991**, *217*, 71–85.
8. Yang, G.; Pidko, E. A.; Hensen, E. J. M. *J. Catal.* **2012**, *295*, 122–132.
9. Kimura, H.; Nakahara, M.; Matubayasi, N. *J. Phys. Chem. A* **2011**, *115*, 14013–14021.
10. Qian, X. *Top. Catal.* **2012**, *55*, 218–226.
11. Chen, X.; Shekiro, J.; Franden, M. A.; Wang, W.; Zhang, M.; Kuhn, E.; Johnson, D. K.; Tucker, M. P. *Biotechnol. Biofuels* **2012**, *5*, 8.
12. Popoff, T.; Theander, O. *Acta Chem. Scand. B Org. Chem. Biochem.* **1976**, *30*, 397–402.
13. Forsskahl, I.; Popoff, T.; Theander, O. *Carbohydr. Res.* **1976**, *48*, 13–21.
14. Kumar, R.; Hu, F.; Sannigrahi, P.; Jung, S.; Ragauskas, A. J.; Wyman, C. E. *Biotechnol. Bioeng.* **2013**, *110*, 737–753.
15. Almeida, J. R. M.; Modig, T.; Petersson, A.; Hahn-Hagerdal, B.; Liden, G.; Gorwa-Grauslund, M. F. *J. Chem. Technol. Biotechnol.* **2007**, *82*, 340–349.
16. Del Rio, J. C.; Marques, G.; Rencoret, J.; Martinez, A. T.; Gutierrez, A. *J. Agric. Food Chem.* **2007**, *55*, 5461–5468.
17. Sannigrahi, P.; Kim, D. H.; Jung, S.; Ragauskas, A. *Energy Environ. Sci.* **2011**, *4*, 1306–1310.
18. Hu, F.; Jung, S.; Ragauskas, A. *Bioresour. Technol.* **2012**, *117*, 7–12.
19. Popoff, T.; Theander, O. *Carbohydr. Res.* **1972**, *22*, 135–149.
20. Luijkx, G.; Vanrantwijk, F.; Vanbekkum, H. *Carbohydr. Res.* **1993**, *242*, 131–139.
21. Dee, S. J.; Bell, A. T. *ChemSusChem* **2011**, *4*, 1166–1173.
22. Patil, S. K. R.; Lund, C. R. F. *Energy Fuels* **2011**, *25*, 4745–4755.
23. Oefner, P.; Lanziner, A.; Bonn, G.; Bobleter, O. *Monatsh. Chem.* **1992**, *123*, 547–556.
24. Qian, X.; Nimlos, M.; Davis, M.; Johnson, D.; Himmel, M. *Carbohydr. Res.* **2005**, *340*, 2319–2327.
25. Nimlos, M. R.; Qian, X.; Davis, M.; Himmel, M. E.; Johnson, D. K. *J. Phys. Chem. A* **2006**, *110*, 11824–11838.
26. Jadhav, H.; Pedersen, C. M.; Soiling, T.; Bols, M. *ChemSusChem* **2011**, *4*, 1049–1051.
27. Alvira, P.; Tomas-Pejo, E.; Ballesteros, M.; Negro, M. J. *Bioresour. Technol.* **2010**, *101*, 4851–4861.
28. Larsen, J.; Haven, M. Ø.; Thirup, L. *Biomass Bioenergy* **2012**, *46*, 36–45.
29. Werpy, T.; Petersen, G.; Aden, A.; Bozell, J.; Holladay, J.; White, J.; Manheim, A.; Eliot, D.; Lasure, L.; Jones, S. Top Value Added Chemicals From Biomass. In *Results of Screening for Potential Candidates From Sugars and Synthesis Gas*, Vol. 1, 2004.
30. Panagiotou, G.; Ximenes, E.; Mosier, N. S.; Ladisch, M. R. *Enzyme Microb. Technol.* **2011**, *48*, 408–415.
31. Du, B.; Sharma, L. N.; Becker, C.; Chen, S.; Mowery, R. A.; van Walsum, G. P.; Chambliss, C. K. *Biotechnol. Bioeng.* **2007**, *96*, 250–258.
32. Tejirian, A.; Xu, F. *Enzyme Microb. Technol.* **2011**, *48*, 239–247.
33. Ximenes, E.; Kim, Y.; Mosier, N.; Dien, B.; Ladisch, M. R. *Enzyme Microb. Technol.* **2011**, *48*, 54–60.
34. Takagi, M. *Biotechnol. Bioeng.* **1984**, *26*, 1506–1507.
35. Klinke, H.; Thomsen, A.; Ahring, B. *Appl. Microbiol. Biotechnol.* **2004**, *66*, 10–26.
36. Larsson, S.; Palmqvist, E.; Hahn-Hagerdal, B.; Tengborg, C.; Stenberg, K.; Zacchi, G.; Nilvebrant, N. *Enzyme Microb. Technol.* **1999**, *24*, 151–159.
37. Du, B.; Sharma, L. N.; Becker, C.; Chen, S.; Mowery, R. A.; van Walsum, G. P.; Chambliss, C. K. *Biotechnol. Bioeng.* **2010**, *107*, 430–440.
38. The National Non-Food Crops Centre (NNFCC) http://www.nnfcc.co.uk, 2013-08-14.
39. KEMI Swedish Chemicals Agency http://apps.kemi.se/flodessok/floden/kemamne_eng/attiksyra_eng.htm, 2013-08-14.
40. Hayes, D. J.; Fitzpatrick, S.; Hayes, M. H. B.; Ross, J. R. H. In *Biorefineries-Industrial Processes and Products*; Nonymous, Ed.; Wiley-VCH GmbH, 2005; pp 139–164.
41. International Programme on Chemical Safety (IPCS) Inchem http://www.inchem.org/pages/sids.html, 2013-08-14.

Paper 2

New degradation compounds from lignocellulosic biomass pretreatment: Routes for formation of potent oligophenolic enzyme inhibitors

Helena Rasmussen[a], David Tanner[b], Hanne R. Sørensen[a] and Anne S. Meyer[c*]

[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark

[b] Dept. of Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark.

[c] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Lyngby, Denmark.

*Corresponding author: e-mail: am@kt.dtu.dk; Tel: (+45) 45 25 2800

Abstract

In this study 28 new oligophenol cellulase inhibitors were discovered in wheat straw pretreatment liquors. By consideration of the reaction mechanisms for their formation it is proposed that these oligophenols are formed during hydrothermal biomass pretreatment by pentose self-condensation reactions involving aldol condensations, 1,4 additions to α,β unsaturated carbonyl compounds, 3-keto acid decarboxylations and oxidations. Furthermore, pentose reactions with phenolic lignin components are suggested. The identification of the central role of xylose in the reaction routes for oligophenolic inhibitor formation led to the solution to protect the reactive anomeric center in xylose. It is shown that protection of the anomeric center in *in situ* generated xylose with ethylene glycol monobutyl ether, during pretreatment of wheat straw, reduces the level of oligophenols by 73 %. The results pave the way for implementation of new types of reactions that hinder inhibitor formation in lignocellulosic biomass processing.

Keywords: reaction mechanisms, degradation compounds, hydrothermal pretreatment, oligophenol, lactone, flavonoid, xylooligosaccharide, LC-MS/MS

1. Introduction

Lignocellulosic conversion processes have the potential to provide the future society with environmentally friendly energy and platform chemicals [1-5]. A challenge is to make the biorefinery processes economically competitive and a cost driver is the enzymes that are utilised in the first lignocellulosic saccharification. Unfortunately, in lignocellulosic biomass conversion, the enzyme catalysed reactions are inhibited by compounds that are generated or liberated during the hydrothermal pretreatment of the biomass [6]. Identification of these inhibitory compounds, and notably an understanding of how they are formed, are crucial in order to develop methods to avoid their formation or to remove them.

Compound types that are reported to be especially inhibitory towards cellulases, include xylooligosaccharides [7-10] and several phenolic compounds [11-13]. Xylooligosaccharides are biomass structural elements that are liberated from xylan during pretreatment. Although phenolics compounds found in biomass liquors can stem from lignin [14] , early reports show that various phenolic compounds may also form as degradation products from glucose, xylose and arabinose [15-17] . Recently this formation of phenolics has been confirmed by demonstration of pseudo-lignin generation from model substrates of cellulose (i.e. Avicel), xylan and xylose [18] .

It can be hypothesised that other still unidentified compounds may form during biomass pretreatment and that these compounds can be potent inhibitors as well, since compounds with multiple chemical functionalities are formed during biomass pretreatment [6], and give rich possibilities for various chemical reactions to take place and hence form many new potential inhibitor compounds.

The objective of this work was to reveal the chemical identity of the most potent cellulase inhibitors present in the liquor of pretreated wheat straw and consider their synthesis routes in order to understand the origin of the compounds and hinder their formation.

2. Experimental section

Chemicals and enzymes

Buffer chemicals, solvents, acids and bases were purchased from Sigma Aldrich. Cellic CTec3 (1920 Biomass Hydrolysis Units/g) obtained from Novozymes A/S (Bagsværd, Denmark) was utilised. It is a commercially available cellulase preparation based on the *Trichoderma reesei* complex. Apart from the cellulolytic enzyme base from *T. reesei* it contains at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, and a β-xylosidase [19], the preparation Cellic CTec3 also contains other proprietary hydrolysis-boosting proteins.

Fractionation of pilot plant liquid from hydrothermal pretreatment (LfHP) of wheat straw

Liquid from pilot scale hydrothermal pretreated (LfHP) wheat straw (183 °C, 18 minutes, 40 % (w/w) dry matter) was extracted with 2-butanone or 2-butanol. 170 g LfHP was extracted with 2 x 160 g solvent. Both extractions were left overnight for complete separation. The emulsion in the middle phase was centrifuged (10 min, 10.000 rcf) for complete separation of water, solvent and insolubles. The water phase and

insolubles were freeze dried separately and the organic phase was evaporated at reduced pressure at 50 ºC and freeze dried.

Determination of cellulase inhibition from the fractions: Enzyme assay

The freeze dried fractions were dissolved in 0.1 M acetate buffer (pH 5.1) to dry matter 13.6 % (w/w) and avicel was added to 12 % (w/w). When the pretreated wheat straw fiber was used as substrate, it was added to a dry matter content of 12 % (w/w) corresponding to 6 % (w/w) cellulose. (The pretreated wheat straw fiber was the fiber fraction corresponding to the utilised LfHP). Incubation was at 50 °C, 160 rpm and Cellic CTec3 (1920 Biomass Hydrolysis Units/g) was added to 0.6 % (w/w). All assays were made as duplicates with displaced sampling. Upon sampling (200 μL) the enzyme was inactivated at 99 °C for 5 minutes.

Mini pretreatments: Protection of the anomeric center in xylose

Experiments were carried out in a Parr reactor (Parr Instrument Company, Moline, Illinois, USA) with internal cooling loop, on 200 mL scale. 18 g of wheat straw milled to 1 mm size were suspended in 200 mL liquid to give a final dry matter of 8 % (w/w). Agitation speed was 100 rpm.

In the statistical experimental central composite face design the following parameters were tested: Temperatures: 100, 130 and 160 ºC. Reaction times: 10, 20, 30 minutes. Solution of glycol ethers with: H_2SO_4 concentrations (% w/w of solution): 0.5, 2.5 and 4.5 %. Water equivalents (to xylose content in the wheat straw): 3.5 (corresponds to the authentic water content in wheat straw i.e. no addition of water), 10.7 (4 mL) and 17.9 (8 mL) equivalents.

In addition to the statistical design, the following parameters were tested in a round bottomed flask equipped with a condenser: Temperatures: 80 and 100 ºC. Reaction times: 15, 30 and 60 minutes. H_2SO_4 concentrations (% w/w of solution): 4.5 and 6.5 %. Water equivalents (to xylose content in the wheat straw): 17.9 and 25.1 equivalents.

Mini pretreatments: Spiking experiments with xylose and wheat arabinoxylan

Experiments were carried out in a Parr reactor (instrumentation as described above) at 183 °C, 18 minutes with 18 g of wheat straw milled to 1 mm size suspended in 200 mL deionised water. In one experiment xylose (4.64 g, Merck, New Jersey, USA) was added. In another experiment wheat arabinoxylan (4.18 g, medium viscosity ~ 22 cST, Megazyme, Wicklow, Ireland) was added. The added amounts of xylose and arabinoxylan correspond to the respective amounts of xylose from xylan in 18 g wheat straw and arabinoxylan in 18 g wheat straw. Agitation speed was 100 rpm. The experiments were compared to a control experiment with no spiking with respect to the amount of oligophenolic compounds measured as total UV count from 3.0-6.0 minutes in the HPLC analysis (evaluation of inhibitor level) described below.

2.1 Analytical methods

HPAEC: Glucose analysis

Analysis was performed on a ICS-5000 Dionex ThermoFisher Scientific (ThermoScientific, Sunnyvale, California, USA) analytical system with a PA1 column, 2 x 250 mm (Thermo Scientific). The method was a 2 step isocratic method with an additional final cleaning step with KOH concentrations held constant at 25, 10 and 100 mM for 5, 14 and 7 minutes respectively. The flow rate was 0.25 mL/min.

HPLC: Xylose protection

Analysis was performed on a Dionex (ThermoScientific, Sunnyvale, California, USA) Ultimate 3000 UHPLC with RI detection and a Rezex RHM monosaccharide H+ (8%) column, 7.80 x 300 mm (Phenomenex) with the method according to [20].

HPLC: Evaluation of inhibitor level

Analysis was carried out on a Dionex (ThermoScientific, Sunnyvale, California, USA) Ultimate 3000 UHPLC equipped with a DAD detector and a pentafluorphenyl column 2.1 x 100 mm, particle size 2.6 μm (Phenomenex). The flow was 6.5 mL/min with a gradient from 100 % water (containing 0.025 % TFA) -100 % acetonitrile (containing 0.025 % TFA) over 10 minutes. 100 % acetonitrile (0.025 % TFA) was kept for 2 minutes and changed to 100 % water (0.025 % TFA) over 0.1 minute. The system was equilibrated 3 minutes with 100 % water (0.025 % TFA).

The inhibitor level was evaluated as the total UV count at 250 nm at retention time 3.0-4.9 minutes, which corresponds to the retention time for the compounds in the 2-butanone phase in this method.

LC-MS and LC-MS/MS

The freeze dried fractions were dissolved in MilliQ water (water fraction and freeze dried LfHP) and 25 % (vol/vol) acetonitrile (2-butanone and 2-butanol fractions). LC-MS and LC-MS/MS analyses were performed on a Thermo (ThermoFisherScientific, Waltham, MA, USA) Orbitrap Fusion instrument with electrospray ionisation with spray voltage 3500 in positive ionisation and 2500 in negative ionisation. HCD energy for MS/MS was 25 %.

The LC conditions were 1.0 mL/min with a gradient as above (eluents containing 0.1 % formic acid).

The column was a pentafluorphenyl (pfp) discovery HS F5, L x I.D. 15 cm x 4.6 mm, 5 μm particle size (Supelco).

3. Results and discussion

3.1 Determination of inhibition from the fractions: Enzyme assay

Liquid from hydrothermal pilot scale pretreatment (LfHP) of wheat straw was extracted with 2-butanone and 2-butanol. These solvents were chosen because they are very polar, but can be separated from water and have low boiling points and are thus suitable for fractionating of compounds solubilised LfHP. Furthermore they were chosen because a protic and an aprotic solvent could possibly result in further selectively *via* solubility. The fractions were evaporated (only organic phases), freeze dried and subsequently compared at the same dry matter base for their enzyme inhibitor potency.

LfHP inhibited the enzymatic glucose release from avicel with 70 % after 22 hours of reaction compared to the control (Figure 1, top). The organic fractions, notably the 2-butanone fraction, were more inhibitory than the LfHP itself (Figure 1, top). The water fractions showed a decrease in inhibition compared to LfHP, which corresponds to that some of the most inhibitory compounds had been extracted into the organic phases.

The 2-butanone extracts also showed significantly inhibitory action on enzymatic glucose release from authentic pretreated wheat straw (Figure 1, bottom).

No difference in inhibition between freeze dried and non-freeze dried LfHP was observed (data not shown) indicating that potentially inhibitory, volatile compounds, such as furfural and formic acid, had not escaped evaluation Thus, in agreement with previous data [13] it was affirmed, that volatile compounds, in the present amounts, where furfural was measured to 0.04 g/l and acetic acid to 5.5 g/L do not contribute to the inhibition from LfHP.

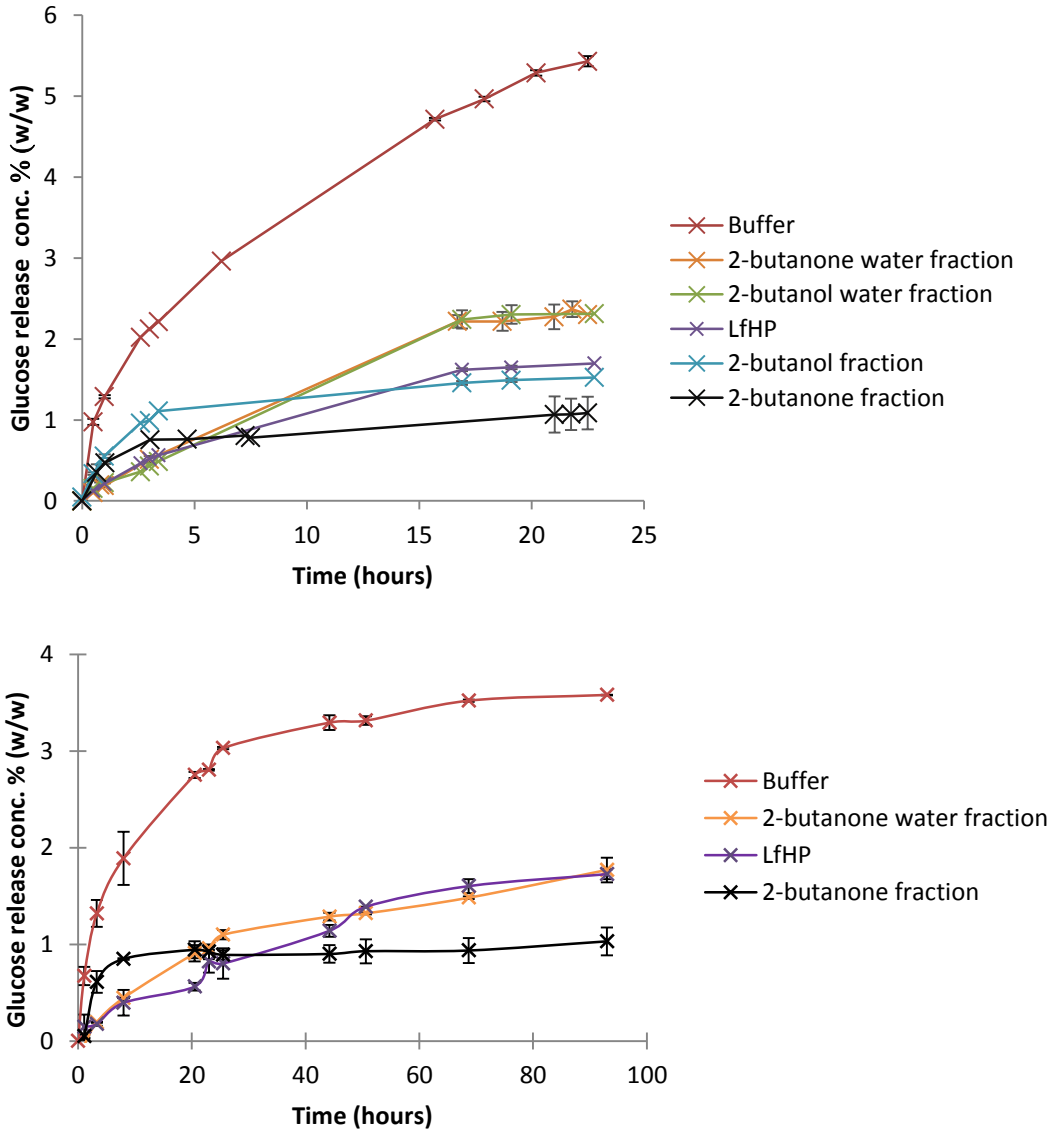


Figure 1. Glucose release during enzymatic hydrolysis of model cellulose substrate (avicel) (top) and authentic pretreated wheat straw (bottom), with compounds added from solvent extractions of liquid from hydrothermal pretreatment (LfHP) of wheat straw. All fractions i.e. compounds from the organic fraction, water fraction or freeze dried LfHP were added to equal concentrations (13.6 % w/w).

3.2 Compound distribution in the fractions

The peaks with retention time from 1 to 8 minutes were distributed differently in the phases after the solvent extractions (Figure 2). Peaks beyond 8 minutes were also observed in blank control samples (data not shown) and thus interpreted as extractables. The compounds with rt from 1 to 3.7 minutes were left in the water phases (Figure 2A and Figure 2B) and the compounds with rt from 5.5 to 8 minutes were extracted

into the organic phases (Figure 2D and Figure 2E) most effectively into the 2-butanone phase (Figure 2E). The 2-butanol phase also contained compounds with retention time 1-3.7 minutes similar to those in the water phases.

The compound distribution in the fractions corresponded well with the enzyme inhibition assay (Figure 1), that showed less inhibition from the 2-butanol fraction than the 2-butanone fraction. Thus, the difference between strong inhibition and relived inhibition is likely due to the compounds with rt 5.5-8 and 1-3.7 minutes respectively. There was an overlap of compounds with rt 3.7-5.5 in the water and organic solvent fractions, indicating that many of the compounds in this region of the chromatogram could be similar in the different fractions.

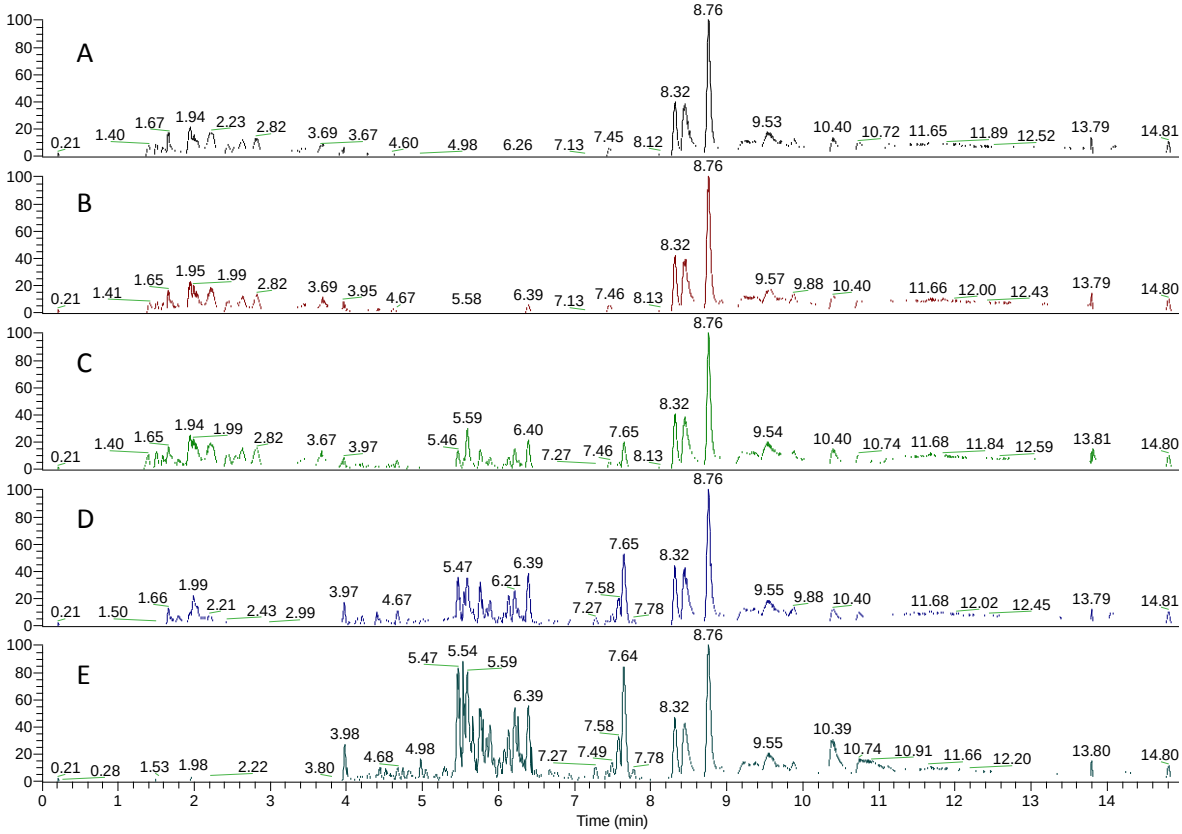


Figure 2. Base peak chromatograms (negative ionisation) of fractions from solvent extractions. A: Water fraction from 2-butanol extraction. B: Water fraction from 2-butanone extraction. C: Non extracted LfHP. D: 2-butanol fraction. E: 2-butanone fraction. The fractions exhibit increasing inhibition from top to bottom

3.3 Identification of compounds with LC-MS/MS

2-butanone fraction

More than 100 different compounds were eluting in the region 5.5-8 minutes in the 2-butanone fraction (Figure 2E). Thus it was found that LC-MS/MS was more suitable for analytical evaluation than NMR, and the approach was to structurally elucidate as many compounds as possible, to gain information of general structures and compound types. 30 compounds had sufficient intensity and quality of fragmentation.

The compounds eluting in the region 5.5-8 minutes in the 2-butanone fraction (Figure 2E) had a high degree of double bond equivalents (DBE) compared to their masses (S1 Table 1) i.e. they were quite condensed structures. Common mass losses that gave rise to fragments (S1 Table 1, S1 Table 3) were 18, 44 and 15, which corresponded to H_2O from elimination of hydroxyl, CO_2 from carboxylic acids and CH3• radical from cleavage of phenol methoxy groups [21] respectively (S1 Table 3). Lactones with hydroxyl substituents were strongly suggested by i) multiple sequential neutral mass losses involving $[M\text{-}H^+\text{-}44]^-$, $[M\text{-}H^+\text{-}18]^-$ and in some cases also $[M\text{-}H^+\text{-}28]^-$ CO loss, as well as by ii) the structures being so condensed, that they cannot contain carboxylic acids and hydroxyl groups to explain the multiple fragmentation pattern. Compounds with fused ring systems were strongly suggested by minor fragmentation, again consistent with a condensed structure. Additional fragmentation (S1 Table 1, S1 Table 3) arose from straightforward heterobond cleavage and cleavage of bonds between non-fused ring systems.

The compounds in the 2-butanone fraction were thus mainly oligophenolic compounds, including lactone and flavonoid like structures, flavonoids in addition to monophenolic compounds (Figure 3). Out of the 30 proposed structures, 26 are new (search in Chemical Abstract Service via SciFinder, similarity search down to 70 % based on molecular formula). Three have been reported earlier (cumaroyl substituted pentose (**8**), feruloyl substituted pentose (**12**) and the flavonoid **15**). A regioisomer (**9**) of a previously described flavonoid structure was also found, but it has not been reported previously from wheat straw. Apart from the structures shown in Figure 3, 10 already known monophenolic compounds, including methoxylated phenols, ferulic and coumaric acids, and several of their regioisomers were also found (data not shown). In relation to cellulase inhibition, the data strongly indicate that the higher inhibition of the 2-butanone fraction is contributed by the new oligophenolic compounds that we propose are a group of "super inhibitory" compounds in LfHP. Many of the proposed structures in Figure 3 are Michael acceptors and they are thus readily susceptible for nucleophilic attack from nucleophiles in amino acids in proteins. This may explain why they are potent enzyme inhibitors.

Due to the presence of the many different compounds, several enzyme activities in the catalytic cascade of enzymatic cellulose conversion are expected to be affected.

The super inhibitory action of oligophenols agree with earlier studies [8], reporting that oligophenols, such as tannic acid and oligomeric proanthocyanidin isolated from a grape seed extract, are more potent cellulase inhibitors than monophenolic compounds.

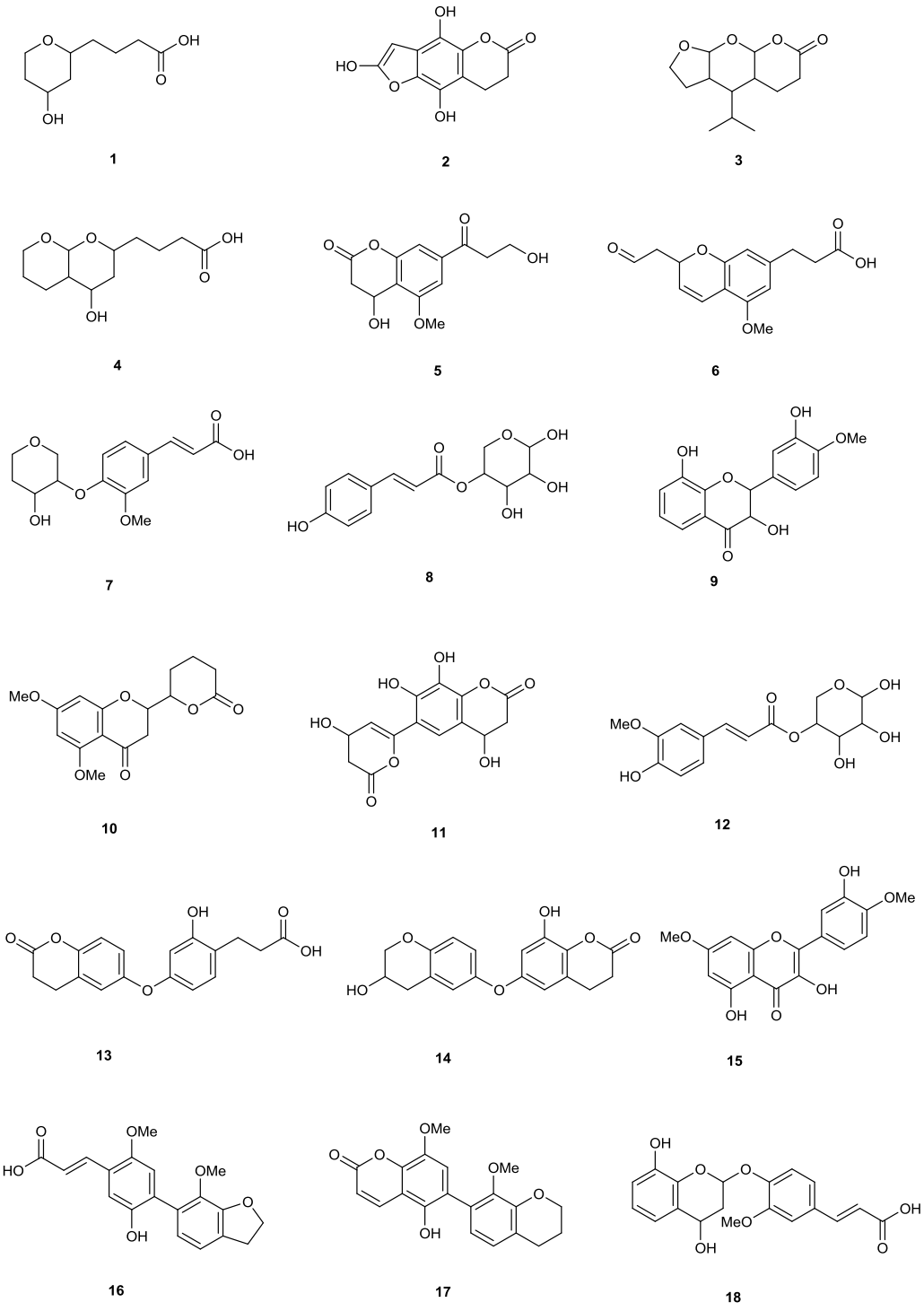


Figure 3. Proposed structures from LC-MS/MS. Note that ring substitution positions are given for clarity, but regioisomerism was not determined *(Figure continues).*

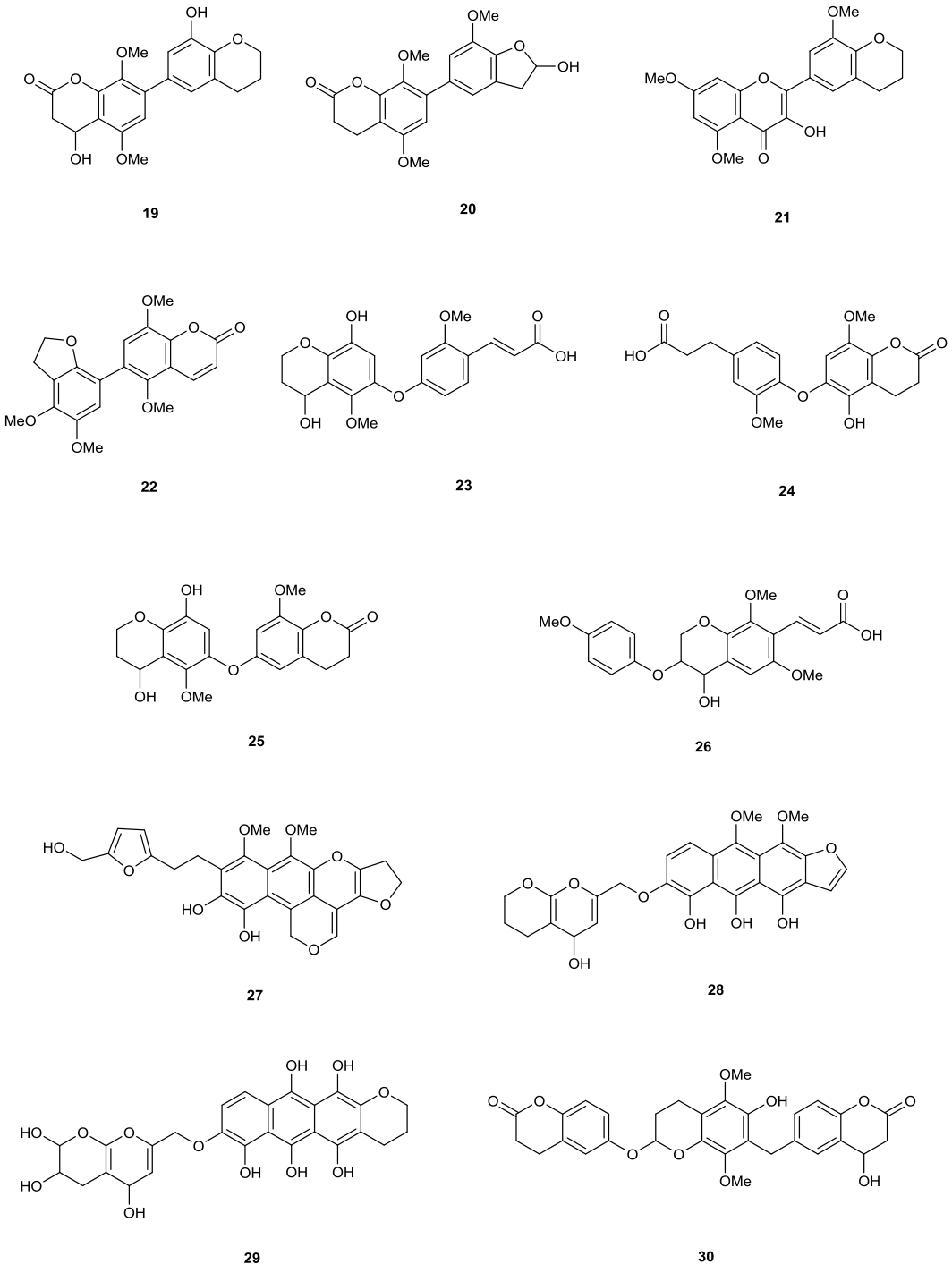


Figure 3 *(continued)*. Proposed structures from LC-MS/MS. Note that ring substitution positions are given for clarity, but regioisomerism was not determined.

Water fraction from 2-butanone extraction

The compounds eluting in the region 1-4 minutes in the water fraction (Figure 2B) were identified as xylooligosaccharides (S1 Table 2), some possibly with arabinose substitution as xylose and arabinose substitution cannot be distinguished with MS. They were identified from their accurate mass and fragmentation pattern, which were compared to xylooligosaccharide standards. Pentoseoligosaccharides up

to DP6 with 0-3 acetyl substituents were identified with MS2, but in the MS1 spectra pentoseoligosaccharides up to DP 12 were also seen - still with only 1-3 acetyl substituents (data not shown). The presence of acetylated xylooligosaccharides in LfHP is in accordance with previously reported data [7]. Feruloyl substituted pentoseoligosaccharides were also found in the water phase as well as the 2-butanone phase *vide infra*.

Other compounds distributed across the 2-butanone and water phase fractions

A closer assessment of the compounds with retention time from 3.7-5.5 minutes, where the 2-butanone fraction and its water fraction had an overlap in the base peak chromatograms (Figure 2), indicated that the majority was feruloyl and/or acetyl substituted pentose oligomers (S1 Table 4). The feruloyl substituted compounds were thus not completely separated in the extraction. From the accurate masses there was a clear tendency, that the 2-butanone fraction contained more of the shorter feruloyl substituted pentoseoligosaccharides, while the water fraction contained more of the longer feruloyl substituted pentoseoligosaccharides (S1 Table 4). In a corresponding manner mono coumaroylated pentose oligomers were found in the overlap region with retention time from 3.7-5.5 (S1 Table 4).

The finding of mono feruloylated pentoseoligomers and feruloylated and acetylated pentoseoligomers (of DP 1-9) agreed with data reported earlier [22] that thermochemically (0.22 w/w H_2SO_4) pretreated corn fiber contains feruloylated xylooligomer analogues with large side chains, ethyl glycosides as well as acetyl substituents.

The MS analysis in positive ionisation mode also revealed the presence of compounds containing nitrogen in both the water fraction and the 2-butanone fraction. The vast majority of these compounds were equal in accurate mass, retention time and had approximately equal intensity and were thus likely not decisive for enzyme inhibition. An initial analysis did show one compound with accurate mass corresponding to acetyl azepinone. This compound was reported previously [23] to be a Maillard reaction product formed in acidic glucose/glycine rich solutions.

3.4 Origin of the identified compounds - reaction mechanisms

Consideration of the possible reaction mechanisms involved in the formation of the identified oligophenols indicates that the oligophenols arise from a wide array of reactions and reactants. Some of the compounds appear to arise from pentose self-condensation reactions alone (Figure 4, Figure 5A), while other compounds are most likely reaction products from pentose reactions with lignin degradation compounds (Figure 5B and Figure 5C) or result from additional routes involving only lignin degradation compounds (Figure 5D). These considerations were confirmed with spiking experiments with xylose and wheat arabinoxylan in pretreatment of wheat straw (Section 2) which increased the amount of oligophenolic compounds with 70 % and 21 % respectively.

Reactions involving only pentoses

The reaction mechanism for formation of **11**, as an example (Figure 4), is proposed to be initiated with an acid catalysed aldol condensation between two pentoses resulting in compound **31**. Xylose was abundant in

the LfHP (data not shown) and is therefore likely to be involved in the reaction (arabinose was present in less amounts, but may also be involved). In the suggested reaction, the aldol condensation is followed by further dehydration and cyclisation, resulting in the aldehyde substituted oxane **32**. The aldehyde is oxidised to the carboxylic acid by molecular oxygen, present during the reactions, presumably catalysed by trace metals present. The oxane hemiacetal is in equilibrium with its open chain form **33**, where the enol function is proposed to tautomerise to its keto form, resulting in the 3-keto acid **34**, that readily undergoes decarboxylation under acidic conditions. A dehydration gives a terminal carbonyl group, that is subject to nucleophilic attack from the enol, that acts as a carbon nucleophile, resulting in the ketone substituted cyclic compound **35**. The ketone is proposed to tautomerise to its enol form, resulting in the possibility of a new ring closure to the bicyclic hemiacetal **36**. Compound **36** is suggested to undergo further dehydration and protonation and subsequently undertake nucleophilic attack on a third pentose molecule. A cyclisation of the pentose moiety leads to a tricyclic compound, that further dehydrates and undergoes an electrocyclic ring opening. Nucleophilic attack from water facilitates ring closure to compound **37**, which undergoes oxidation to the final product **11** due to atmospheric oxygen present during pretreatment. Previously reported data [24], show that phenols readily undergo oxidation under similar reaction conditions.

Many variations of the reaction mechanism described for **11,** as an example, are possible. For example the first cyclisation of compound **31**, will lead to a six membered ring with a different substituent pattern, if the nucleophilic attack takes place as a 1,4 addition to an α,β unsaturated carbonyl compound (Figure 5A). In this case the molecule will lack oxygen in the 2-position. Furthermore, a five membered ring i.e. an oxolane can be formed by nucleophile attack from the appropriate hydroxyl group (Figure 5A). Variations of the mechanisms in the proposed reaction schemes and reaction with other nucleophiles as for example lignin degradation compounds (Figure 5B and Figure 5C), explain the many different reaction products formed.

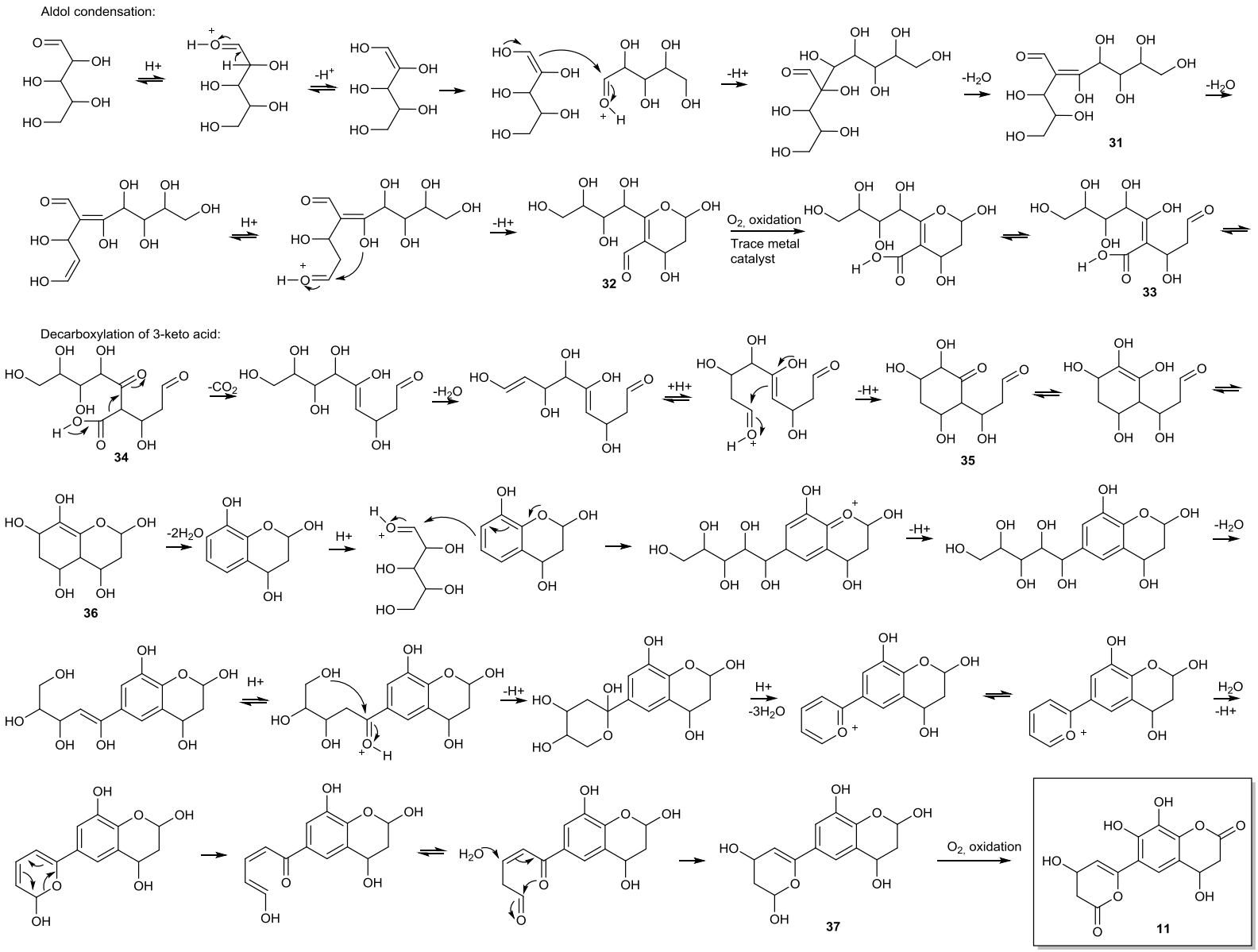


Figure 4. Proposed reaction mechanisms involving only pentose self-condensation reactions.

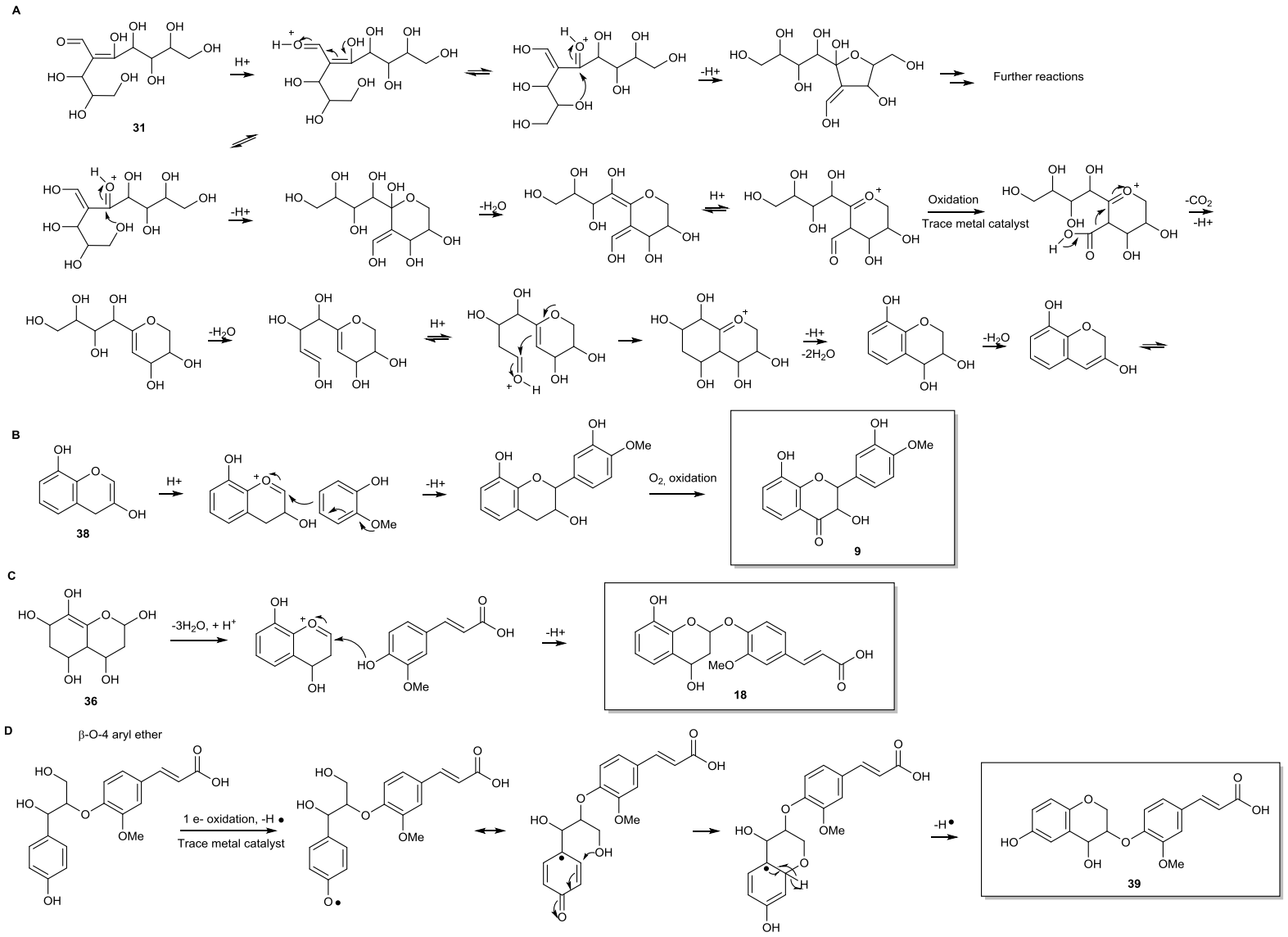


Figure 5. Examples of reactions leading to other of the discovered compounds. A) Variations in pentose self-condensation reactions. B) Reaction with methoxylated phenol resulting in methoxylated compounds and C) Reaction with ferulic acid resulting in the common (methoxyphenyl)prop-2-enoic acid motif. D) The common β-O-4 aryl ether in lignin - here the aryl is ferulic acid.

Reactions involving pentoses and lignin degradation compounds

In addition to pentose self-condensation reactions, reactions involving other compounds in the reaction mixture such as methoxylated phenols, ferulic and coumaric acids arising from lignin degradation may take place (Figure 5B and Figure 5C). Methoxylated phenols explain the common methoxylations as exemplified with **9** (Figure 5B). It is worth noting that flavonoids can actually be formed as a reaction product during pretreatment.

Another common structural repeater was (methoxyphenyl)prop-2-enoic acid, which can be explained by reaction of ferulic acid with pentoses (Figure 5C) as exemplified with **18**. The ferulic acid moiety can in turn give rise to the corresponding bicyclic lactone by intramolecular esterification when a hydroxyl substituent is in *ortho* position. The lactone in the bicyclic moiety can be further dehydrated and/or react with other components in the reaction mixture.

Furthermore non methoxylated (phenyl)prop-2-enoic acid moieties are also proposed to be present and can, apart from reactions involving coumaric acid from lignin degradation, be explained by acid catalysed ring opening of lactones origination from pentose reactions alone (Figure 4).

Another possible isomer of compound **18** was found. Compound **39** can be explained by lignin degradation, where a β-O-aryl ether, which was also found in the mass spectra, is proposed to undergo a one electron oxidation catalysed by trace metals as depicted in Figure 5D. A radical mechanism is involved in the ring closure resulting in the bicyclic compound with a ferulic acid substituent.

3.5 Protection of pentoses at the anomeric position

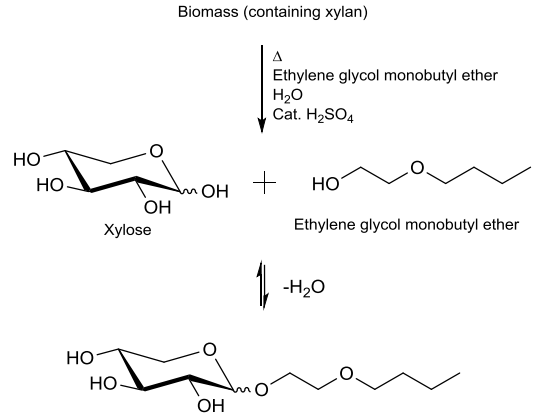
The above findings make it very clear that pentoses are heavily involved in formation of the identified inhibitors. Many of the new oligophenol compounds are possibly also precursors to pseudo lignin. This proposition is supported by the findings from [25] where pretreated holocellulose, isolated from bamboo, was found to generate pseudolignin which was more rich in alicyclic and hydroxyl substituted structures, than reference lignin. In addition the pseudolignin went from being rich in aliphatic structures, to being rich in aromatic structures, with increasing pretreatment time. Furthermore [18] have shown that pseudo lignin is formed from carbohydrates and that especially xylan and xylose are prone to undergo degradation, even at low severities, and precipitate as spheres of pseudo lignin on cellulose surfaces.

The degradation of pentoses is a double negative, because pentoses are now also becoming desired products in biorefineries. The pentoses are thus both lost, and at the same time unwanted products as inhibitors and/or pseudolignin are formed.

On this basis we speculated, that protection of the anomeric center could reduce the level of pentose degradation, even with no protection of the other hydroxyl groups. Anomeric protection could be possible in an industrial process of biomass pretreatment with a Fischer-type glycosylation with glycol ethers as protection groups. Glycol ethers have the desired high boiling points for biomass pretreatment and are water miscible, which is also important, because some water has to be present in order to hydrolyse the pentose oligosaccharides in the biomass to monomers, but water may also obstruct the protection reaction. An acid catalyst must be present in order for the protection reaction to take place, but the presence of acid will only additionally facilitate structural breakage of the biomass, which is the purpose of the biomass pretreatment.

Ethylene glycol monobutyl ether (Figure 6) was tested in a pretreatment of wheat straw in the presence of water. It showed good results with respect to protection of the anomeric center and in reduction in the level of degradation compounds.

The optimal parameters for the pretreatment with anomeric protection results from an interplay of several factors in the pretreatment reaction mixture. 1) Disruption of xylan in the biomass to xylooligosaccharides. 2) Hydrolysis of xylooligosaccharides to xylose. 3) Reaction of xylose with the glycol ether to both the α and β anomer of the xyloside. 4) Possibly also reaction at the reducing ends of xylooligosaccharides with the glycol ether to form both anomers, followed by hydrolysis/cleavage of a protected xylose from the particular xylooligomer. As such the parameters were optimised toward the maximum amount of protected xylose (sum of both anomers evaluated from HPLC, Supplementary Information, Figure 1), rather than the actual yield, based on the strategy that optimal protection would also result in minimum oligophenolic inhibitor formation. A statistical experimental design was conducted in order to identify the optimal parameters for the protection reaction. Protection of the anomeric center (100 °C, 30 minutes, 18 equivalents of water (to xylose), 4.5 % H_2SO_4 (w/w of solution)) reduced the level of degradation compounds by 73 % compared to the original pretreatment method investigated in this paper (183 °C, 18 minutes, 100 % water) (Figure 6). The control experiment to the ethylene glycol monobutyl ether pretreatment, with no glycol ether present, only reduced the level of degradation compounds by 54 %. These are promising results with respect to protection of xylose to hinder xylose degradation to the group of degradation compounds/inhibitors identified in this paper. However, the deprotection of ethylene glycol monobutyl ether protected xylose, by addition of water, as well as the separation of ethylene glycol monobutyl ether from the aqueous reaction mixture need further optimisation. Only after a full technology development of the separation can the xylose recovery and options for ethylene glycol monobutyl ether recycling be fully quantitatively evaluated, but quantitative estimates are given in the next section.



Pretreatment	Degradation compound/inhibitor reduction compared to original pretreatment (%)
Original: 183 °C, 18 minutes, 100 % water	0
Ethylene glycol monobutyl ether: 100 °C, 30 minutes, 18 equivalents of water (to xylose), 4.5 % H_2SO_4 (w/w of solution)	73
Control: 100 °C, 30 minutes, 95.5 % water, 4.5 % H_2SO_4 (w/w of solution)	54

Figure 6. Protection of the anomeric center in *in situ* generated xylose from biomass with ethylene glycol monobutyl ether and reduction in the level of degradation compounds/inhibitors during a pretreatment of wheat straw.

3.6 Quantitative considerations and perspectives

The current pilot plant biomass pretreatment (183 °C, 18 minutes, 100 % water) has been optimised towards minimum pentose degradation and minimum enzyme inhibitor formation, whilst at the same time aiming at enhancing the cellulose degradability. As such the oligophenolic enzyme inhibitor compounds in the 2-butanone fraction constituted 9 % (w/w) of the total weight of compounds in the 2-butanone and water fractions (the mass balance of the 2-butanone extraction was 94 %). However, the trade-off with optimisation towards minimum pentose degradation and minimum enzyme inhibitor formation is that cellulose digestibility for the enzymes is not optimal, due the mild pretreatment conditions. For the pretreatment with anomeric protection, calculations based on the reduced level of inhibitors of 73 % determined above and an estimated increase in cellulose digestibility implies an increase in glucose yield of approximately 15 % after the enzymatic hydrolysis. The pretreatment with anomeric protection can be increased further towards cellulose digestibility without the negative effect of pentose degradation to oligophenolic inhibitor compounds due to the protected pentose. Another, at least as important consideration is the loss of xylose, which for example is utilised in mixed fermentation of xylose and glucose to ethanol in biorefineries. A quantitative scenario as follows may be realistic when considering that in the current pilot plant pretreatment process, approximately 25 % of the xylose is lost due to degradation to inhibitor compounds during pretreatment. Calculation based on the reduced level of inhibitor compounds in pretreatment with anomeric protection, implies a xylose recovery of up to 93 %. Thus approximately 20 % more xylose can be deployed in a mixed glucose-xylose fermentation to ethanol which adds up to a total increase in ethanol from both xylose and glucose

fermentation to 30 %. Even if only half of these estimated levels are attained in practice, the gains would be significant.

These calculations illustrates the concept of pretreatment with anomeric protection, which is illustrated in Figure 7.

Pretreatment outcome	Hydrothermal pretreatment	Pretreatment with anomeric protection
Cellulose digestibility for the enzymes	Low	High
Inhibitor level	High	Low
Xylose degradation	High	Low

Figure 7. Concept of pretreatment with anomeric protection compared to the current pilot plant hydrothermal pretreatment (please refer to Figure 6 for pretreatment parameters).

4. Conclusions

The formation of inhibitors during pretreatment of lignocellulosic feedstocks is a persistent problem, and notably the compounds that retard enzymatic cellulose conversion represent an obstacle for achieving optimal enzymatic productivity and high glucose yields.

A solvent extraction approach was employed for liquid separation of compounds of different polarity from pilot scale hydrothermal pretreated wheat straw. The compounds extracted into 2-butanone were found to exert particularly profound inhibition of cellulolytic glucose release on both pure cellulose and pretreated wheat straw fibers, but also the compounds left in the aqueous phase exerted profound inhibition. The inhibitors were identified with LC-MS/MS as oligophenols and xylooligosaccharides respectively. Although the xylooligosaccharide were more abundant, the oligophenols were significantly more potent cellulase inhibitors when compared on a dry matter basis.

In total, 26 new inhibitory oligophenol compounds were identified from hydrothermally pretreated wheat straw liquor and their reaction mechanisms for formation were considered. The most important conclusion from this work was that reactions arising from xylose (or pentoses in general) are heavily involved in formation of the new compounds. The solution to avoid degradation of pentoses to form inhibitors is to protect the reactive anomeric center. The amount of degradation compounds, identified in this study, was reduced with 73 % by pretreatment with ethylene glycol monobutyl ether, compared to the original pretreatment. The results obtained not only provided new information of the compounds present in pretreated wheat straw biomass liquor, but pave the way for implementation of new types of reactions that prevent inhibitor formation in biomass processing.

Acknowledgement

This work was supported by the Danish National Advanced Technology Foundation *via* the Technology Platform “Biomass for the 21st century – B21st”.

REFERENCES

1. M. J. Climent, A. Corma and S. Iborra. *Green Chem.*, 2014, **16**, 516–547.
2. J. Han, S. M. Sen, D. M. Alonso, J. A. Dumesic and C. T. Maravelias. *Green Chem.*, 2014, **16**, 653–661.
3. J. S. Luterbacher, J. M. Rand, D. M. Alonso, J. Han, J. T. Youngquist, C. T. Maravelias, B. F. Pfleger and J. A. Dumesic. *Science*, 2014, **343**, 277-280.
4. T. D. Matson, K. Barta, A. V. Iretskii and P. C. Ford,. *J. Am. Chem. Soc.*, 2011, **133**, 14090–14097.
5. R. Rinaldi. *Angew. Chem. Int. Ed.*, 2014, **53**, 8559–8560.
6. H. Rasmussen, H.R. Sørensen and A.S. Meyer. *Carbohydr. Res.*, 2014, **385**, 45-57.
7. R. Kont, M. Kurasin, H. Teugjas and P. Väljemäe. *Biotechnol. Biofuels*., 2013, **6**, 135.
8. J. Zhang and L. Viikari. *Bioresour. Technol*., 2012, **117**, 286–291.
9. M. J. Baumann, K. Borch and P. Westh. *Biotechnol. Biofuels*, 2011, **4**, 45.
10. Q. Qing, B. Yang, and C. E. Wyman, C. E. *Bioresour. Technol.,* 2010, **101**, 9624–9630.
11. A. Tejirian and F. Xu. *Enzyme Microb. Technol.*, 2011*,* **48**, 239-247.
12. S. I. Mhlongo, R. d. Haan, M. Viljoen-Blooma and W. H. van Zyla. *Enzyme Microb. Technol.*, 2015, **81**, 16–22.
13. Y. Kim, E. Ximenes, N. S. Mosier and M. R. Ladisch. *Enzyme Microb. Technol.*, 2011, **48**, 408–415.
14. V. D. Mitchell, C. M. Taylor and S. Bauer. *Bioenerg. Res.*, 2014, **7**, 654–669.
15. I. Forsskåhl, T. Popoff and O.Theander. *Carbohydr. Res*., 1976, **48**, 13-21.
16. T. Popoff and O. Theander. *Carbohydr. Res.*, 1972, **22**, 135-149.
17. T. Popoff and O. Theander. *Acta Chem. Scand., Ser. B*, 1976, **30**, 397-402.
18. R. Kumar, F. Hu, P. Sannigrahi, S. Jung, A. J. Ragauskas and C.E. Wyman. *Biotechnol. Bioeng.*, 2013, **110**, 737-753.
19. L. Rosgaard, S. Pedersen, J. Langston, D. Akerhielm, J.R. Cherry and A.S. Meyer. *Biotechnol. Prog.,* 2007, **23**, 1270–1276.
20. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. 2006, NREL - Biomass Program.
21. F. Sánchez-Rabaneda, O. Jáuregui; I. Casals, C. Andrés-Lacueva, M. Izquierdo-Pulido and R. M. Lamuela-Raventós. *J. Mass Spectrom.*, 2003, **38**, 35-42.
22. M. M. Appeldoorn, P. de Waard, M. A. Kabel, H. Gruppen and H. A. Schols. *Carbohydr. Res.*, 2013, **381**, 33-42.
23. H. Wang, H. Qian and W. Yao. *Food Chem.*, 2011, **128**, 573-584.
24. H. R. Devlin and I J. Harris. *Ind. Eng. Chem. Fundam.*, 1984, **23**, 387-392.
25. X. Ma, X. Yang, X. Zheng, L.Chen, L. Huang, S. Cao and H. Akinosho. *Cellulose*, 2015, **22**, 1687–1696.

Supplementary Information

New degradation compounds from lignocellulosic biomass pretreatment: Routes for formation of potent oligophenolic enzyme inhibitors

Helena Rasmussen[a], David Tanner[b], Hanne R. Sørensen[a] and Anne S. Meyer[c*]

[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark

[b] Dept. of Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark.

[c] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Lyngby, Denmark.

*Corresponding author: e-mail: am@kt.dtu.dk; Tel: (+45) 45 25 2800

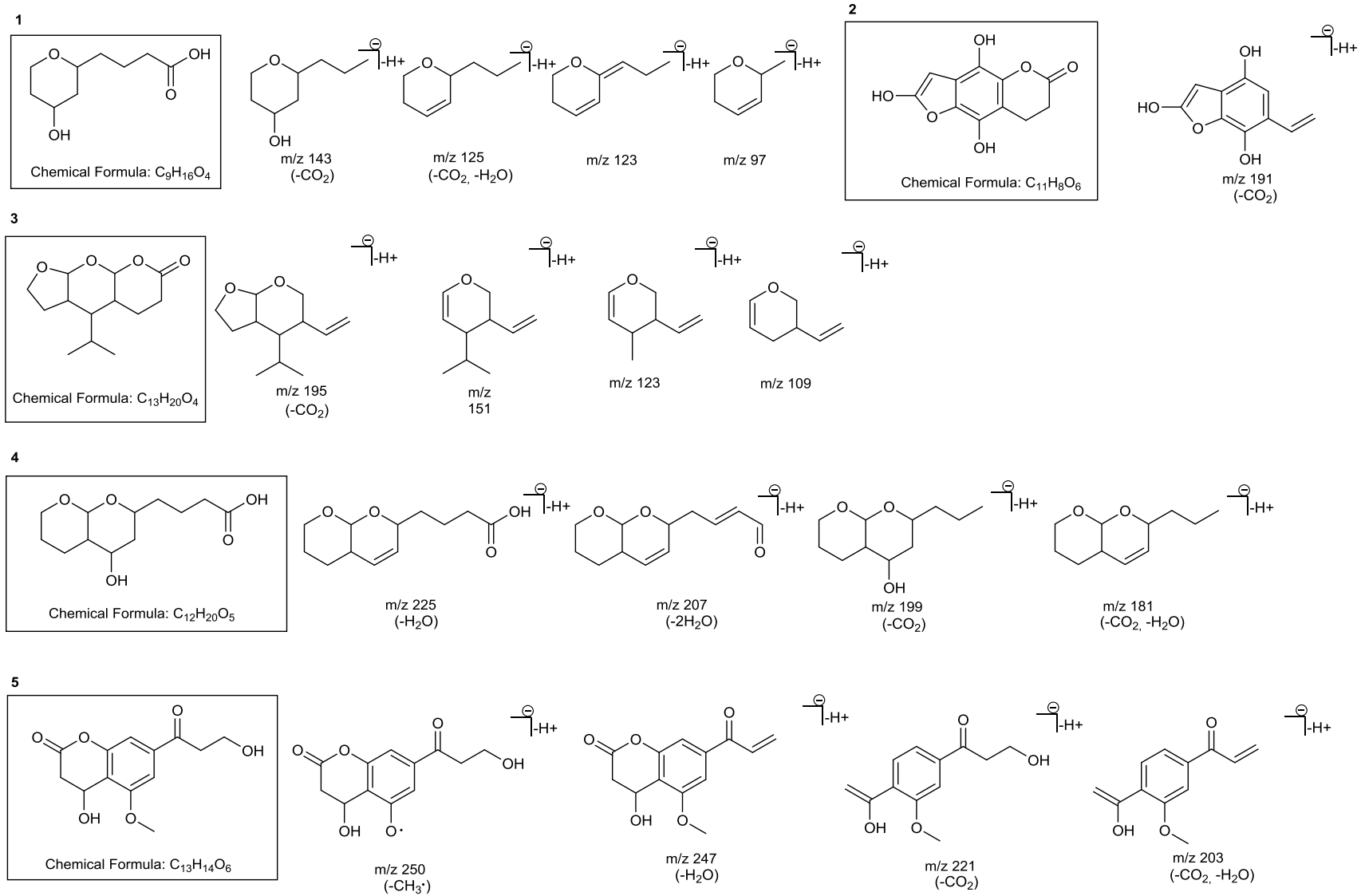
S1 Table 8. LC-MS/MS negative ionisation, 2-butanone fraction. Fragment structures are proposed in Table 3.

Entry	Retention time (min)	Accurate mass	C	H	O	DBE	Precursor ion (intensity)	Fragments (intensity)													
1	6.25-6.27	188,10433	9	16	4	2	187,09705 (14)	143,10754 (2)	125,09683 (100)	123,08135 (8)	97,06552 (18)										
2	6.31-6.33	236,03176	11	8	6	8	235,02448 (38)	191,03442 (100)													
3	6.37-6.42	240,13576	13	20	4	4	239,12848 (38)	195,13854 (100)	151,1123 (36)	123,08097 (18)	109,06533 (14)										
4	6.46-6.48	244,13060	12	20	5	3	243,12332 (30)	225,11264 (100)	207,10204 (62)	199,13336 (18)	181,12275 (30)	163,11216 (10)	99,00808 (20)								
5	5.53-5.63	266,07894	13	14	6	7	265,07166 (10)	250,04776 (3)	247,06071 (4)	221,08136 (12)	203,07075 (8)	193,04996 (100)	178,02649 (66)	175,03944 (14)	160,01592 (8)	149,06008 (40)	134,0366 (68)				
6	6.28-6.30	276,09953	15	16	5	8	275,09225 (24)	231,1023 (100)	216,07859 (30)	215,07114 (66)	203,07103 (10)	188,04753 (9)	107,04969 (10)								
7	6.04-6.09	294,10997	15	18	6	7	293,10269 (4)	275,09254 (8)	249,11306 (12)	231,10243 (24)	216,07891 (28)	163,07601 (10)	149,06028 (40)	134,03677 (18)	131,03439 (26)	130,02661 (88)	125,06032 (20)	123,04469 (34)	113,06021 (100)	99,0446 (20)	
8	5.44-5.52	296,08956	14	16	7	7	295,08228 (2)	235,06073 (16)	205,05010 (4)	163,03936 (100)	145,02881 (38)	119,04955 (36)									
9	7.26-7.30	302,07866	16	14	6	10	301,07138 (66)	286,04786 (100)	268,03753 (8)	257,04529 (4)											
10	6.25-6.27	306,11006	16	18	6	8	305,10278 (26)	290,07926 (8)	261,11277 (100)	246,0893 (18)	245,08158 (20)	231,0658 (82)									
11	6.06-6.09	308,05290	14	12	8	9	307,04562 (100)	289,03492 (18)	263,05571 (36)	245,04506 (18)	219,06572 (48)	201,05522 (4)									
12	5.53-5.63	326,09981	15	18	8	7	325,09253 (1)	265,07184 (20)	235,06097 (7)	193,05008 (100)	175,03971 (32)	160,01625 (6)	149,06035 (14)	134,03681 (16)							
13	5.78-5.80	328,09429	18	16	6	11	327,08701 (10)	283,09729 (20)	239,10756 (8)	163,03944 (100)	119,04959 (42)										
14	5.92-5.97	328,09429	18	16	6	11	327,08701 (100)	309,07652 (20)	283,09722 (22)	265,08674 (74)	237,09171 (20)	211,07598 (16)	163,03945 (29)	143,03945 (29)	119,04957 (15)	93,03396 (21)					
15	7.63-7.70	330,07509	17	14	7	11	329,06781 (40)	314,04321 (100)	299,02028 (40)												
16	6.56-6.60	342,10994	19	18	6	11	341,10266 (40)	326,07909 (82)	323,09197 (8)	311,05567 (12)	309,07647 (48)	297,11269 (36)	294,05306 (62)	282,08942 (100)	267,06603 (72)						
17	6.58-6.64	354,11003	20	18	6	12	353,10275 (20)	338,07918 (100)	323,05553 (60)	310,08417 (10)	294,08935 (36)	279,06586 (38)									
18	5.98-6.02	358,10515	19	18	7	11	357,09785 (100)	339,08689 (10)	313,10771 (44)	295,09717 (25)	280,07378 (17)	254,09451 (12)	193,05011 (10)	163,03949 (12)	119,04959 (16)						
19	6.60-6.65	372,12044	20	20	7	11	371,11316 (26)	356,09000 (65)	341,06621 (26)	339,08698 (30)	327,12339 (29)	324,06356 (25)	312,10001 (100)	297,07654 (64)	294,08957 (9)	279,06603 (6)					
20	5.92-5.97	372,12053	20	20	7	11	371,11325 (21)	356,08998 (14)	353,10282 (42)	341,10258 (100)	327,12328 (88)	326,07928 (31)	312,09995 (87)	309,11293 (36)	297,11276 (12)	294,08948 (66)	282,08945 (24)	279,06596 (40)	173,06023 (27)	123,04453 (12)	
21	7.41-7.43	384,12044	21	20	7	12	383,11316 (62)	368,08932 (100)	353,06612 (9)	229,05020 (9)											
22	6.60-6.65	384,12062	21	20	7	12	383,11334 (14)	368,08959 (54)	353,06676 (100)	352,09528 (60)	338,07910 (32)	337,07167 (92)	324,09976 (7)	309,07644 (16)							
23	5.78-5.80	388,11531	20	20	8	11	387,10803 (100)	369,09787 (100)	354,07428 (30)	343,11832 (35)	325,10786 (70)	310,08443 (65)	295,06082 (12)	282,08945 (43)	193,05017 (65)	173,06131 (21)	159,04464 (15)	149,06022 (24)	134,03673 (23)	123,0445 (14)	
24	5.84-5.91	388,11540	20	20	8	11	387,10812 (20)	343,11857 (30)	299,12878 (4)	284,10534 (9)	193,05013 (100)	178,02676 (5)	149,06028 (12)	134,03679 (22)							
25	5.92-5.97	388,11546	20	20	8	11	387,10818 (100)	369,09748 (18)	354,07416 (5)	343,11805 (6)	325,10755 (30)	310,08415 (25)	282,08933 (15)	271,09741 (5)	193,05001 (20)	173,06023 (17)	159,04457 (8)	134,03663 (5)	123,04451 (7)		
26	5.63-5.73	402,13097	21	22	8	11	401,12369 (26)	386,10059 (17)	383,11344 (45)	371,11343 (100)	357,13426 (95)	342,11047 (87)	339,12345 (36)	327,12299 (10)	324,09995 (66)	312,10007 (25)	309,07664 (43)	217,05026 (10)	203,03457 (3)	173,06027 (24)	
27	7.77-7.80	480,13978	26	24	9	15	479,1325 (100)	464,11117 (10)	449,08776 (4)	355,08239 (41)	340,05855 (6)										
28	7.29-7.36	508,13628	27	24	10	16	507,12900 (100)	492,10619 (36)	477,08256 (5)	341,06650 (24)											
29	6.40-6.45	528,12630	26	24	12	15	527,11902 (69)	509,10795 (6)	329,06609 (100)	185,04494 (24)	167,03436 (2)										
30	6.94-6.99	548,16805	30	28	10	17	547,16077 (3)	503,17078 (6)	401,12355 (44)	383,11315 (100)	371,11332 (16)	368,0899 (13)	357,13427 (12)	339,12312 (60)	324,09977 (56)	309,07643 (8)	163,03948 (15)	145,02882 (79)	119,04960 (6)	117,03393 (9)	

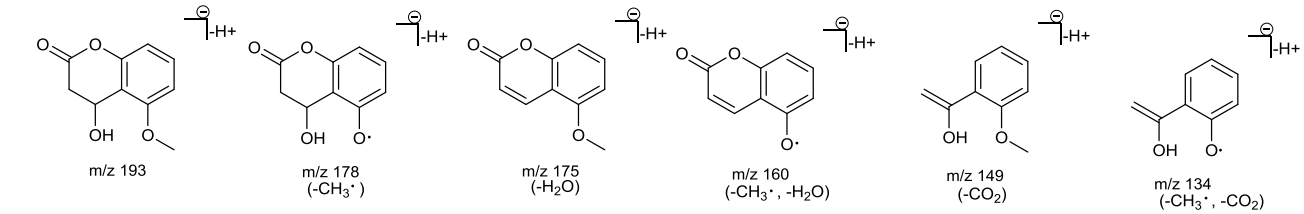
S1 Table 9. LC-MS/MS negative ionisation, water fraction from 2-butanone extraction. Shaded fragments are in common with LC-MS/MS fragments from xylooligosaccharide standards(xylobiose to xylohexaose).

Retention Time (min)	Accurate mass	C	H	O	DBE	Compound	Fragments														
1.97-1.99	150,05203	5	10	5	1	xylose	131,03508	129,01977	101,02457	85,02951											
1.85-1.87	180,06282	6	12	6	1	glucose	161,04527	150,95383	134,98767	131,03461	122,95918	113,02392	101,02410	97,02912	89,02398	85,02911					
1.79-1.81	282,09505	10	18	9	2	xylobiose	203,05728	131,03503	112,98583	101,02453	85,02950										
1.91-1.96	460,14241	16	28	15	3	xylotriose	335,09823	263,07716	203,05581	149,04506	131,03442	113,02387	101,02386	85,02897							
3.94-3.96	502,15434	18	30	16	4	1 acetyl xylotriose	377,11137	335,10167	305,08741	263,07894	149,04559	131,03513	113,02467	101,02460	85,02948						
2.75-2.77	546,17928	20	34	17	4	xylotetraose	467,14017	395,11896	335,09780	263,07701	203,05563	149,04497	131,03436	113,02380	101,02379	85,02890					
3.94-3.96	634,19679	23	38	20	5	1 acetyl xylotetraose	527,16246	509,15079	467,14007	437,12956	395,11893	377,10861	335,09898	305,08789	263,07776	203,05563	149,04497	131,03436	113,02380	101,02379	85,02890
3.13-3.21	678,22157	25	42	21	5	xylopentaose	527,16135	467,14013	395,11896	263,07702	131,03437	101,02381									
3.94-3.96	766,23934	28	46	24	6	1 acetyl xylotepentaose	641,19406	599,18158	569,17220	509,15079	467,14007	437,12956	395,11893	377,10861	305,08789	263,07776					
3.31-3.35	810,26436	30	50	25	6	xylohexaose	731,22514	719,22522	659,20411	641,19357	623,18283	611,18273	599,18294	527,16164	509,15109	467,14041	395,11922	377,10893	263,07721		
4.00-4.03	894,28456	34	54	27	8	2 acetyl xylohexaose	731,22514	719,22522	659,20411	641,19357	623,18283	611,18273	599,18294	527,16164	509,15109	467,14041	395,11922	377,10893	263,07721		

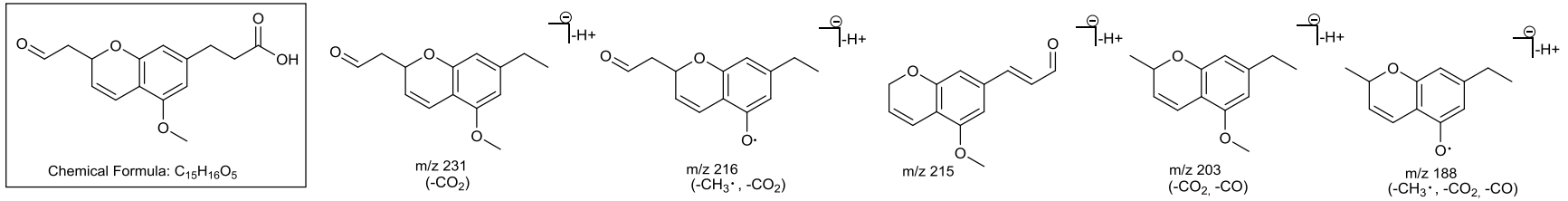
S1 Table 10. Proposed MS/MS fragments (*continued*).



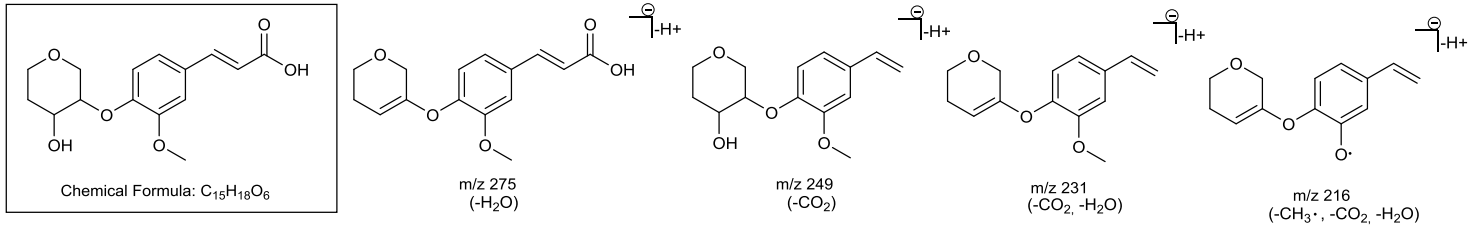
S1 Table 3 (*continued*). Proposed MS/MS fragments (*continued*).

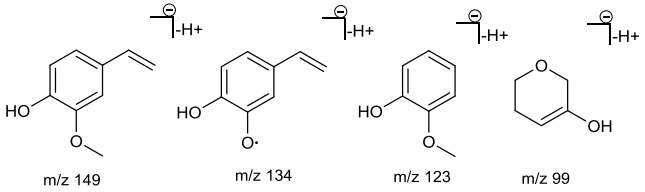


6



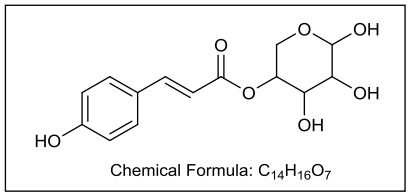
7

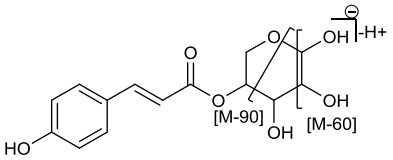


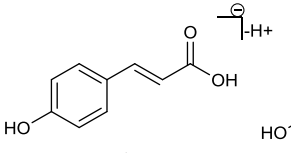


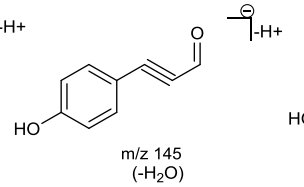
S1 Table 3 (*continued*). Proposed MS/MS fragments (*continued*).

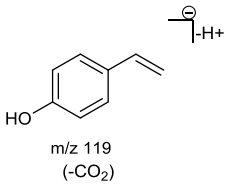
8



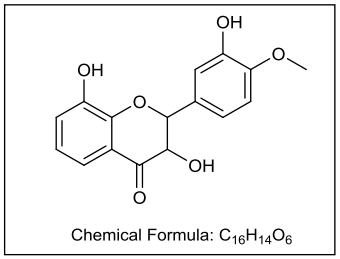


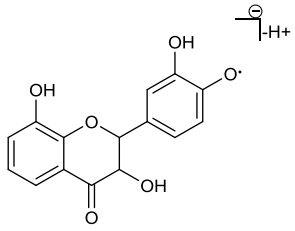


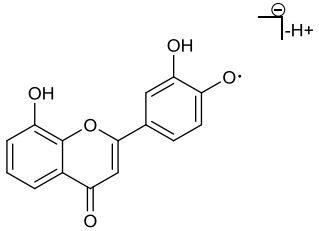




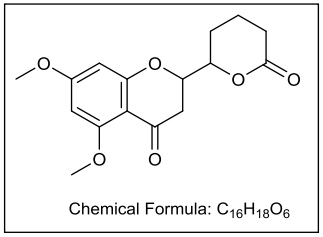
9

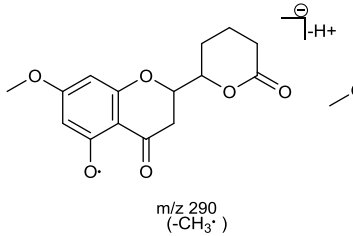


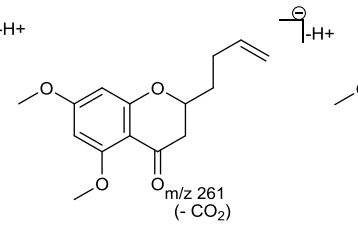


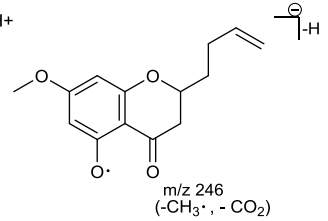


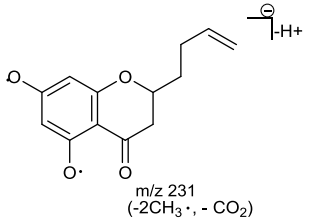
10



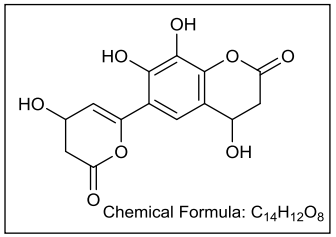


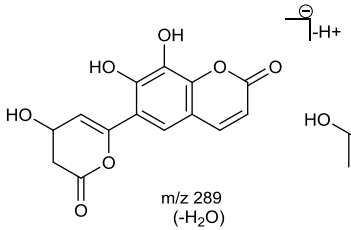


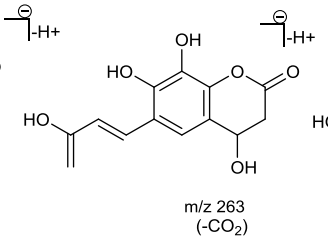


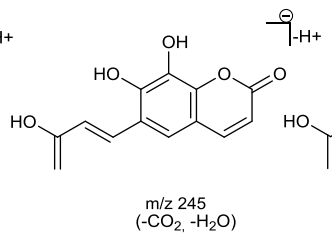


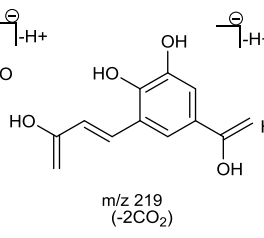
11

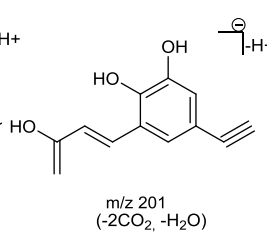






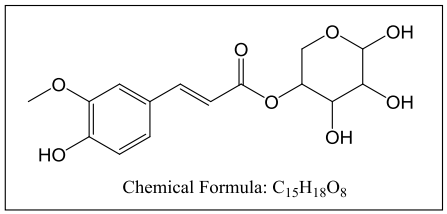


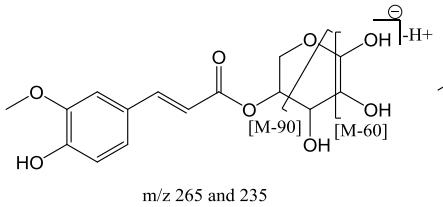




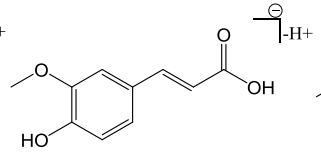
S1 Table 3 (*continued*). Proposed MS/MS fragments (*continued*).

12

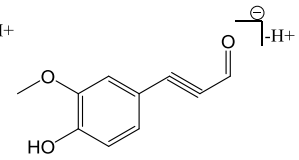




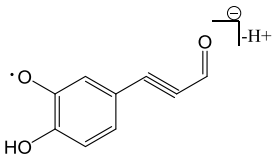
m/z 265 and 235



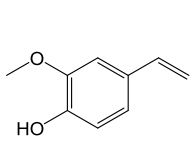
m/z 193



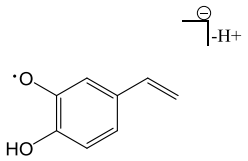
m/z 175
($-H_2O$)



m/z 160
($-CH_3^{\bullet}$, $-H_2O$)

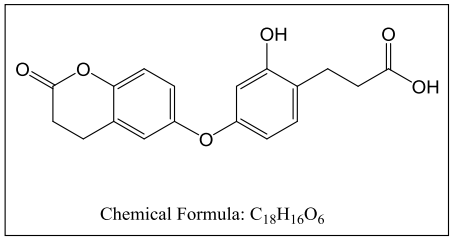


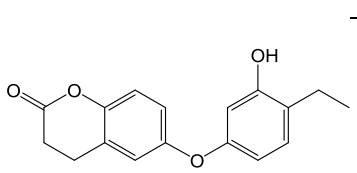
m/z 149
($-CO_2$)



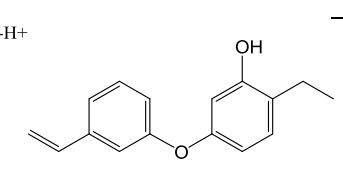
m/z 134
($-CH_3^{\bullet}$, $-CO_2$)

13

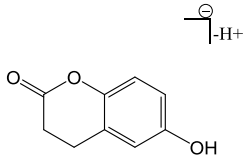




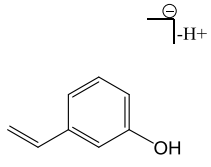
m/z 283
($-CO_2$)



m/z 239
($-2CO_2$)

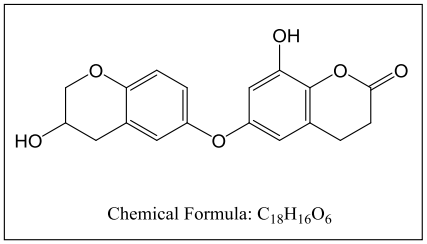


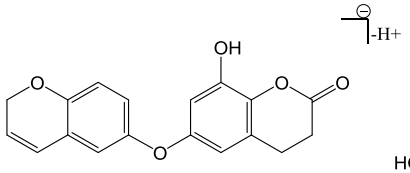
m/z 163



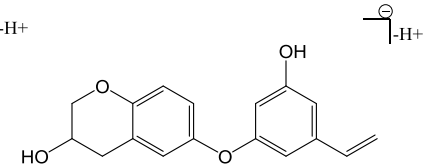
m/z 119

14

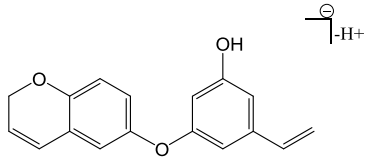




m/z 309
($-H_2O$)

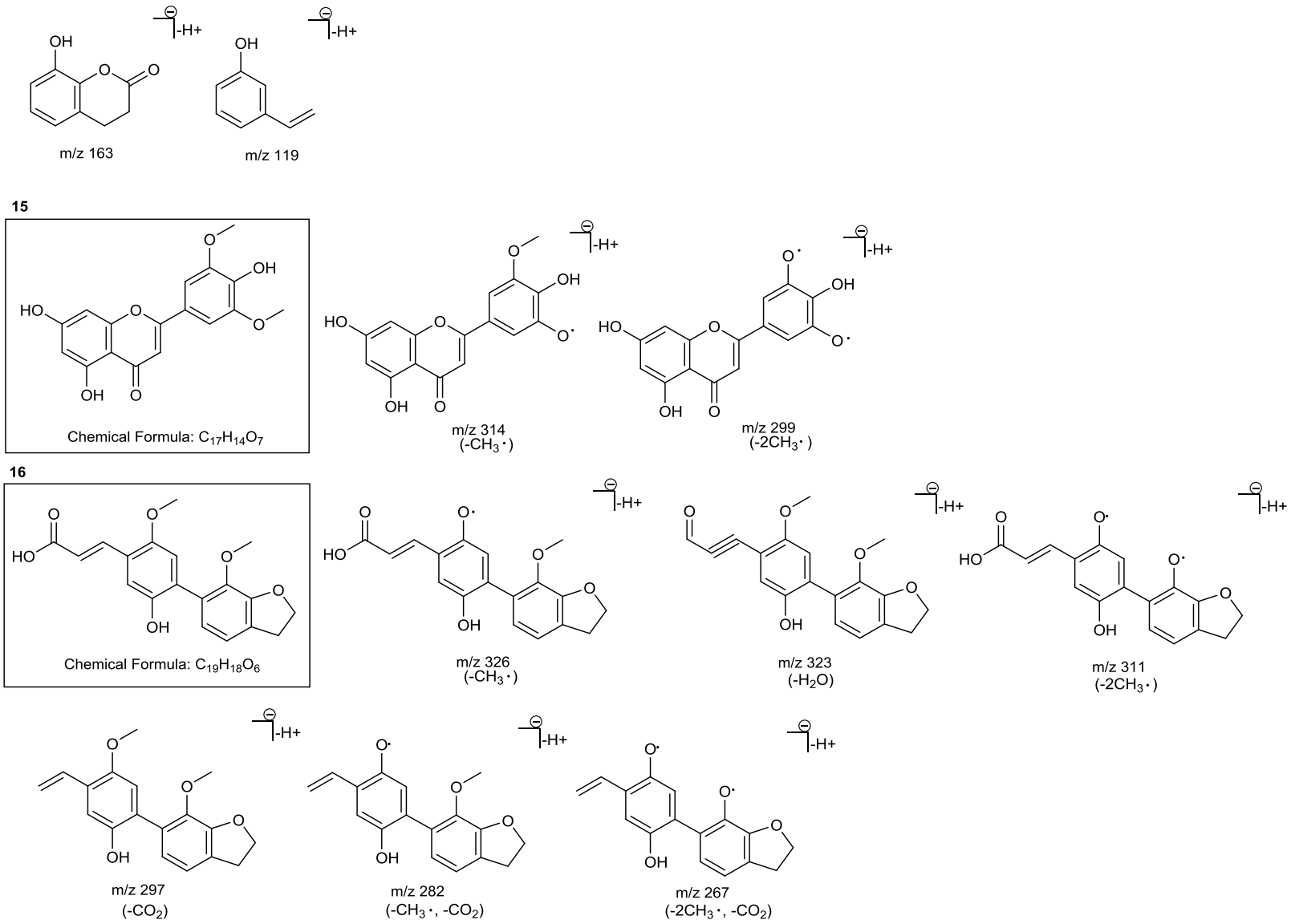


m/z 283
($-CO_2$)

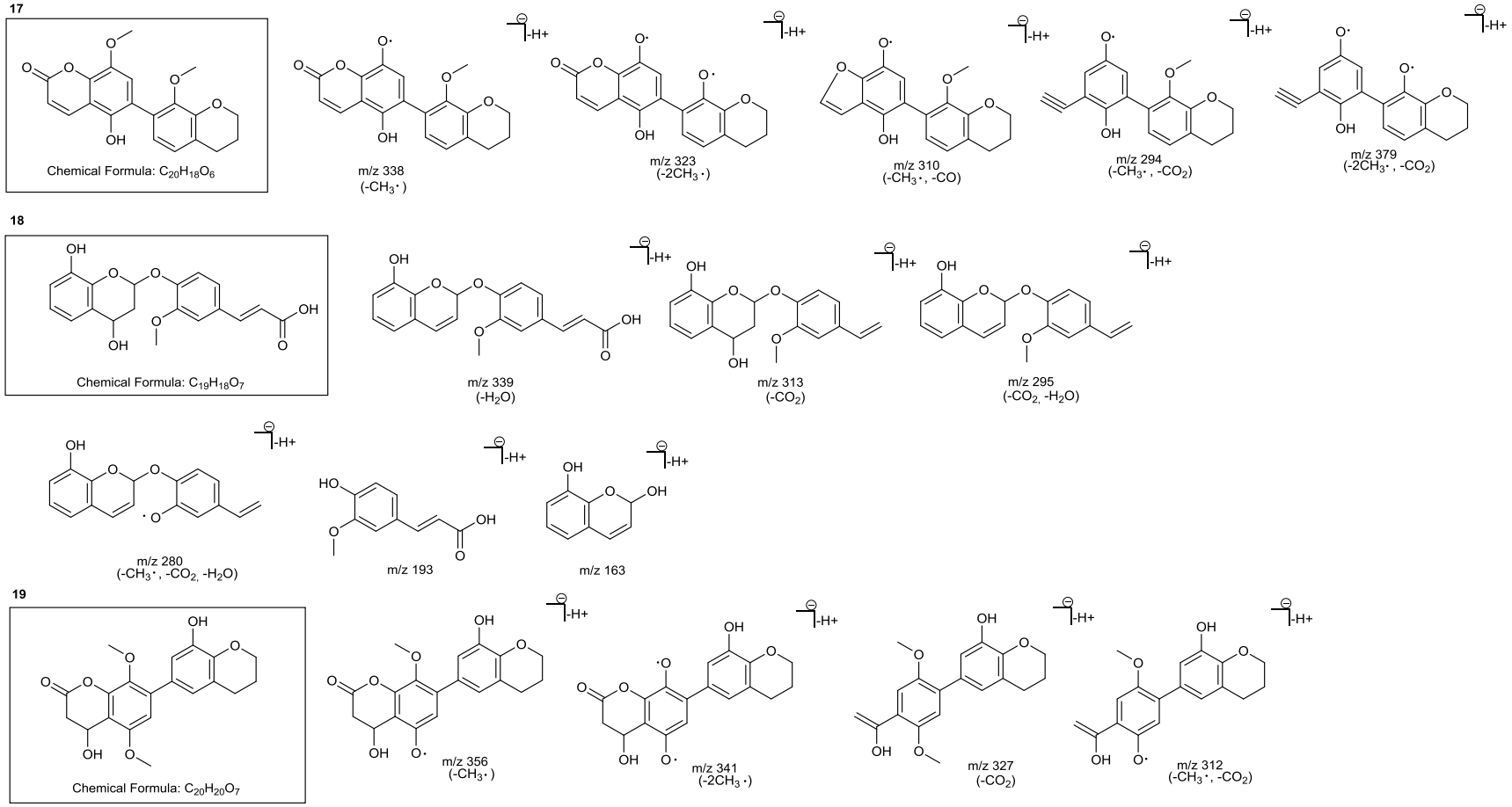


m/z 265
($-CO_2$, $-H_2O$)

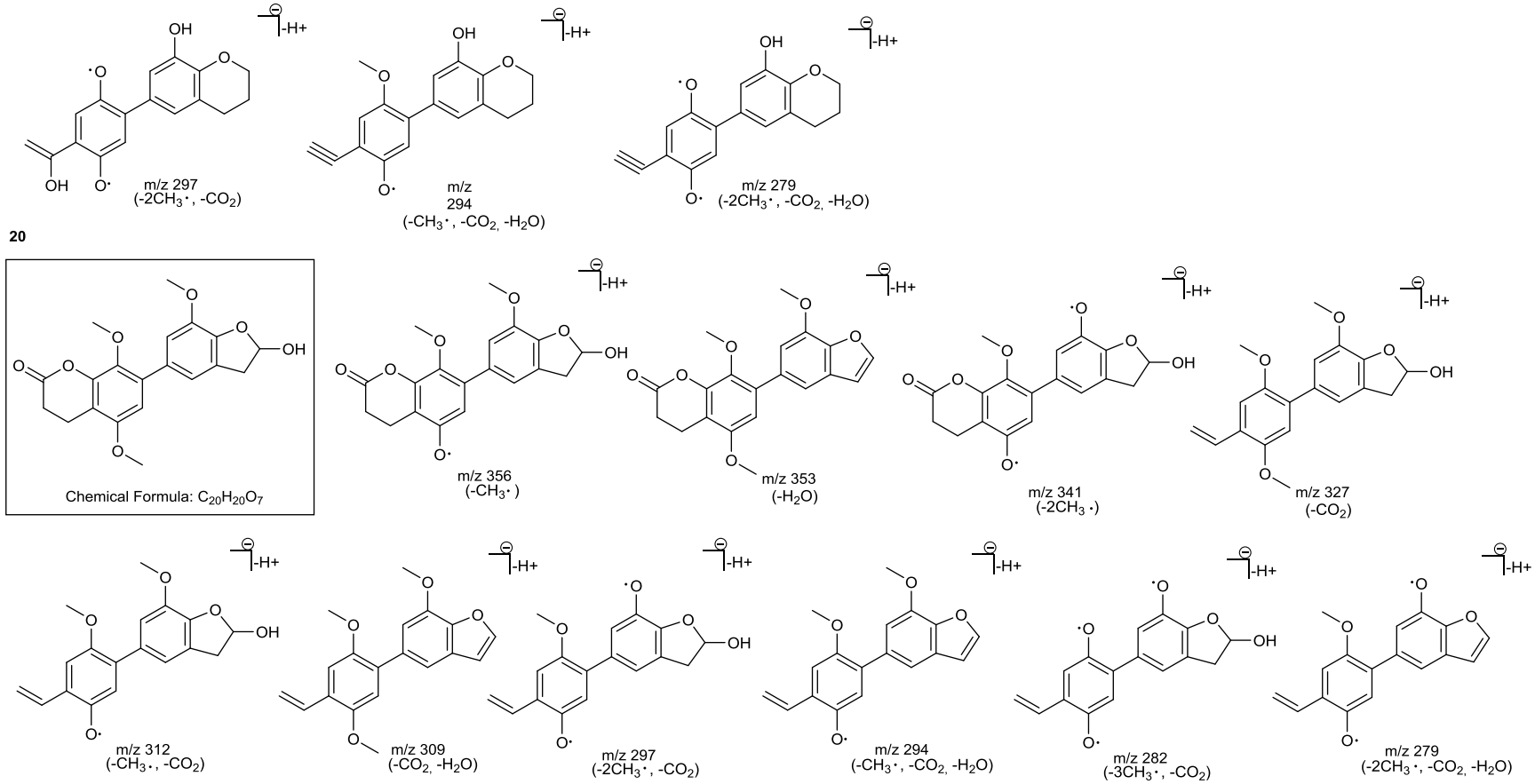
S1 Table 3 (*continued*). Proposed MS/MS fragments (*continued*).



S1 Table 3 (*continued*). Proposed MS/MS fragments (*continued*).

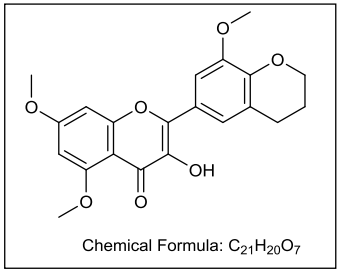


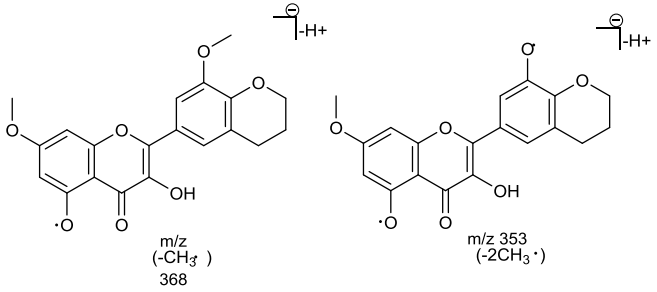
S1 Table 3. (*continued*). Proposed MS/MS fragments (*continued*).



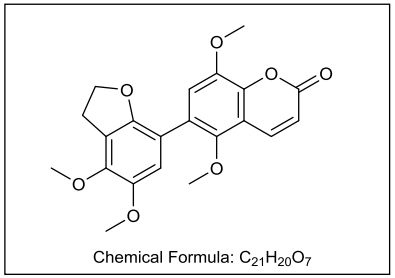
S1 Table 3 (*continued*). Proposed MS/MS fragments (*continued*).

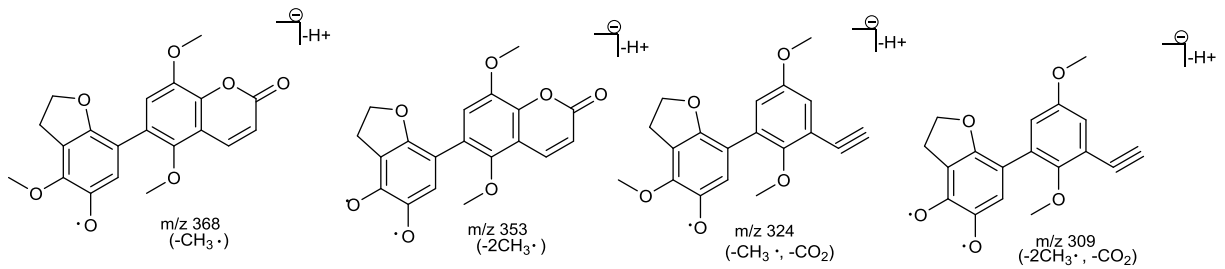
21



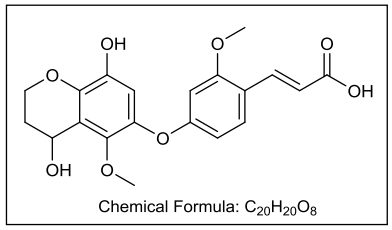


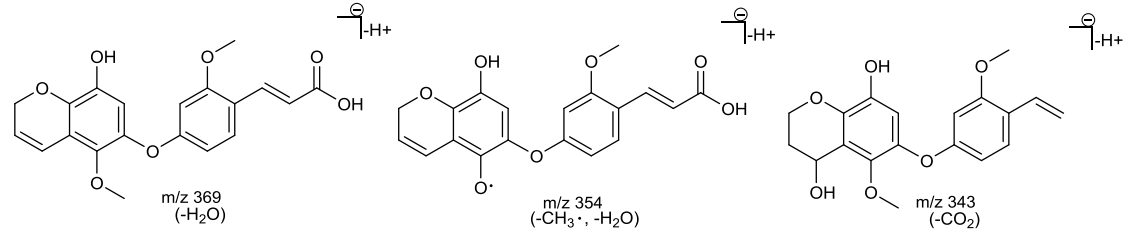
22

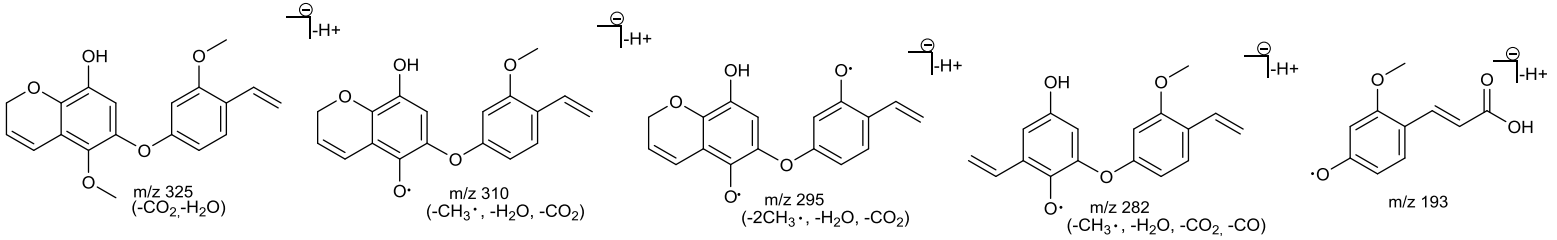




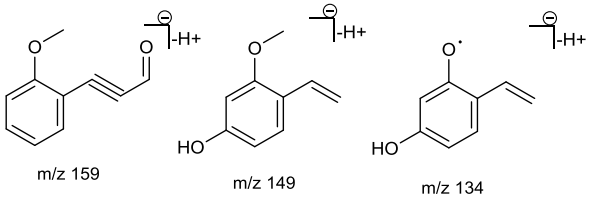
23



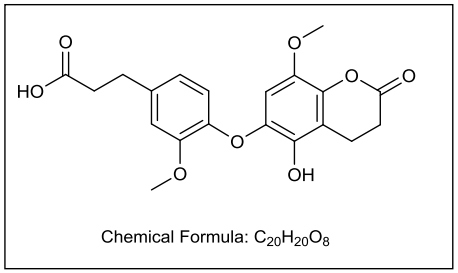


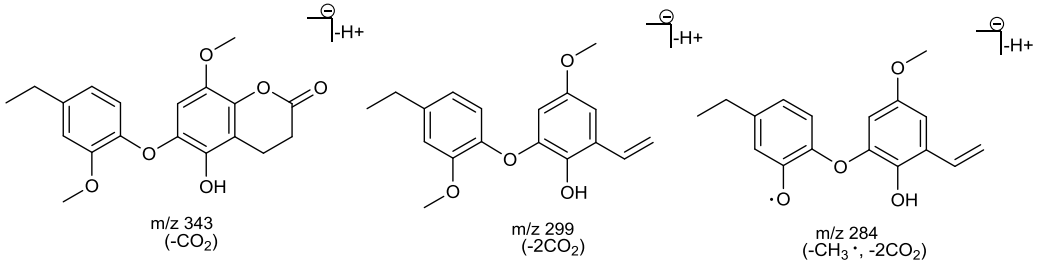


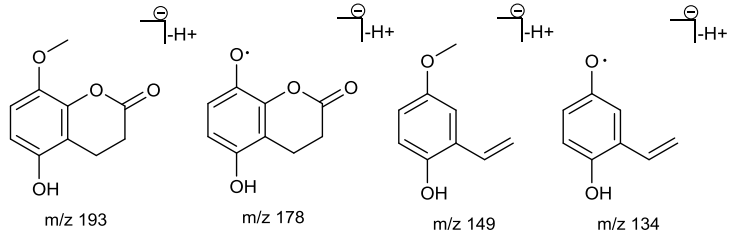
S1 Table 3 (*continued*). Proposed MS/MS fragments.



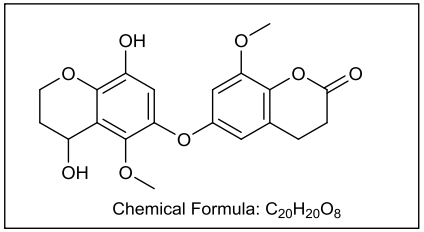
24

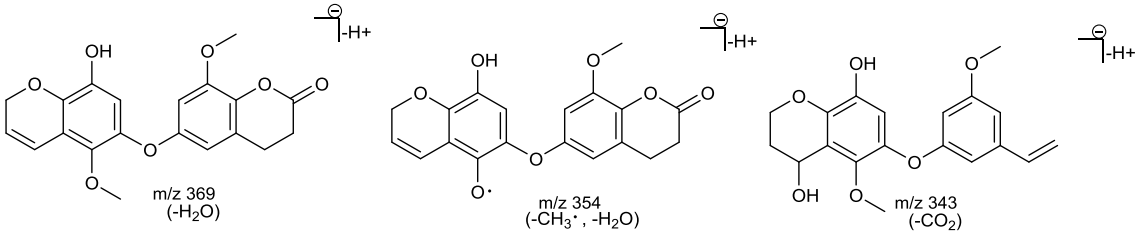




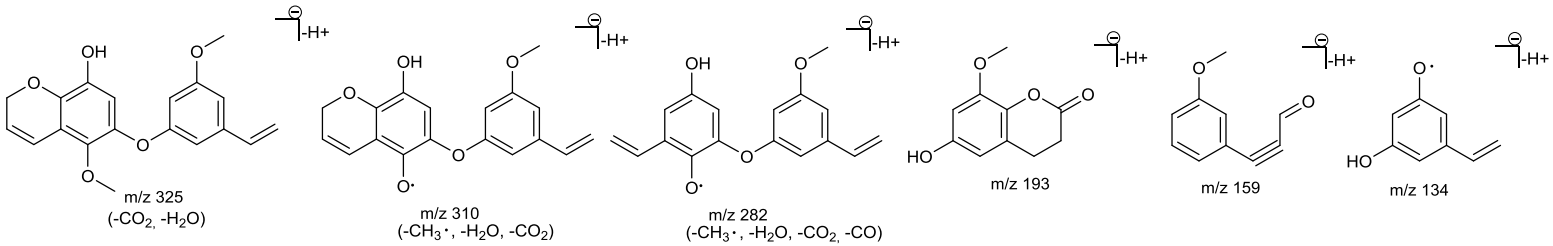


25

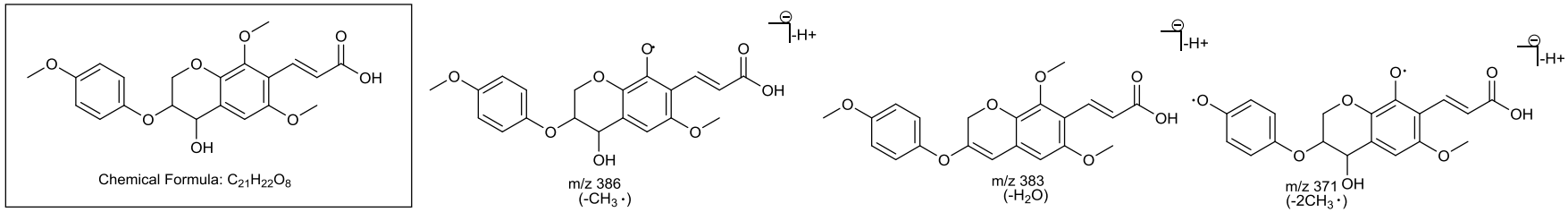


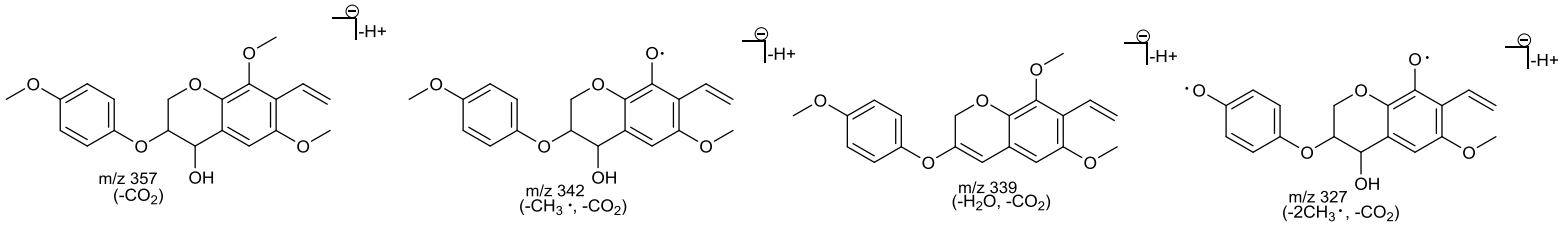


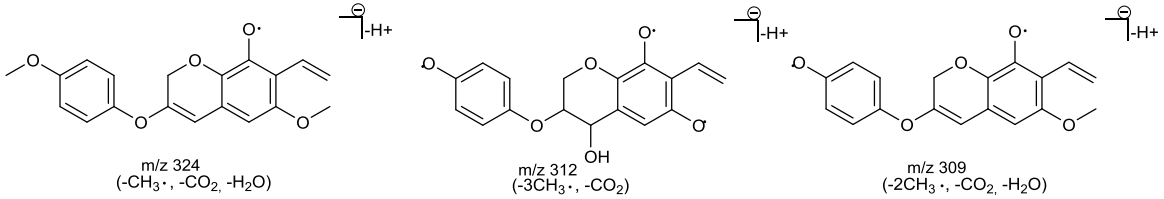
S1 Table 3 (*continued*). Proposed MS/MS fragments.



26

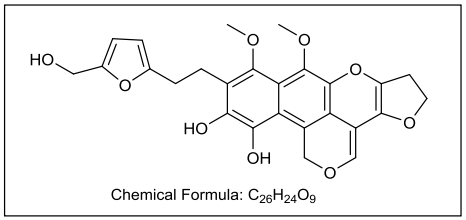


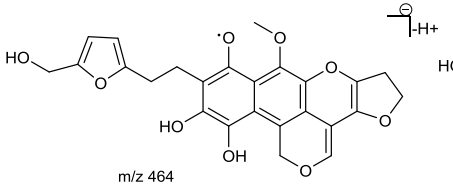


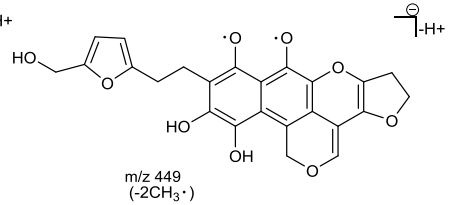


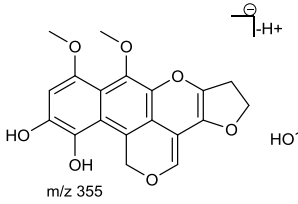
S1 Table 3 (*continued*). Proposed MS/MS fragments.

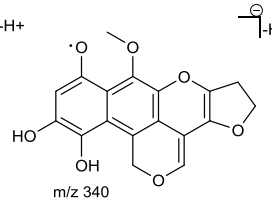
27



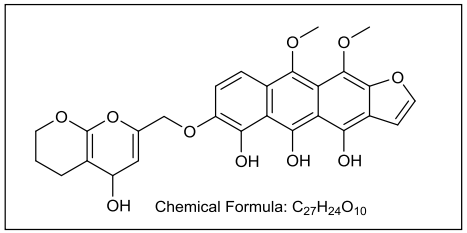


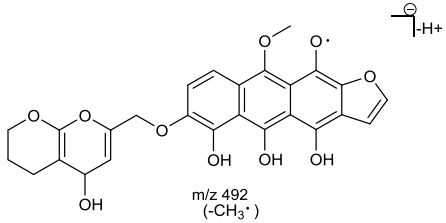


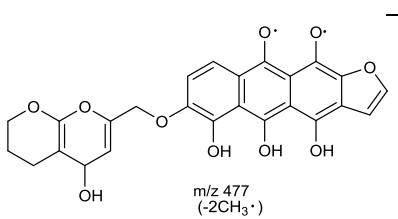


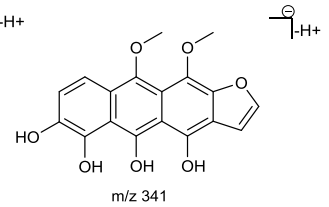


28

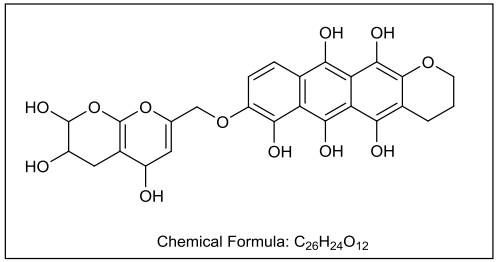


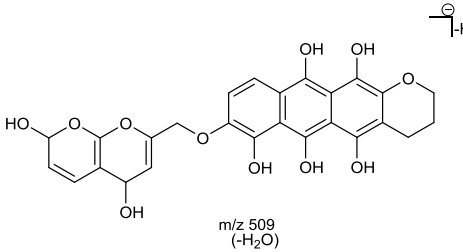


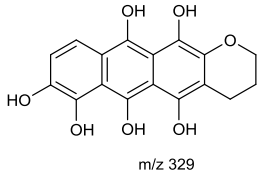


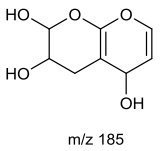


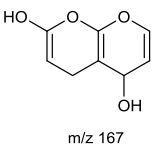
29





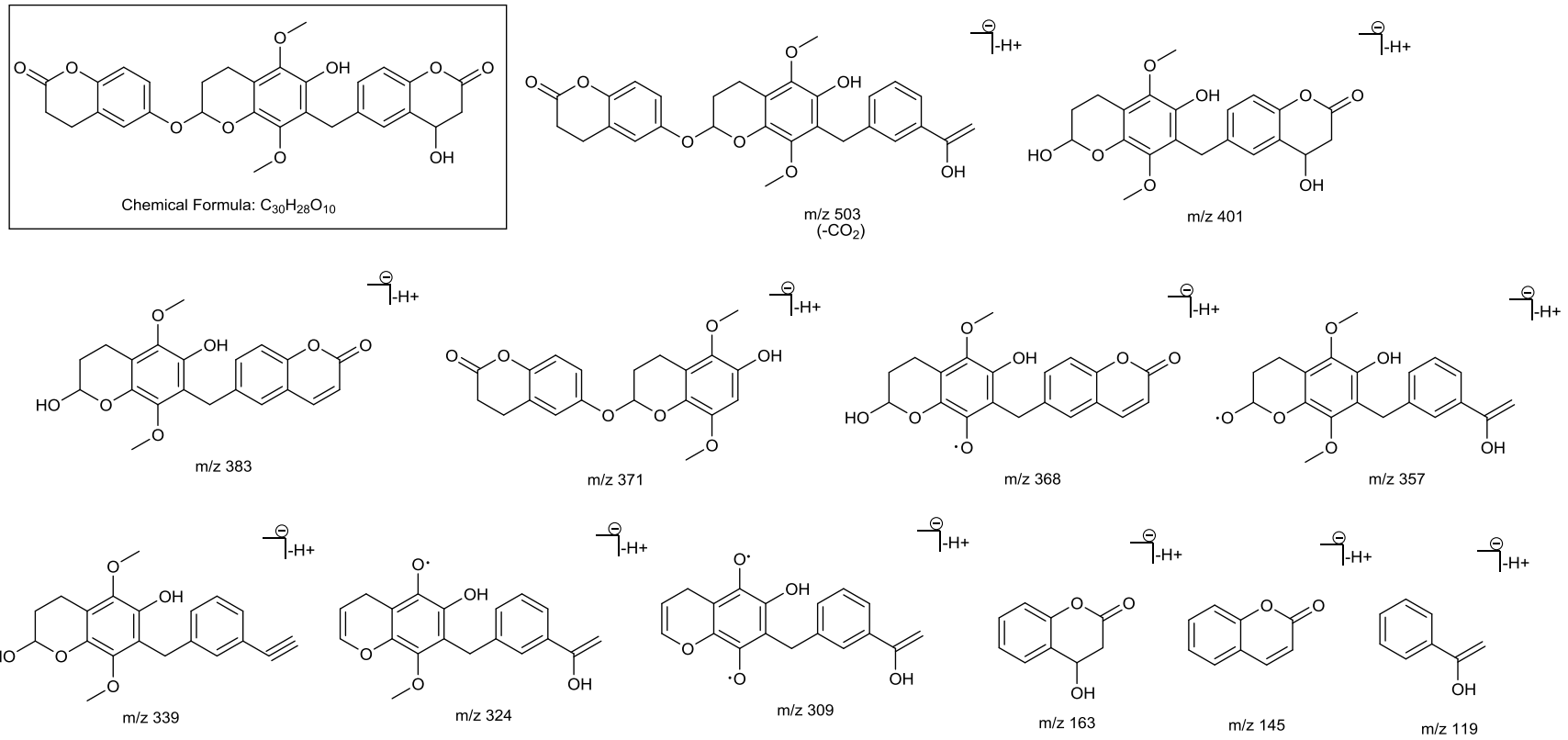






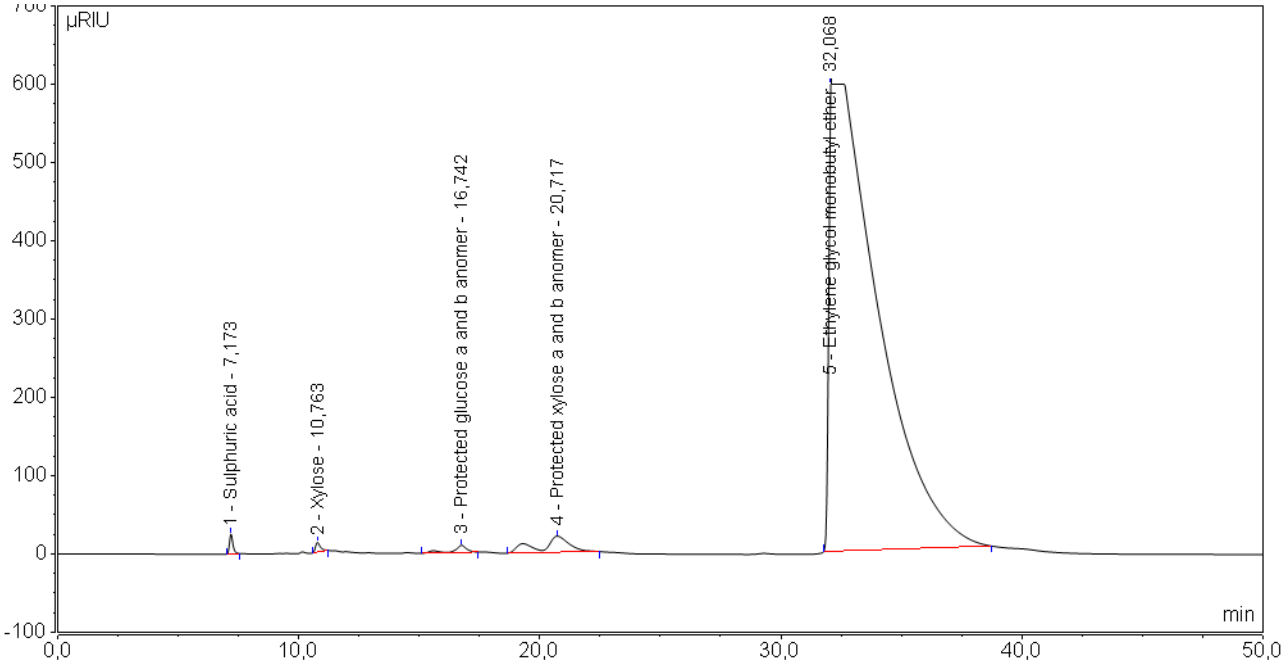
S1 Table 3 (*continued*). Proposed MS/MS fragments.

30



S1 Table 11. Compounds in the water fraction from 2-butanone extraction and 2-butanone extraction in the retention time region from 3.7-5.5 minutes. WF: Water Fraction. 2-BF: 2-Butanone Fraction.

Retention time (min)	Ions, *m/z* $[M- H^+]^-$ (relative intensity)	Fraction	Compounds
3.95	455.14203 (3), 587.18378 (12), 719.22626 (14), 851.26862 (9), 983.31140 (6),1115.35315 (2)	WF	Pentoses + 1 acetyl, DP 3, DP 4, DP 5, DP 6, DP 7, DP 8.
3.95	455.14233 (4), 587.18451 (6)	2-BF	Pentoses + 1 acetyl, DP 3, DP 4.
4.14	629.19318 (16), 761.23517 (29), 893.27692 (20), 1025.31921 (12), 1157.36060 (5), 1199.37134 (8), 1331.41345 (6), 1463.45447 (4)	WF	Pentoses + 2 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8. Pentoses + 3 acetyl, DP 8, DP 9, DP 10.
4.14	629.19604 (0.1)	2-BF	Pentose + 2 acetyl, DP 4
4.54	803.24573 (4), 935.28717 (4), 1249.38831 (7), 1381.43054 (3)	WF	Pentoses + 3 acetyl, DP 5, DP 6. Feruloyl substituted pentoses, DP 8, DP 9.
4.68	985.30377 (20), 1117.34460 (4), 1249.38843 (4)	WF	Feruloyl substituted pentoses, DP 6, DP 7, DP 8.
4.88	691.20862 (24), 721.21936 (32), 853.26135 (100), 1027.31409 (8), 1159.35547 (12), 1291.39734 (4)	WF	Cumaroyl substituted pentose DP 4. Feruloyl substituted pentose DP 4, DP 5. Feruloyl + 1 acetyl substituted pentose DP 6, DP 7, DP 8.
4.98	559.16693 (6), 721.21985 (5)	2-BF	Cumaroyl substituted pentose DP 3. Feruloyl substituted pentose DP 4.
5.00	721.21936 (100), 1027.31445 (9)	WF	Feruloyl substituted pentose DP 4. Feruloyl + 1 acetyl substituted pentose DP 7.
5.05	589.17706 (100), 721.21906 (16), 853. 26074 (7), 1117.34448 (10) 895.27173 (16), 1027.31494 (8), 1159.35559 (5), 1291.39893 (4), 1423.43689 (3)	WF	Feruloyl substituted pentoses, DP 3, DP 4, DP 5, DP 7. Feruloyl + 1 acetyl substituted pentoses, DP 5, DP 6, DP 7, DP 8, DP 9.
5.05	589.17737 (30)	2-BF	Feruloyl substituted pentose DP 3.
5.27	325.09247 (100), 559.16699 (17), 763.23035 (6),	2-BF	Feruloyl substituted pentose DP 1. Cumaroyl substituted pentose DP 3. Feruloyl + 1 acetyl substituted pentose DP 4.
5.47	295.08221 (15), 457.13538 (8)	2-BF	Cumaroyl substituted pentose DP 1. Feruloyl substituted pentose DP 2.



S1 Figure 32. HPLC chromatogram with refractive index detection, anomeric protection.

Paper 3

Pentose dimers with bicyclic moieties from pretreated biomass

Helena Rasmussen[a], Hanne R. Sørensen[a], David Tanner[b] and Anne S. Meyer[c*]

[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark

[b] Dept. of Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark.

[c] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Lyngby, Denmark.

*Corresponding author: e-mail: am@kt.dtu.dk; Tel: (+45) 45 25 2800

Abstract

In lignocellulosic biorefinery processes involving enzyme catalysed reactions it is a challenge that enzyme inhibiting compounds are generated and liberated during pretreatment of the biomass. In this study the contribution to cellulase inhibition from xylooligosaccharides and newly discovered oligophenolic compounds from pilot scale pretreated wheat straw was assessed at two different pretreatment severities. An increase in severity of the pretreatment led to more oligophenol compounds and a change in the relative amounts of the different oligophenolic compounds and in turn the total overall cellulase inhibition increased.

When the xylooligosaccharides were enzymatically degraded prior to cellulose hydrolysis, a relief in cellulase inhibition was observed, but some inhibition remained, suggesting that other components also played a role in inhibition. We propose that these components include dipentoses with bicyclic moieties and feruloylated tripentoses, because LC-MS/MS analysis revealed the presence of these components in the liquid from hydrothermal pretreated wheat straw after enzymatic treatment.

The reaction mechanisms for synthesis of the new dipentoses having hydroxylated oxane bicyclic residues are considered and they are proposed to be formed as reaction products from either the isomerisation product xylulose or fructose reacting with glyceraldehyde during pretreatment.

The data show that the main cellulase inhibition from hydrothermally pretreated wheat straw liquors is due to xylooligosaccharides followed by oligophenolic compounds and the new dipentose with bicyclic moieties and feruloylated tripentoses. The relative amounts and hence contribution to inhibition from each class of compounds changes with severity of the pretreatment.

Keywords: Pretreatment, inhibitors, reaction mechanisms, degradation compounds, biorefinery, enzymatic hydrolysis, wheat straw, sugar cane bagasse, hydrothermal pretreatment, oligophenol, xylooligosaccharide, enzyme resistance, fractionation, extraction, LC-MS/MS.

1 Introduction

Plant biomass biorefinery processes can be tuned to produce environmentally friendly energy or important chemicals [1-6]. In processes applying enzyme catalysed reactions, it remains a challenge that enzyme inhibiting compounds are generated and liberated during pretreatment of biomass [7,8]. Degradation compounds such as phenolic compounds, but also xylooligosaccharides have previously been identified as cellulase inhibitors in lignocellulosic degradation reactions [9,10].

It complicates the picture that both the amounts and type of degradation compounds as well as the biomass structural elements that are liberated during pretreatment, can vary with pretreatment process parameters [8]. Both the inhibitor potency, but also the actual amount of a compound determines its contribution to overall inhibition from the pretreatment liquors and this may in turn change when process parameters during pretreatment are changed. To shed light on these relations an "inhibitor mass balance" method was applied to account for inhibition contributions from different compounds in LfHP and in turn account for all inhibition exerted by LfHP.

Our previous work revealed that even though soluble oligophenols produced during hydrothermal pretreatment of wheat straw (LfHP) are potent enzyme inhibitors, the simultaneously released xylooligosaccharides are more abundant [11].

The aim of the present work was to investigate 1) if pretreatment severity would change the relative level of oligophenolic compounds and xylooligosaccharides in the liquid from pretreated wheat straw and in turn affect the contribution to total inhibition from LfHP. 2) If other compounds in the LfHP beyond oligophenols and enzymatically degradable xylooligosaccharides may exert inhibitory action on cellulose degrading enzymes.

2 Materials and Methods

Chemicals and enzymes

Buffer chemicals, solvents, acids and bases were purchased from Sigma Aldrich. Cellic CTec3 (1920 BHU(2)/g) obtained from Novozymes A/S (Bagsværd, Denmark) was utilised. This is a commercially available cellulase preparation based on the Trichoderma reesei complex. Apart from the cellulolytic enzyme base from T. reesei containing at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, and a β-xylosidase [12], the preparation Cellic CTec3 also contains other proprietary hydrolysis-boosting proteins.

Fractionation of pilot plant liquid from hydrothermal pretreatment (LfHP) of wheat straw

Fractionation was carried out as previously described [11], in short:

Liquid from pilot scale hydrothermal pretreated wheat straw (18 minutes, 40 % (w/w) dry matter, LfHP 1 = 183 °C, LfHP 2 = 191 °C) was extracted with 2-butanone. 170 g liquid from pretreatment was extracted with 2 x 160 g solvent. The water phase was freeze dried and the organic phase was evaporated and freeze dried.

Determination of inhibition from the fractions: Enzyme inhibition assay

Enzymatic hydrolysis was carried out as previously described [11], in short:

The freeze dried fractions were dissolved in 0.1 M acetate buffer (pH 5.1) to dry matter 13.6 % (w/w) and avicel was added to 12 % (w/w). The flasks were incubated at 50 °C, 160 rpm and the enzyme preparation Cellic CTec3 was added to 0.6 % (w/w). Upon sampling (200 μL) the enzyme was inactivated at 99 °C for 5 minutes.

Glucose release was measured with High Pressure Anion Exchange Chromatography (HPAEC) as described below.

Enzyme pre-hydrolysis of water phase from 2-butanone extraction and LfHP (prior to enzyme assay)

The freeze dried compounds from the water phase from 2-butanone extraction was dissolved in 0.1 M acetate buffer (pH 5.1) to dry matter 16.6 % (w/w). The 15 g scale flasks were incubated at 50 °C, 160 rpm and an enzyme preparation (Cellic CTec3) was added to 2.4 % (w/w). An additional enzyme dose (to a total of 4.8 % (w/w) was added after 3 days and the mixture was allowed to react for another 5 days until the enzyme was inactivated at 99 °C for 15 minutes.

At the end of the prehydrolysis the glucose and xylose release were 1 and 0.7 % (w/w) respectively. Thus glucose and xylose were added to these concentrations to control experiments with 12 % (w/w) avicel in the enzyme inhibition assay above, to account for product inhibition from glucose and xylose in the inhibition mass balance from the prehydrolysed experiments.

Determination of xylooligosaccharides in LfHP

Weak acid hydrolysis was carried out according to Sluiter *et al* [13] and analysed by HPLC as described below.

2.1 Analytical methods

HPLC monosaccharide analysis (weak acid hydrolysis)

Analysis was performed on a Dionex (ThermoScientific, Sunnyvale, California, USA) Ultimate 3000 UHPLC with RI and dual wavelength detection (210 and 250 nm) with a Rezex RHM monosaccharide H+ (8%) column, 7.80 x 300 mm (Phenomenex). Column temperature was 65 °C. The method was a 50 minutes isocratic method with 5mM sulphuric acid in water and 0.6 mL/min flow [13].

HPAEC glucose and xylose analysis (enzyme inhibition assay)

Analysis was performed on a ICS-5000 Dionex ThermoFisher Scientific (ThermoScientific, Sunnyvale, California, USA) analytical system with a PA1 column, 2 x 250 mm (Thermo Scientific). The method was a 2 step isocratic method with an additional final cleaning step with KOH concentrations held constant at 25, 10 and 100 mM for 5, 14 and 7 minutes respectively. The flow rate was 0.25 mL/min.

LC-MS and LC-MS/MS

LC-MS and LC-MS/MS analyses were performed on a Thermo (ThermoFisherScientific, Waltham, MA, USA) Orbitrap Fusion instrument with electrospray ionisation as previously described [11]. In short, spray voltage was 2500 in negative ionisation and HCD energy for MS/MS was 25 %. LC conditions were a gradient from 100 % water (containing 0.025 % TFA) -100 % acetonitrile (containing 0.025 % TFA) over 10 minutes followed by wash and equilibration.

The column was a pentafluorphenyl (pfp) discovery HS F5, L x I.D. 15 cm x 4.6 mm, 5 μm particle size (Supelco).

3 Results and Discussion

3.1 Consequences of increased severity in pretreatment

Hydrothermal pretreatments were performed at 183 ºC for 12 minutes and 191 ºC for 12 minutes and inhibitory action of the liquid fractions LfHP 1 (183 ºC for 12 minutes) and LfHP 2 (191 ºC in 12 minutes) and their fractions from extraction were compared. It was found that LfHP 2 was slightly more inhibiting toward cellulases than LfHP 1 (Figure 1) when compared at the same dry matter level, but both LfHPs retarded the glucose release with ~75 % after ~24 hours.

With the 2-butanone extraction method previously described [9] it was established that highly potent inhibitory oligophenols were extracted into the 2-butanone organic phase, whereas xylooligosaccharides were left in the water phase (xylooligosaccharides include acetyl and feruloyl substituted xylooligosaccharides).

In the present study the amount by weight of compounds in the 2-butanone fraction increased by 14 % with the more severe pretreatment (LfHP 2), whereas the amount in the water fraction decreased correspondingly. This indicates that more oligophenolic inhibitors were formed with increased pretreatment severity and more xylooligosaccharides were degraded to xylose and in turn resulted in oligophenolic compounds. Also the 2-butanone fraction from LfHP 2 was profoundly more inhibiting than the corresponding LfHP 1 2-butanone fraction when compared at the same dry matter basis. The LfHP 2 2-butanone fraction retarded glucose release by more than 90 % compared to the control (Figure 1) whereas the LfHP 1 2-butanone fraction retarded glucose release by ~80 %. These data suggest that the more severe pretreatment had induced the formation of either new more potent inhibitory compounds or changed the profile of oligophenolic compounds extracted into the 2-butanone fraction.

The inhibitory effect exerted by the water fractions from the 2-butanone extraction were similar for LfHP 1 and LfHP 2 and were ~60 % compared to the control (Figure 1).

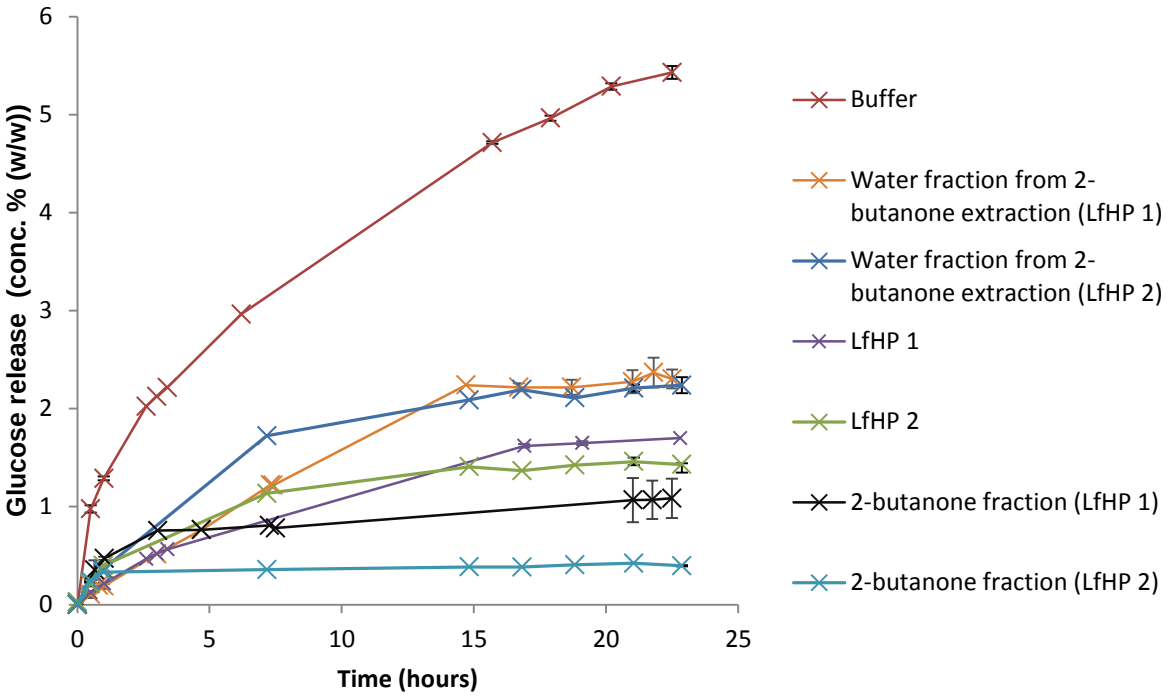


Figure 33. Enzymatic cellulose (avicel) hydrolysis assay with LfHP from pretreatment in 12 minutes at 183 ºC (LfHP 1) and 191 ºC (LfHP 2) and corresponding fractions from extractions.

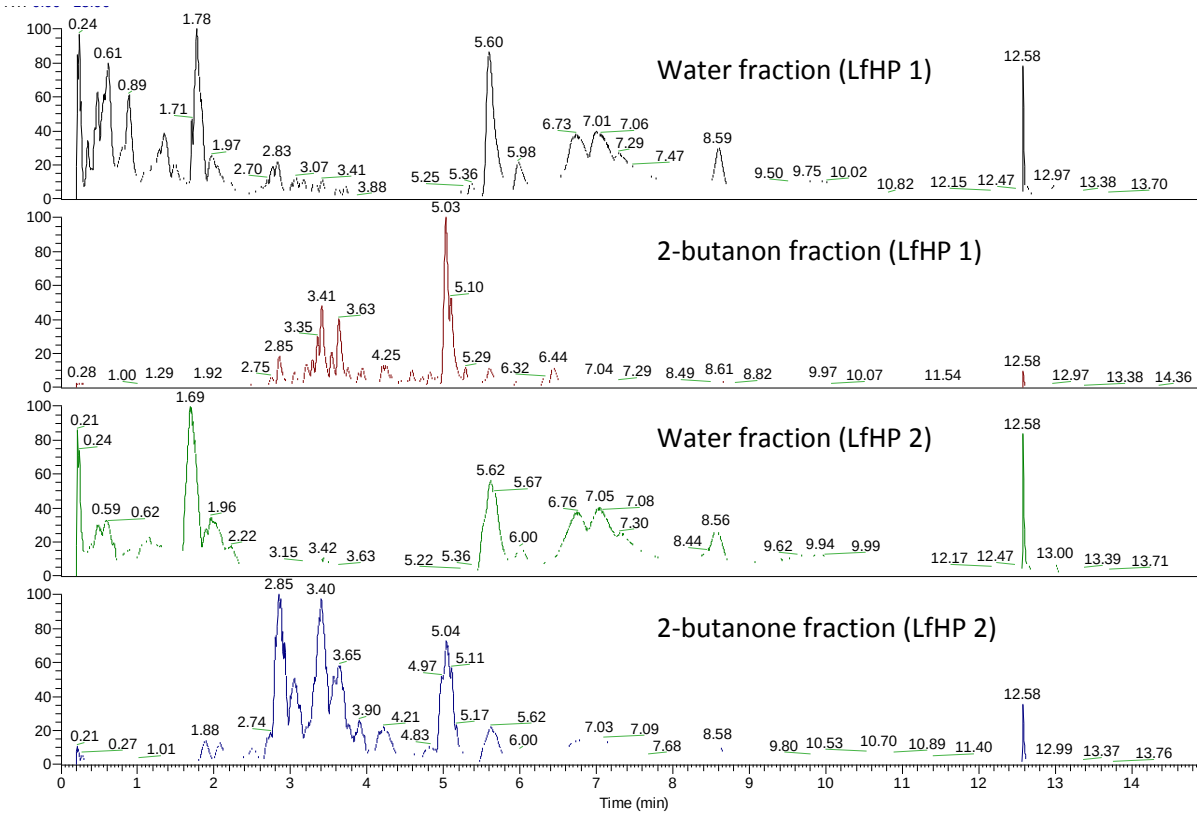
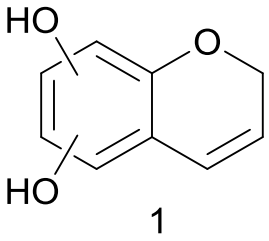


Figure 34. Base peak chromatograms (negative ionisation) of water fractions and 2-butanone fractions from LfHP 1 and LfHP 2.

3.1.1 Compound distributions in the fractions

From the base peak chromatograms, a different distribution of the compounds abundance can be observed in the water fractions and 2-butanone fractions from LfHP 1 and LfHP 2 (Figure 2). The compounds in the 2-butanone and water fractions were analysed in detail, regarding retention time (Figure 2), accurate mass and fragmentation pattern (Supplementary material, Table 1 and Table 2), and they were found to be similar in LfHP 1 and LfHP 2, even though the relative abundance had changed: In the water fractions the amount of xylooligosaccharides mainly without ester substitution (rt 0.21-1.69, Figure 2) were less abundant with more severe pretreatment. However, isomers of tetrapentoses and pentapentoses with 1 acetyl substituent (rt 1.78 Figure 2 and rt 1.69 Figure 2) were present at similar abundance in the water fractions from LfHP and LfHP 2. In the 2-butanone fraction of LfHP 2, the shift in distribution as compared to LfHP 1 was towards earlier retention times and especially the compound at rt 2.85 had an increased relative abundance compared to its abundance in the 2-butanone fraction from LfHP 1 (Figure 2, pane 2 and 4). This ion, rt 2.85, *m/z* 163.03938 ($[M- H^+]^-$) was subjected to fragmentation with HCD energies 25 % and 50 %, but did not result in any fragmentation, suggesting a condensed compound with no functionalities readily available for fragmentation. The accurate mass corresponds to the molecular formula $C_9H_8O_3$ with 6 double bond equivalents. On this basis compound **1** (Figure 3) is suggested. Compound **1** and other compounds eluting at the earlier retention time in the 2-butanone fraction from LfHP 2 may be responsible for the increased inhibition towards cellulases of this fraction (both in relation to the corresponding 2-butanone fraction of LfHP 1 and the water fraction of LfHP 2) (Figure 1).



1

Figure 35. Proposed structure of the compound (*m/z* 163.03938), that is present at elevated relative amounts, when the pretreatment temperature of wheat straw is increased from 183 ºC to 191 ºC.

3.2 Inhibition mass balance

To compare the significance of the pretreatment severity in relation to overall inhibition from LfHP 1 compared to LfHP 2 a compound mass balance was performed. To evaluate how large a part of the total LfHP inhibition in the two cases each of the fractions 2-butanone and the residual water fraction comprised, the actual amounts of dry matter extracted into the fractions were considered. Despite the high inhibitor potency of the oligophenolic compounds per mass unit, the total mass of compounds in the organic fraction was much less than in the water fraction regardless of the severity of the pretreatment. Hence the contribution to the total LfHP inhibition from the compounds in the organic fraction (containing oligophenols) was smaller in total than the contribution from the compounds in the water fraction (containing xylooligosaccharides both without substitution and with acetyl, feruloyl and arabinose substitutions) in both LfHP 1 and LfHP 2 cases (Figure 4).

The water fraction containing the xylooligosaccharides (with and without substitutions) thus accounted for approximately 80% of the total inhibition exerted by the LfHP 1 (Figure 4). The content of xylooligosaccharides in LfHP 2 decreased to 22 % (w/w of dry matter) from 37 % in LfHP 1 and the inhibition from the 2-butanone fraction increased from 11 % in LfHP 1 to 18 % in LfHP 2.
Even though the more severe pretreatment of LfHP 2 resulted in formation of more oligophenolic compounds and their potency increased (Figure 1), the majority (75 %) of the overall inhibition from LfHP 2 when compared on an equal mass base to LfHP 1 was still from the water fraction containing the xylooligosaccharides (with and without substitutions) (Figure 4).
The above results and the recent finding, that xylose is heavily involved in inhibitor formation by several synthesis routes [11] stress, that pretreatment of biomass is a balance between degradation of xylooligosaccharides and xylose to oligophenols. One solution, to have xylooligosaccharides hydrolysed to xylose and avoid xylose degradation at the same time, is to protect the anomeric center in xylose as earlier reported [11].

3.3 Enzymatic removal of xylooligosaccharides and subsequent evaluation of cellulase inhibition

To investigate if all inhibition from the water phase was exerted solely by enzymatically degradable xylooligosaccharides (with and without substitution), they were enzymatically removed prior to test in the avicel assay.
To remove xylooligosaccharides, the water fraction from the 2-butanone extraction were prehydrolysed with the same enzyme preparation (Cellic CTec3) as used in the enzyme inhibition assay, but at a higher dose and prolonged time: After 8 days 90 % of the xylooligosaccharides were degraded to xylose (data not shown). However, even though the majority of xylooligosaccharides were removed, the prehydrolysates were still inhibiting compared to buffer, which suggests that some other components in the prehydrolysed water fraction were inhibitory to the cellulose degradation (Figure 4).
The compounds in the water fraction still contributed with 81 % of the total inhibition from the LfHP 1, whereof 70 % were contributions from xylooligosaccharides, that were hydrolysable in the prehydrolysis, and 11 % were contributions from other components (Figure 4). Thus the vast majority of inhibition from the water fraction was confirmed to be due to hydrolysable xylooligosaccharides. However, other components also played a role. 10 % of the xylooligosaccharides (including various substituted xylooligomers) were not degraded by the enzymatic treatment and are accounted for in the category of other components in the water fraction. Hence, these compounds most likely contributed to inhibition in this category.

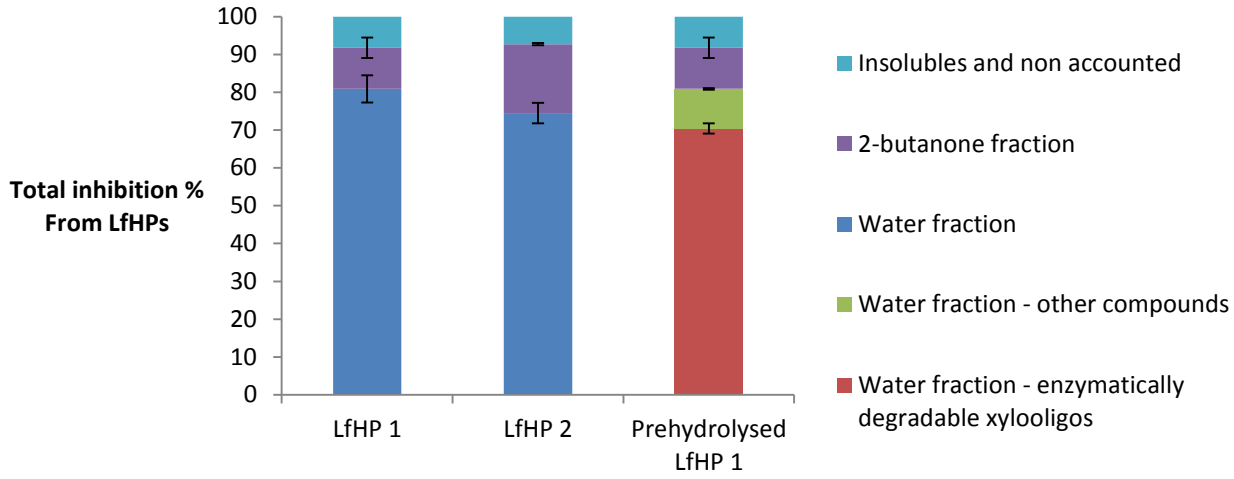


Figure 36. Inhibition of cellulases exerted by the compounds in LfHP 1, LfHP 2 and LfHP 1 with enzymatic removal of xylooligosaccharides (Prehydrolysed LfHP 1).

3.4 Residual compounds after enzymatic hydrolysis

In order to investigate the structures of the residual compounds after enzymatic hydrolysis, the intensively enzymatically prehydrolysed water fraction was analysed with LC-MS/MS.

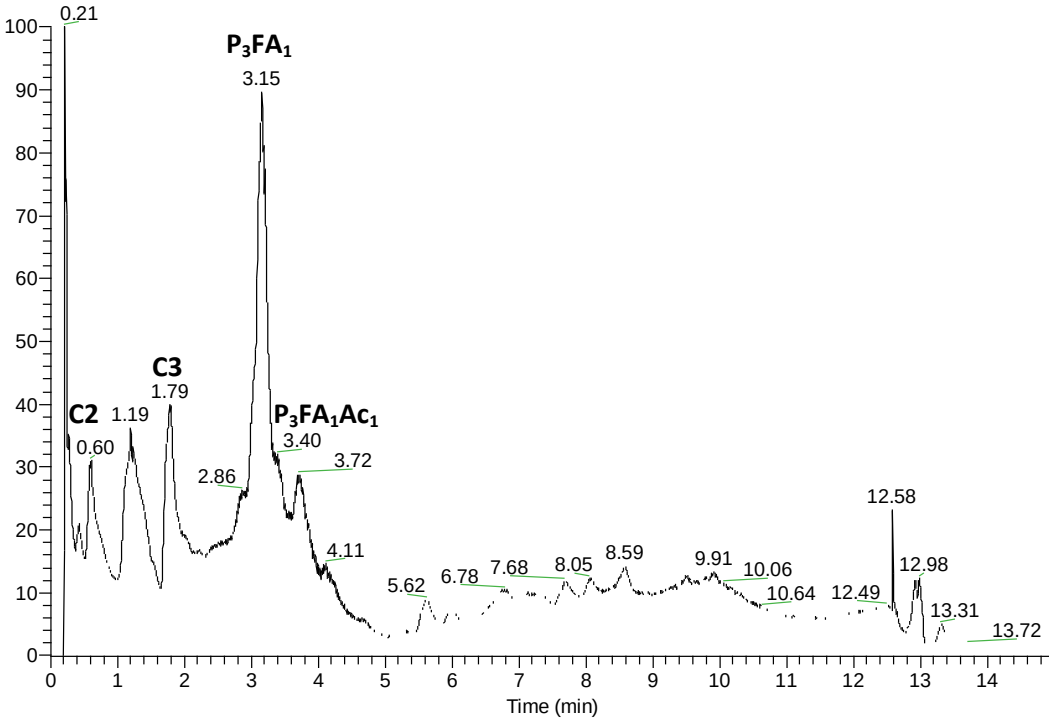


Figure 37. Total Ion Chromatogram (negative ionisation) of prehydrolysed water fraction from 2-butanone extraction of LfHP 1. C2: Compound 2 (Figure 6), C3: Compound 3 (Figure 6), P_3FA_1: Feruloylated tripentose, $P_3FA_1Ac_1$: Feruloylated acetylated tripentose.

The major compound in the TIC from the enzymatically prehydrolysed water fraction corresponded to a feruloylated tripentose, which was present as different isomers with retention times around 3.15 minutes (Figure 5). Upon fragmentation (Table 1) it formed fragments, corresponding to feruloylated dipentose (*m/z*

457), feruloylated pentose (*m/z* 325) and ferulic acid (*m/z* 193) together with neutral mass losses of 90 and 60, which are characteristic for glycosides [14]. Water loss was also evident and especially profound for the feruloylated dipentose fragment (*m/z* 439) and for the $[M- H^+-60]^-$ fragment.

Feruloylated pentose oligomers from mild acid pretreated corn fiber, have previously been reported to accumulate after treatment with enzyme mixtures not containing feruloyl esterase activities [15].

In the present study, feruloylated acetylated tripentoses were also present (Table 1). They exhibited the same type of fragmentation pattern as feruloylated tripentoses i.e. breakdown to its substructures and neutral mass losses of 18, 60 and 90. One important fragment from the precursor ion 631.18663 is *m/z* 367.10300 (Table 1), which corresponds to a pentose substituted with both an acetyl group and a feruloyl group, thus suggesting that tripentoses with acetyl and feruloyl at the same pentose were present.

Two of the other major compounds in the total ion chromatogram (Figure 5) were found to be a new type of residual compounds after enzymatic hydrolysis (Table 1 and Figure 6). They are dipentoses, which have a highly hydroxylated bicyclic residue consisting of 2 oxanes with a double bond, attached at the unprotected anomeric center of the dipentose (Figure 6). Both compounds displayed the same type of fragmentation pattern with fragments corresponding to the bicyclic residue with either none, one or two pentoses (Table 1). Within these substructures neutral mass losses of 60 and 18 were also observed (Table 1).

3.5 Proposed reaction mechanism for formation of bicyclic residues

The bicyclic residues are proposed to be reaction products from pretreatment, where xylose and glucose have undergone isomerisation to xylulose and fructose respectively [16,17], followed by aldol condensation with glyceraldehyde **4** (Figure 7), which is present due to degradation of hexoses[18]. Two ring closures lead to the final bicyclic residues **5** and **6** (Figure 7).

To form the pentose dimers with the bicyclic moieties (compounds **2** and **3** Figure 6), compounds **5** and **6** are suggested to have reacted at the reducing end of an oligosaccharide, at any length, which is capable of forming an oxocarbenium ion. The oxocarbenium ion is readily available for nucleophile attack from the hydroxylated bicyclic compounds. It is likely that the enzymes can degrade pentose oligomers with the bicyclic residue down to dipentose level, because up to pentapentose levels with bicyclic moieties were observed in the mass spectra before enzymatic treatment, but not after.

Table 12. Compounds in prehydrolysed water fraction from 2 butanone extraction of LfHP 1.

Retention time (min)	Mass (*m/z*) $[M- H^+]^-$	Fragments MS/MS (relative intensity)	Compound
0.60	499.13094	499.13094 (100), 439.10976 (27), 421.09933 (8), 367.08855 (6), 307.06721 (3), 235.04579 (0.1), 175.02422 (2)	Compound 2 (Figure 6)
1.19	513.14941	Could not be found in MS/MS. Possibly due to double charged ion (1027 ion observed)	Not determined
1.79	513.14649	513.14649 (100), 453.12512 (25), 435.11457 (10), 381.10398 (8), 363.09342 (4), 321.08258 (2), 317.08769 (4), 249.06123 (9), 189.03979 (4)	Compound 3 (Figure 6)
3.15	589.17976	589.17976 (0.5), 529.15801 (1), 511.14736 (18), 499.14763 (4), 457.13649 (17), 439.12602 (58), 325.09332 (100), 265.07214 (52), 235.06125 (10), 193.05033 (14), 175.03972 (4)	Feruloylated tripentose
3.40	631.18663	631.18663 (3), 571.16618 (38), 553.15579 (100). 541.15589 (44), 511.14512 (26), 499.14525 (16), 493.13464 (12), 481.13468 (90), 457.13479 (14), 439.12414 (88), 421.11369 (9), 367.10300 (14), 325.09243 (86), 307.08182 (8), 265.07131 (51), 235.06051 (11), 193.04979 (35), 175.03918 (18)	Feruloylated acetylated tripentose

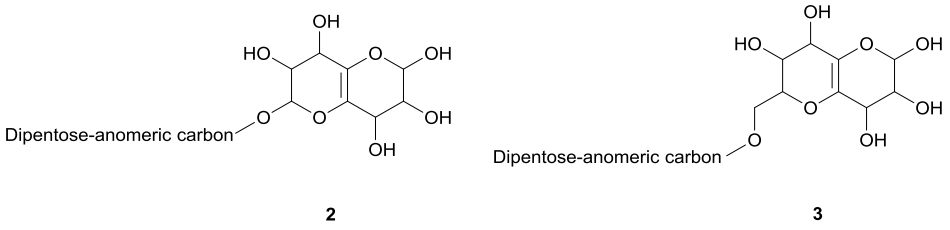


Figure 38. Proposed structures for two new residual compounds after enzymatic hydrolysis. Note that substitution position is given for clarity, but regioisomerism was not determined.

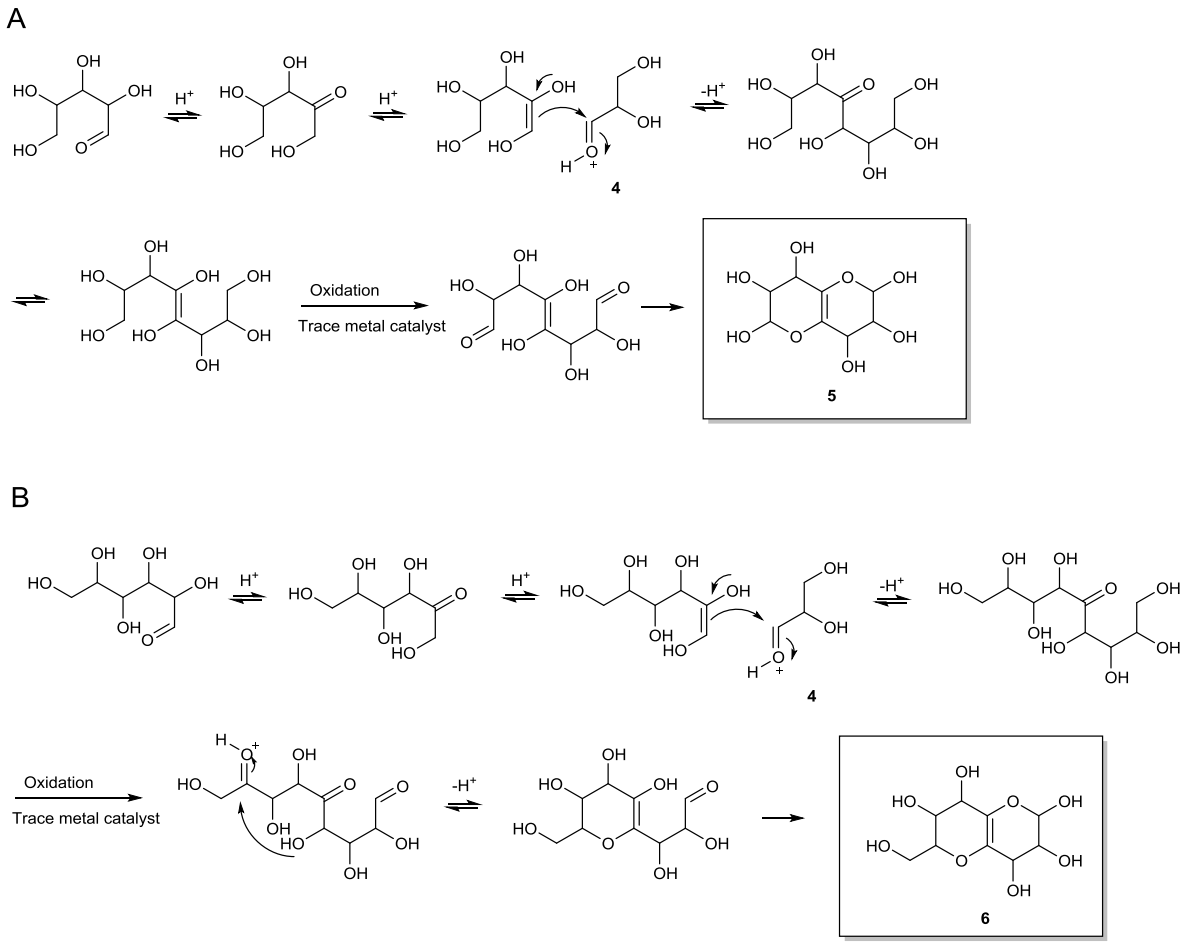


Figure 39. Proposed mechanisms for formation of the bicyclic residues in A) dipentose 2 and B) dipentose 3 (Figure 6).

It is unknown at the present point in time, if enzymes activities that catalyse cleavage of pentoses from the bicyclic residue in compound **2** and **3** exist.

In addition to their accumulation after enzymatic hydrolysis, it is also possible that both compounds **2** and **3** as well as acetylated and/or feruloylated tripentoses are inhibitors towards enzymes. The bicyclic residues in compound **2** and **3** have structural similarities with compounds found in the 2-butanone fraction [11], which contained the most inhibitory compounds and shorter feruloyl substituted pentose oligomers were found in the very inhibiting 2-butanone fraction, whereas the longer feruloyl substituted pentose oligosaccharides were in the water fraction [11].

Protection of the anomeric center in xylose [11], as well as the anomeric center at the reducing end of a xylooligosaccharide, will hinder formation of the enzyme resistant compounds **2** and **3**.

4 Conclusions

In processes applying enzyme catalysed reactions, a challenge is that enzyme inhibiting compounds are generated and liberated during pretreatment of biomass and some compounds are not degraded by enzymatic hydrolysis.

A solvent extraction approach was applied to separate xylooligosaccharides and oligophenolic compounds and determine contribution from oligophenolic compounds and enzyme hydrolysable xylooligosaccharides to total inhibition from hydrothermal pilot scale pretreated wheat straw. An increase in severity of the pretreatment lead to more oligophenol compounds and thus the overall inhibition increased, but compared on an equal dry matter base the xylooligosaccharides still contributed most to overall inhibition at the investigated pretreatment severities. Although the relative abundance of different compounds changed with pretreatment severity, the compounds present were similar.
After enzymatic removal of xylooligosaccharides from the water fraction from 2-butanone extraction some inhibition remained compared to the control, and also two major types of residual compounds after enzymatic hydrolysis were found; feruloylated tripentoses and pentose dimers with bicyclic moieties. The latter are novel dipentose compounds with a hydroxylated oxane bicyclic residue and reaction mechanisms for their synthesis is proposed.

Acknowledgement

This work was supported by the Danish National Advanced Technology Foundation *via* the Technology Platform “Biomass for the 21st century – B21st”.

REFERENCES

1. D. M. Alonso, S. G. Wettstein and J. A. Dumesic, *Chem. Soc. Rev*., 2012, **41**, 8075–8098
2. T. D. Matson, K. Barta, A. V. Iretskii and P. C. Ford, *J. Am. Chem. Soc*., 2011, **133**, 14090–14097
3. X. Wang and R. Rinaldi, *Angew. Chem., Int. Ed*., 2013, **52**, 11499–11503.
4. P. Gallezot, *Chem. Soc. Rev*., 2012, **41**, 1538–1558
5. M. Besson, P. Gallezot and C. Pinel*, Chem. Rev.*, 2014, **114**, 1827–1870
6. R. Rinaldi, *Angew. Chem., Int. Ed.*, 2014, **53**, 8559–8560.
7. L. J. Jönsson and C. Martín. *Bioresour. Technol.*, 2016, **199**, 103–112
8. H. Rasmussen, H.R. Sørensen and A.S. Meyer. *Carbohydr. Res,.* 2014, **385**, 45-57.
9. A. Tejirian and F. Xu. *Enzyme Microb. Technol.,* 2011*,* **48**, 239-247.
10. R. Kont, M. Kurasin, H. Teugjas and P. Väljemäe.. *Biotechnol. Biofuels*., 2013, **6**,135.
11. H. Rasmussen, D. Tanner, H.R. Sørensen and A.S. Meyer. Unpublished work.
12. L. Rosgaard, S. Pedersen, J. Langston, D. Akerhielm, J.R. Cherry and A.S. Meyer. *Biotechnol. Prog.,* 2007, **23**, 1270–1276.
13. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. 2006, NREL - Biomass Program.
14. A.G.A.W. Alakolanga, A.M.D.A. Siriwardane, N. S. Kumar, L. Jayasinghe, R. Jaiswal and N. Kuhnert. *Food Res. Int.,* 2014, **62**, 388–396.
15. M. M. Appeldorn, P. de Waard, M. a. Kabel, H. Gruppen and H. A. Schols. *Carbohydr. Res.*, 2013, **381**, 33-42.
16. O. Ershova, J. Kanervo, S. Hellsten and H. Sixta. *RSC Adv.*, 2015, **5**, 66727
17. H. Kimura, M. Nakahara and N. Matubayasi. *J. Phys. Chem. A,* 2011, **115**, 14013–14021.
18. T. Flannelly, M. Lopes, L. Kupiainen, S. Dooley and J. J. Leahy. *RSC Adv*., 2016, **6**, 5797.

SUPPLEMENTARY INFORMATION

Pentose dimers with bicyclic moieties from pretreated biomass

Helena Rasmussen[a], Hanne R. Sørensen[a], David Tanner[b] and Anne S. Meyer[c*]

[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark

[b] Dept. of Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark.

[c] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Lyngby, Denmark.

*Corresponding author: e-mail: am@kt.dtu.dk; Tel: (+45) 45 25 2800

Table 13. Comparison of water fractions from 2-butanone extraction of LfHP 1 and LfHP2.

Retention time (min)	Ions, *m/z* $[M- H^+]^-$	Source	Compounds
0.34	281.08670, 413.12897, 545.17053	LfHP 1	Pentoses DP2, DP 3, DP 4.
0.34	281.0864, 413.12854, 545.17017	LfHP 2	Pentoses DP2, DP 3, DP 4.
0.47	281.08667, 413.12878, 545.17102, 677.21277	LfHP 1	Pentoses DP2, DP 3, DP 4, DP 5
0.47	281.0849, 413.12857, 545.17041, 677.21246	LfHP 2	Pentoses DP2, DP 3, DP 4, DP 5
0.61	545.17047, 677.21411, 809.25598	LfHP 1	Pentoses DP 4, DP 5, DP6.
0.58	545.17053, 677.21307, 809.25549	LfHP 2	Pentoses DP 4, DP 5, DP6.
0.89	677.21252, 809.25641, 941.29730	LfHP 1	Pentoses DP 5, DP 6, DP 7.
0.88	677.21240, 809.25648, 941.29651	LfHP 2	Pentoses DP 5, DP 6, DP 7.
1.15	455.13885, 809.25500, 941.29755	LfHP 1	Pentoses + 1 acetyl, DP 3. Pentoses DP 6, DP 7.
1.15	455.13895, 809.25488, 941.29688	LfHP 1	Pentoses + 1 acetyl, DP 3. Pentoses DP 6, DP 7.
1.34	941.29785, 1073.33887	LfHP 1	Pentoses DP 7, DP 8.
1.34	941.29651, 1073.33862	LfHP 2	Pentoses DP 7, DP 8.
1.48	587.18073, 941.29657	LfHP 1	Pentoses + 1 acetyl, DP 4. Pentose DP 7.
1.48	587.18073, 941.29669	LfHP 2	Pentoses + 1 acetyl, DP 4. Pentose DP 7.
1.70	941.29742, 1073.34097, 1205.38257	LfHP 1	Pentoses DP 7, DP 8, DP 9
1.70	587.18115, 719.22363, 851.26581, 983.30768	LfHP 2	Pentoses + 1 acetyl, DP 4, DP 5, DP 6, DP 7
1.78	587.18103, 719.22339, 851.26563, 983.30780, 1115.35010, 1247.39148, 1379.43311	LfHP 1	Pentoses + 1 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9, DP 10
1.78	587.18109, 719.22333, 851.26569, 983.30768, 1115.34949, 1247.39160, 1379.43335	LfHP 2	Pentoses + 1 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9, DP 10
1.96	629.19171, 761.23376, 893.27631, 1025.31848, 1157.36035, 1289.40210, 1421.44312	LfHP 1	Pentoses + 2 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9.
1.96	629.19183, 761.23413, 893.27649, 1025.31824, 1157.36023, 1289.40283, 1421.44348	LfHP 2	Pentoses + 2 acetyl, DP 4, DP 5, DP 6, DP 7, DP 8, DP 9.

Table 14. Comparison of 2-butanone fractions from 2-butanone extraction of LfHP 1 and LfHP2. Please refer to ref 11 for structures of compounds marked in italic. Compounds marked with * were subjected to fragmentation and showed the same fragmentation pattern in LfHP 1 and LfHP 2.

Retention time (min)	Ions, *m/z* $[M- H^+]^-$	Source	Compounds
2.85	163.03880*	LfHP 1	Compound **1**
2.85	163.03870*	LfHP 2	Compound **1**
3.04	181.04932	LfHP 1	Not determined
3.04	181.04929	LfHP 2	Not determined
3.20	*265.07108, 325.09229*, 457.13498, 589,17682	LfHP 1	Pentose + feruloyl, DP 2, DP 3.
3.20	*265.07068, 325.09167*, 457.13583, 589,17706	LfHP 2	Pentose + feruloyl, DP 2, DP 3.
3.28	*265.07095, 327.08679, 357.09720, 387.10751**	LfHP 1	
3.28	*265.07071, 327.08627, 357.09689*, *387.10736**	LfHP 2	
3.35	*327.08643, 357.09790, 387.10760**	LfHP 1	
3.35	*327.08630, 357.09698, 387.10760**	LfHP 2	
3.41	*327.08655, 387.10889**	LfHP 1	
3.40	*327.08624, 387.09686**	LfHP 2	
3.52	*357.09763, 387.10760**, 601.17664	LfHP 1	Pentose + 1 acetyl + cumaroyl, DP 3
3.52	*307.04510*, 357.09689, 387.10736**, 601.17670	LfHP 2	Pentose + 1 acetyl + cumaroyl, DP 3
3.63	*187. 09630*, *307.04520*, 323.11258, *387.10849**, 631.18738	LfHP 1	Pentose + 1 acetyl + feruloyl, DP 3
3.63	*187. 09613, 307.04489** 323.11227, *387.10745**, 631.18695	LfHP 2	Pentose + 1 acetyl + feruloyl, DP 3
3.75	*239.12767, 275.09158, 305.10199, 387.10757**	LfHP 1	
3.75	*239.12756, 275.09137, 305.10172, 387.10721**	LfHP 2	
3.89	425.08713	LfHP 1	Not determined
3.89	425.08713	LfHP 2	Not determined
4.21	*371.11252, 383.11255 401.12302,*	LfHP 1	
4.21	*371.11218, 383.11212 401.12259,*	LfHP 2	
4.58	*301.07056*	LfHP 1	
4.58	*301.07047*	LfHP 2	

Paper 4

4-Hydroxybenzoic acid from hydrothermal pretreatment of oil palm empty fruit bunches - its origin and influence on biomass conversion

Helena Rasmussen,[a] Kit H. Mogensen,[a] Martin D. Jeppesen,[a] Hanne R. Sørensen[a] and Anne S. Meyer[b*]

[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark

[b] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 229, DK-2800 Lyngby, Denmark.

*Corresponding author: e-mail: am@kt.dtu.dk; Tel: (+45) 45 25 2800

Abstract

In the present work an unknown major compound, characteristically occurring during processing of oil palm empty fruit bunches was identified with LC-DAD-ESI-MS/MS to be 4-hydroxybenzoic acid. A hypothesis to its origin, other than lignin degradation, was tested. The route considered involves degradation of rhamnose to 5-methylfuran-2-carbaldehyde followed by reaction with formic acid. Experimental hydrothermal pretreatment of pure rhamnose in the presence of formic acid revealed that 5-methylfuran-2-carbaldehyde is in fact a degradation product from rhamnose, analogous to glucose degradation to 5-(hydroxymethyl)-2-furaldehyde (HMF). However, the carboxylation with formic acid to form 4-hydroxybenzoic acid was found not to take place in practice at realistic biomass hydrothermal pretreatment conditions. It was therefore concluded that the 4-hydroxybenzoic acid liberated during hydrothermal pretreatment of oil palm empty fruit bunches, originates from the lignin in the oil palm empty fruit bunches. This interpretation is in line with that 4-hydroxybenzoic acid is a structural element in lignin, readily accessible for cleavage and characteristic to oil palm empty fruit bunches.

Assessment of the influence of 4-hydroxybenzoic acid in the enzymatic hydrolysis of pretreated oil palm empty fruit bunches as well as its presence during fermentation showed that 4-hydroxybenzoic acid is not inhibitory neither on the enzymatic hydrolysis or fermentation in the quantified range from 0.1 g/L to 1 g/L. However, methylfuran-2-carbaldehyde may be an important degradation compound from biomasses rich in deoxysugars such as rhamnose or fucose, e.g. pectin rich biomasses such as sugar beet pulp or similar feedstocks applied in bioethanol production.

Keywords: 5-methylfuran-2-carbaldehyde, rhamnose, fucose, reaction mechanism, degradation compound, pectin.

1. Introduction

A wealth of biomasses as well as processing methods are available for biorefinery solutions - whether the goal is to supply environmentally friendly energy or valuable compounds available for further synthesis [1-5]. In order to avoid formation of undesirable products during processing and thus to attain optimal product yields it is a key prerequisite to know not only the structural composition of the biomass, but also to understand the reactions of the structural elements during processing. - Especially seen in the light of that undesired compounds that inhibit the enzymes and yeast can be formed during pretreatment in the production of 2nd generation bioethanol [6].

The empty fruit bunches left over from palm oil processing have been prospected as an abundantly available lignocellulosic feedstock for cellulosic ethanol production, notably in the South East Asia and South America [7]. In order to implement oil palm empty fruit bunches (EFB) as a successful feedstock in 2nd generation bioethanol production much work is going on to obtain efficient conversion processes [8-10]. In the present work, the appearance of an unknown compound was consistently observed in HPLC chromatograms when monitoring EFB processing. The unknown compound was formed as a major product during hydrothermal pretreatment and was characteristic for EFB. The compound was thus not present after hydrothermal pretreatment of wheat straw or corn stover at similar severity (Figure 1). In order to rule out any undesirable effects and to understand the nature and origin of this compound, the objective of this work was to identify the compound and trace its possible origin.

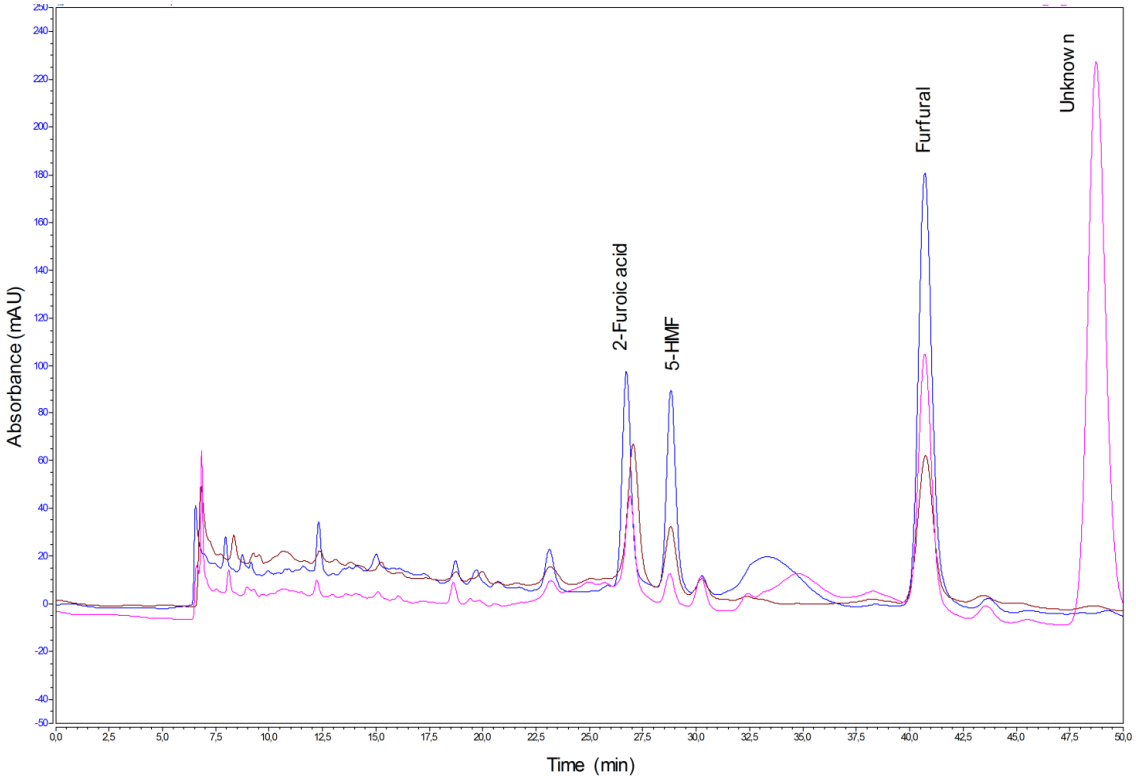


Figure 40. HPLC analysis from pretreatment at a severity of approx. 4.0 of wheat straw (blue), corn stover (brown) and EFB (pink). Unknown compound rt 49.5 minutes.

2. Materials and Methods

2.1 Chemicals

Buffer chemicals, solvents, acids and bases, avicel (avicel® PH-101) as well as 4-hydroxybenzoic acid were purchased from Sigma Aldrich (Steinheim, Germany). 5-Methylfuran-2-carbaldehyde was from Vitas-M Laboratory (Apeldoorn, The Netherlands).

2.2 Biomass

EFB was acquired by DONG Energy from Malaysia fresh from an oil mill, transported cooled (< 5 °C) and stored at -18 °C. Prior to use it was thawn over 2 days to reach ambient temperature.

2.3 Enzymes and yeast

Cellic CTec3 (1920 BHU(2)/g) was obtained from Novozymes A/S (Bagsværd, Denmark). Apart from the cellulolytic enzyme base from *Trichoderma reesei* containing at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, and a β-xylosidase [11], the Cellic CTec3 enzyme preparation also contains other proprietary hydrolysis-boosting proteins.

Dry yeast *Saccharomyces cerevisiae* was acquired from Lallemand, (Montreal, Canada).

2.4 Pilot plant pretreatment

Hydrothermal biomass pretreatment was performed at 200 °C for 18 minutes at 40 % (w/w) dry matter with continuous feeding at a rate of approximately 50 kg (dry matter) EFB per hour. The pretreatment was performed in the Inbicon pilot pretreatment plant in Fredericia, Denmark. The pretreated material was separated into a fiber fraction and a liquid fraction. The liquid fraction was analysed as described below and both the fiber and liquid fractions were stored at -18 °C until enzymatic hydrolysis.

2.5 Isolation of 4-hydroxybenzoic acid from the pretreatment liquid

Preparative chromatographic purification was performed on an Äkta Purifier 100 system (GE Healthcare, Uppsala, Sweden) with a 6 mL Resource S column from GE Healthcare. Elution was carried out with 20 mM BIS-Tris (pH = 6.5) at 0.6 mL/min with isocratic conditions and fraction collection. The selected fraction was extracted with dichloromethane under acidic conditions (1 % (vol/vol) formic acid) and the solvent was evaporated under reduced pressure at 45 °C.

2.6 Enzyme inhibition assessment

Avicel

4-hydroxybenzoic acid was dissolved in 0.1 M acetate buffer (pH 5.1) to a concentration of 1 g/L and avicel was added to 12 % (w/w). The Cellic CTec3 enzyme dose was 53 g/kg glucan and incubation was at 50 °C, 160 rpm. All assays were made as duplicates with displaced sampling. Upon sampling (200 μL) the enzyme was inactivated at 99 °C for 5 minutes.

Pretreated EFB

The experiments were carried out in 100 g scale with 12 % (w/w) dry matter of the pilot process pretreated EFB (as described above). The hydrolysis was carried out in 50 mM citrate buffer (pH 5.1) or in the pilot process liquid from the hydrothermal pretreatment (LfHP) of EFB (adjusted to pH 5.1 with NaOH) or a 1:1

mixture of the two. The Cellic CTec3 enzyme dose was 53 g/kg glucan. The flasks were incubated at 50 °C for 100 hours, upon sampling the enzyme was inactivated at 99 °C for 15 minutes. All enzymatic hydrolysis runs were prepared as duplicates.

Glucose release was measured with High Pressure Anion Exchange Chromatography (HPAEC) as described below.

2.7 Fermentation

The fermentation was carried out in 25 g scale with the hydrolysis liquid from the pilot EFB hydrolysis process. 4-HBA was added to final concentrations 0, 1 and 10 g/L. 100 mg dry yeast (*S. cerevisiae* from Lallemand) was suspended in 5 mL 0.08 M citrate-phosphate buffer (pH 5.7) and incubated for 30 minutes and subsequently added to 20 g hydrolysis liquid to a final yeast dry matter concentration of 4 g/L. The fermentation was incubated at 32 °C for 30 hours and fermentation progress was monitored as weight loss measurements. All flasks were prepared in duplicates. At the end of the experiment, ethanol concentration was measured with HPLC as described below.

2.8 Extraction of empty fruit bunches before pretreatment

The extraction was carried out both at room temperature and at 50 °C: 6.2 g un-pretreated empty fruit bunch was placed in 70 mL water at room temperature and 70 mL water at 50 °C respectively for 2 days. The aqueous extracts were analysed with HPLC as described below (section 2.11.2).

2.9 4-hydroxybenzoic acid release during hydrothermal pretreatment - mini pretreatment

The test was performed in a loop autoclave [12,13] at 1 L scale at 6 % (w/w) dry matter empty fruit bunches milled to 1 mm. To validate the presence of 4-HBA the suspension was spiked with 4-HBA to a concentration of 2.5 mg/L (which was subtracted from the final results) prior to hydrothermal pretreatment at 200 °C. The pretreatment was continued for 33 minutes with sampling every 2-5 minutes. The samples were analysed with HPLC as described below (section 2.11.2).

2.10 Rhamnose degradation - mini pretreatment

The test was performed in a loop autoclave (see section 2.9) at 1 L scale with 100 g rhamnose and 3 equivalents (1.83 g) formic acid at 200 °C for 20 minutes. The reaction mixture was analysed with HPLC as described below.

2.11 Analytical methods

HPLC

Analysis was performed on a Dionex (ThermoScientific, Sunnyvale, California, USA) Ultimate 3000 UHPLC with RI and dual wavelength detection (210 and 250 nm) with a Rezex RHM monosaccharide H+ (8%) column, 7.80 x 300 mm (Phenomenex, Torrance, CA, USA)). Column temperature was 65 °C. The method was a 50 minutes isocratic method with 5mM sulphuric acid in water and 0.6 mL/min flow [14].

HPLC - quantification of 4-hydroxybenzoic acid

Quantification of 4-HBA was carried out on a Dionex (ThermoScientific, Sunnyvale, California, USA) Ultimate 3000 UHPLC equipped with a DAD detector and a pentafluorphenyl column 2.1 x 100 mm, particle size 2.6 μm (Phenomenex). The method had the eluent composition: 100 % water (containing 0.025 % TFA) for the

first 15 minutes, where after the composition was changed to 80 % acetonitrile, 20 % water (containing 0.025 % TFA) over 4 minutes. This eluent composition was kept for 3 minutes where after the composition was changed back to starting conditions over 0.1 minutes and the system was equilibrated for 2.9 minutes giving a total analysis time of 25 minutes. The flow rate was 0.65 mL/min.

HPAEC

Glucose analysis was performed on a ICS-5000 Dionex ThermoFisher Scientific (ThermoScientific, Sunnyvale, California, USA) analytical system with pulsed amperometric detection and a PA1 column, 2 x 250 mm (Thermo Scientific). The method was a 2 step isocratic method with an additional final cleaning step with KOH concentrations held constant at 25, 10 and 100 mM for 5, 14 and 7 minutes respectively. The flow rate was 0.25 mL/min.

LC-(DAD)-ESI-MS/MS (identification of 4-hydroxybezoic acid)

LC-MS and LC-MS/MS analyses were performed on an Agilent (Santa Clara, California) 6550 quadropole time of flight instrument with electrospray ionisation in negative ionisation. CID energies for MS/MS were 10, 20 and 40 eV.

The LC conditions were 0.35 mL/min with a gradient from 100 % water containing 20 mM formic acid to 50 % methanol over 12 minutes. The eluent composition was changed to 100 % methanol over the next 2 minutes followed by a change to 100 % water (20 mM formic acid) over 1 minute. The system was equilibrated 2 minutes with 100 % water (20 mM formic acid).

The column was an Agilent Poroshell Phenyl, L x I.D. 10 cm x 2 mm, 2.6 μm particle size.

The DAD spectrum wave length range was from 190 nm to 640 nm.

Library fragmentation patterns and UV spectra was from an earlier run of 2-hydroxybenzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid respectively.

LC-MS/MS (origin of 4-hydroxybenzoic acid)

LC-MS and LC-MS/MS analyses were performed on a Thermo (ThermoFisherScientific, Waltham, MA, USA) Orbitrap Fusion instrument with electrospray ionisation. Spray voltage was 2500 in negative ionisation and HCD energy for MS/MS was 25 %. LC conditions were a gradient from 100 % water (containing 0.025 % TFA) -100 % acetonitrile (containing 0.025 % TFA) over 10 minutes. 100 % acetonitrile (0.025 % TFA) was kept for 2 minutes and changed to 100 % water (0.025 % TFA) over 0.1 minute. The system was equilibrated 3 minutes with 100 % water (0.025 % TFA).

The column was a pentafluorphenyl (pfp) discovery HS F5, L x I.D. 15 cm x 4.6 mm, 5 μm particle size (Supelco, Sigma-Aldrich, Steinheim, Germany).

3. Results and Discussion

3.1 Purification and isolation of the unknown compound

In order to identify the unknown compound it was isolated from other compounds in the mixture by preparative chromatographic purification. Different eluent systems were tested: Water/formic acid, water/acetic acid, water/MES, water/BIS-Tris and water/Tris and in addition water with sulphuric acid, which corresponded to the eluent in the analytical chromatographic method. Water/BIS-Tris elution was found to

give the best chromatographic separation in the preparative method (data not shown) and was used in the further purification. Three fractions were collected and fraction number 3 was found to contain the unknown compound (rt 50.5 min) at highest purity and concentration (S1, Figure 1). Thus fraction 3 was extracted with dichloromethane and used in the subsequent LC-(DAD)-ESI-MS/MS analysis.

3.2 Identification of the unknown compound by LC-(DAD)-ESI-MS/MS

In accordance with the HPLC data (S1, Figure 1) one major compound with UV absorbance at 250 nm was observed with diode array detection (Figure 2, middle pane). This compound was also the most abundant compound in the TIC scan (Figure 2, top pane) and gave rise to the masses 137.02465 and 93.03509 (Figure 2, bottom pane) in negative ionisation.

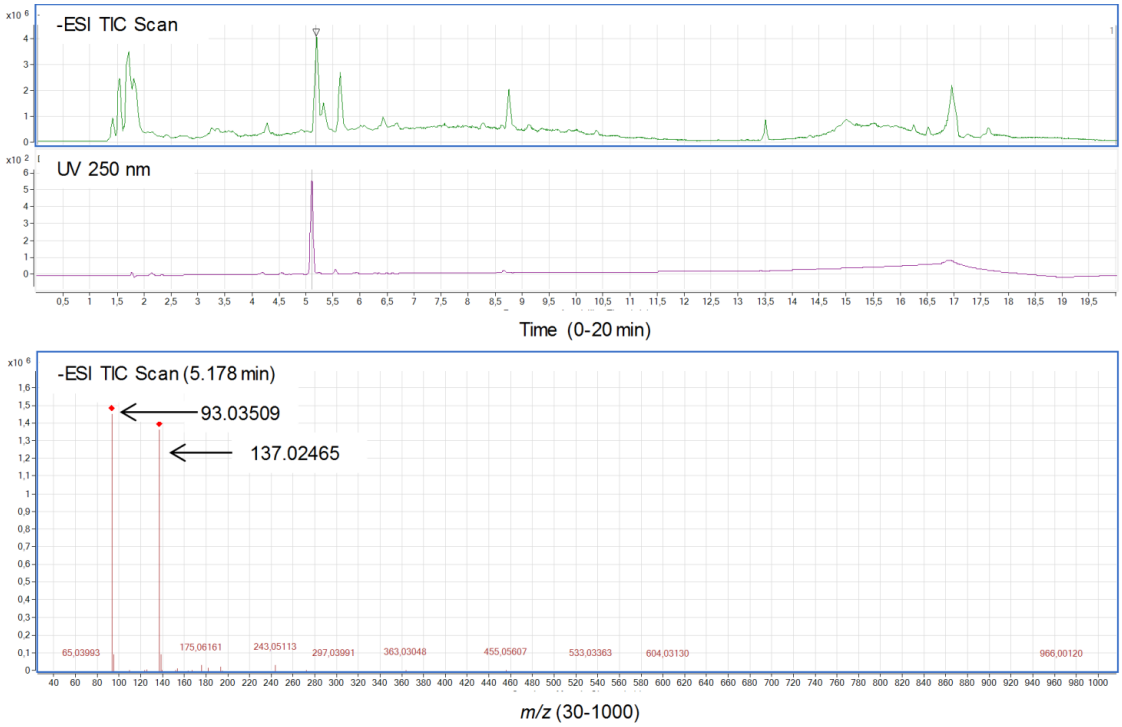


Figure 41. Total ion chromatogram, negative ionisation. Middle: Diode array detection. Bottom: Ions at the retention time appointed in the top pane.

The former mass corresponds to molecular formula $C_7H_6O_3$ and the latter to its loss of CO_2 i.e. a decarboxylation. When the 137.02465 ion was subjected to fragmentation at 10, 20 and 40 eV (S1, Figure 2) the major fragment was confirmed to correspond to loss of CO_2, strongly suggesting the presence of a carboxylic acid. With five double bond equivalents, the most likely compound was thus interpreted to be a hydroxyl substituted benzoic acid. This interpretation was further evaluated by comparison of library UV spectra and fragmentation patterns of 2-hydroxybenzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid, respectively. It was found that the experimentally obtained UV spectrum and fragmentation patterns did not correspond well with either 2-hydroxybenzoic acid or 3-hydroxybenzoic acid library data (data not shown), but a good match was obtained for 4-hydroxybenzoic acid (Figure 3 and S1, Table 1). Thus the unknown compound was concluded to be 4-hydroxybenzoic acid (4-HBA).

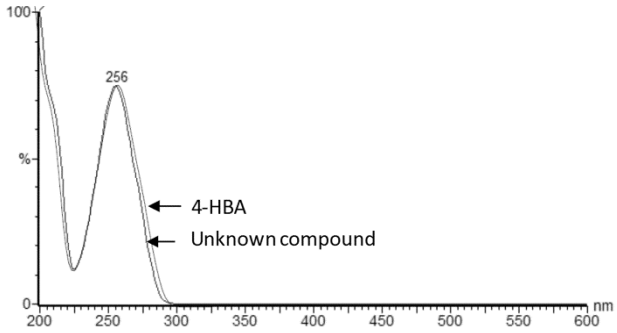


Figure 42. UV spectra of 4-hydroxybenzoic acid and the unknown compound concluded to be the same

Quantification of 4-hydroxybenzoic acid

In order to test if 4-HBA had an inhibitory effect on enzymatic hydrolysis and fermentation in the present pilot process concentrations the level of 4-HBA present in the liquid from the hydrothermally pretreated EFB was quantified and found to be in the range 0.1-1 g/L.

4-HBA has previously been identified in other biomasses as corn stover, hybrid poplar and pine in ranges from 0-0.1 g/l [15] (values converted for comparability to this study) as well as in EFB as the most potential source of 4-HBA [16].

3.3 Origin of 4-hydroxybenzoic acid

An experimental pretreatment of EFB confirmed that 4-HBA is released during hydrothermal pretreatment of the empty fruit bunches (Figure 4). However, 4-HBA has also been reported to be present in the oil palm fruits [17]. To test if the empty fruit bunches contained 4-HBA prior to pretreatment, either as a native compound or as contamination from the fruit, an empty fruit bunch was extracted with water. However, no 4-HBA was detected in the extracts (data not shown).

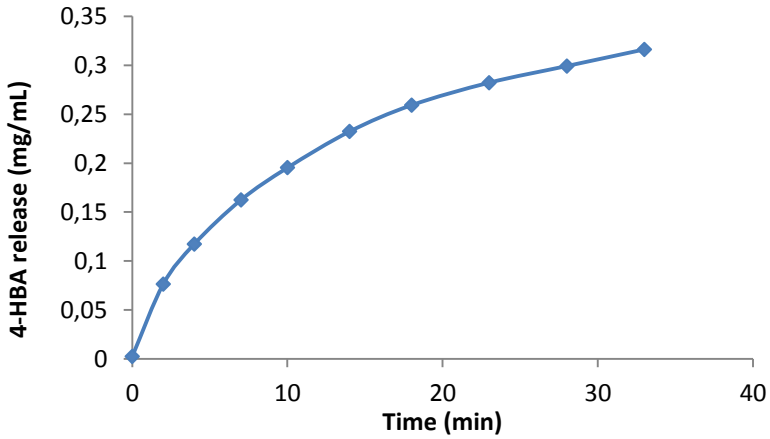


Figure 43. Release of 4-hydroxybenzoic acid (4-HBA) during hydrothermal pretreatment of efb (200 °C, 6 % (w/w) dry matter.

Lu *et al* [16] has recently demonstrated that 4-HBA is a structural lignin component characteristic for EFB and that it is readily accessible for cleavage due to its position on lignin unit side chains. The release of 4-HBA during pretreatment (Figure 4, Figure 5) in is in line with the findings of Lu and coworkers.

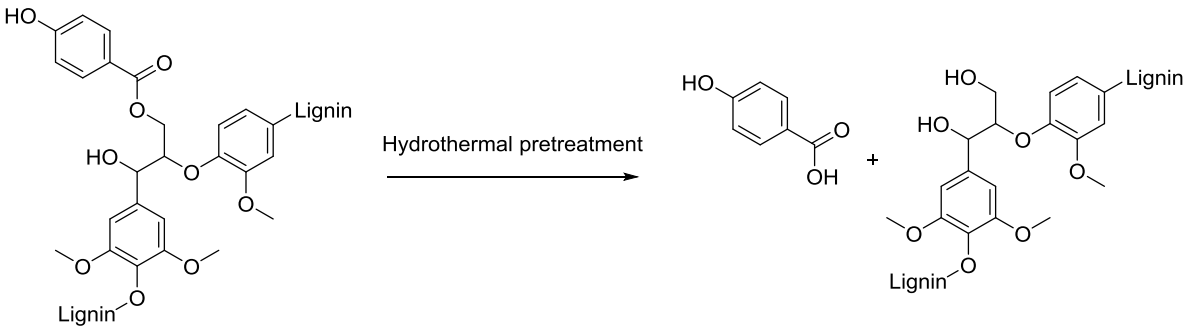


Figure 44. Release of 4-hydroxybenzoic acid during hydrothermal pretreatment.

It can be hypothesised, that 4-HBA can be produced from an additional route arising from biomass degradation compounds reacting with each other as reported earlier [6]. Slightly higher rhamnose levels have been reported in EFB compared to wheat straw [18,19] and a route involving rhamnose degradation to 5-methylfuran-2-carbaldehyde by a degradation route analogues to HMF formation from glucose [6], followed by a carboxylation with formic acid can be suggested (Figure 6). In the proposed mechanism 5-methylfuran-2-carbaldehyde is cleaved to give an 1,4 dione as other furans and the aldehyde function facilitates intramolecular cyclisation to a six membered ring, which undergo dehydration to an activated dihydroxylated benzene. The carboxylation may take place as a Koch-Haaf type reaction with *in situ* formation of CO. The carboxylation may be speculated to take place, due to the high temperature and pressure during the pretreatment, even though no mineral acid is present.

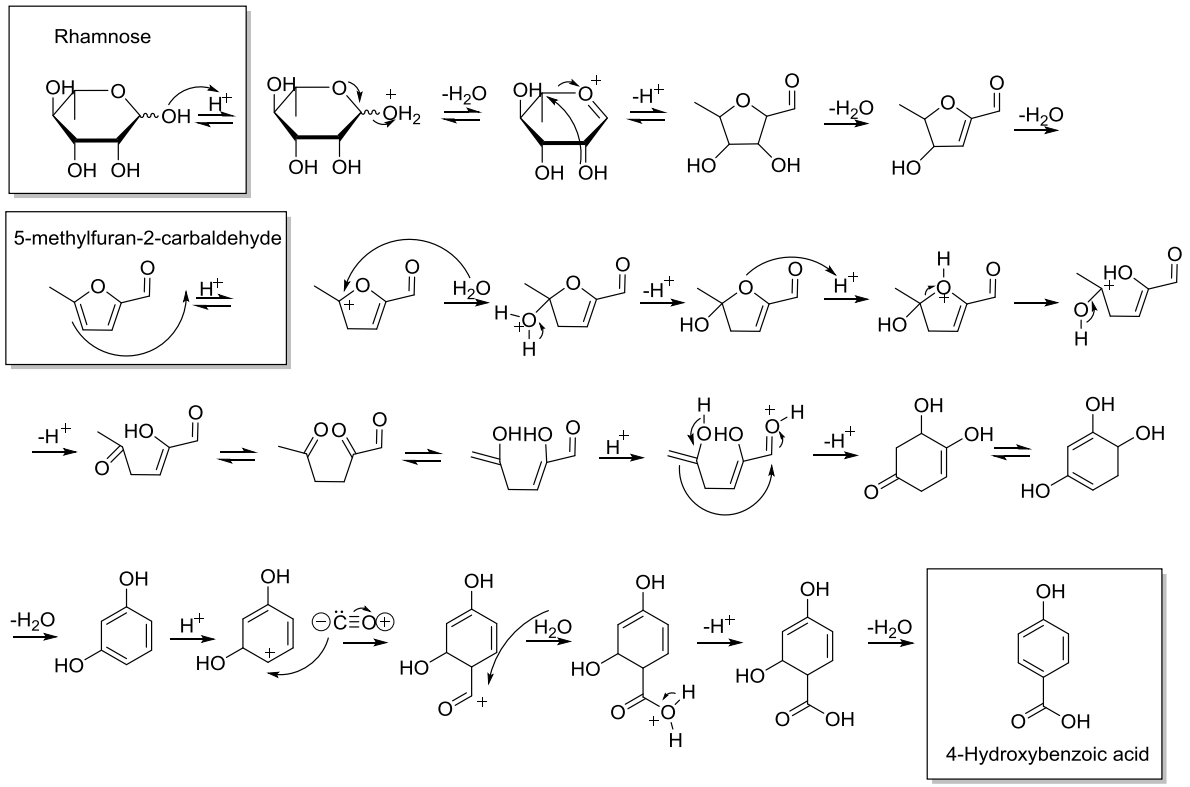


Figure 45. Hypothesised mechanism for formation of 4-hydroxybenzoic acid from rhamnose and formic acid.

To test the hypothesis, rhamnose and formic acid were reacted at pretreatment conditions (200 °C, 20 minutes), where it was found that 5-methylfuran-2-carbaldehyde was actually formed as a reaction product (S1, Figure 3) (confirmed with LC-MS/MS of 5-methylfuran-2-carbaldehyde standard). No 4-HBA was formed, thus suggesting that 5-methylfuran-2-carbaldehyde does not react further with formic acid to form 4-HBA, which may be due to the lack of a mineral acid catalyst *vide supra*.

In the pilot process sample from hydrothermal pretreatment, 5-methylfuran-2-carbaldehyde was found in small amounts (S1, Figure 3) (only a small amount of the peak at rt 6.19 min in the base peak chromatogram is due to 5-methylfuran-2-carbaldehyde), suggesting that rhamnose is actually degraded to 5-methylfuran-2-carbaldehyde in the real biomass pretreatment process, analogously to the degradation of glucose to HMF. Thus 5-methylfuran-2-carbaldehyde may be an important degradation compound in biomasses containing deoxysugars such as rhamnose or fucose. Such biomasses include pectin rich biomasses as sugar beet pulp, apple and citrus agricultural waste products that may be used for bioethanol production [20,21].

3.4 4-hydroxybenzoic acid effect on enzymatic hydrolysis and fermentation of glucose to ethanol

Enzymatic hydrolysis

When 4-HBA was added to a final concentration of 1 g/L in an enzymatic hydrolysis of avicel in buffer, no effect was seen (Figure 7). This addition level was similar to the highest concentration of 4-HBA resulting in the EFB liquid from the pilot process pretreatment. It may be speculated, that 4-HBA exerts inhibition as an additive and/or exerts a synergetic effect together with other components in the liquid from hydrothermal pretreatment (LfHP) and/or the pretreated EFB. In order to test this hypothesis, the pretreated EFB was suspended in buffer and LfHP respectively and in a 50:50 mixture of LfHP and buffer. The mixtures were prepared from the authentic LfHP (containing 4-HBA at 1 g/L), which was diluted 50 % with buffer to obtain a relief in inhibition and compared to a 50 % mixture whereupon 4-HBA was added corresponding to a final concentration of 1.5 g/L (Figure 8).

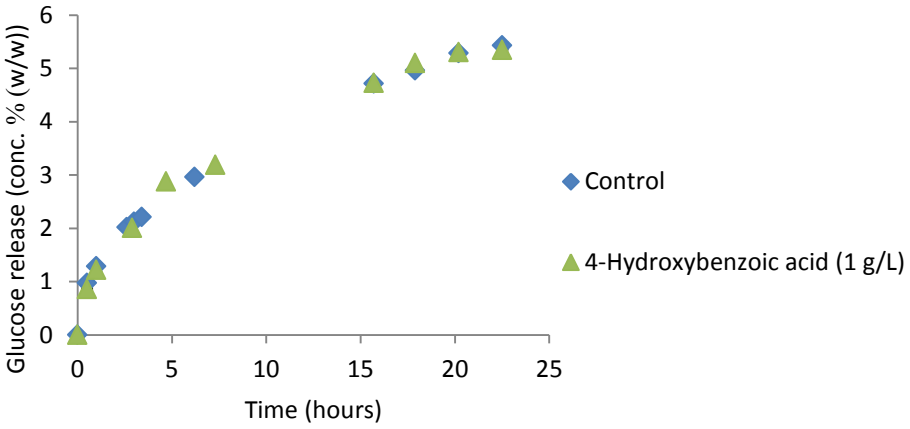


Figure 46. Enzymatic hydrolysis of avicel in buffer (control) and with addition of 4-hydroxybenzoic acid (1g/L).

A slight relief in inhibition could be observed with dilution of LfHP, but addition of 4-HBA to the diluted LfHP did not increase inhibition suggesting that 4-HBA does not inhibit the enzymatic hydrolysis when present at

concentrations equivalent to those in a pretreated EFB liquid.. Furthermore addition of 4-HBA to undiluted LfHP to a final concentration of 2 g/L did not increase inhibition. This supports that 4-HBA alone does not affect inhibition at the measured pilot process conditions.

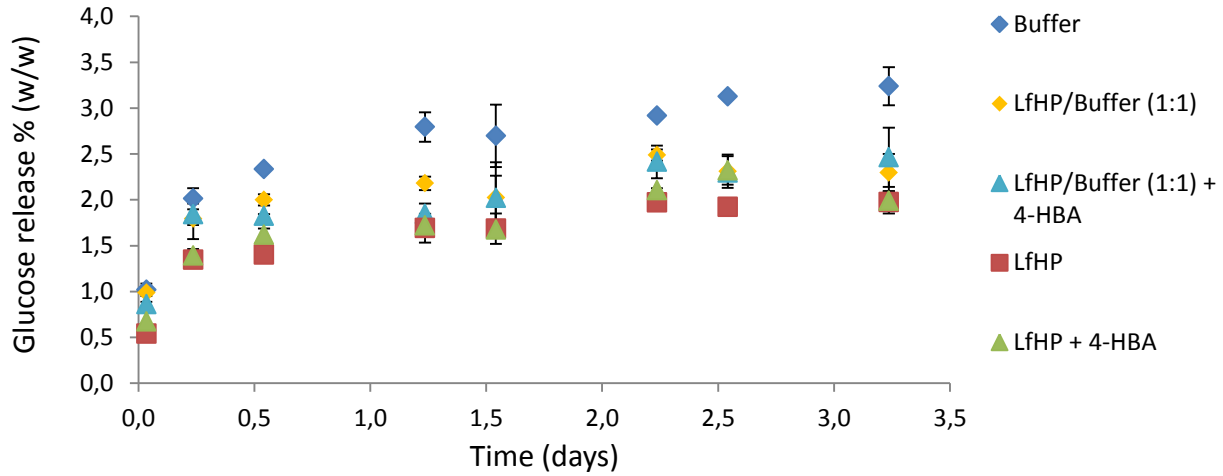


Figure 47. Glucose release during enzymatic hydrolysis of pretreated EFB. LfHP: Liquid from hydrothermal pretreatment, 4-HBA: 4-hydroxybenzoic acid.

Ximenes *et al* [22] have also reported that 4-HBA does not inhibit enzymatic hydrolysis (using the enzyme preparation Spezyme CP). However, if the enzyme preparation is pre incubated with 4-HBA 24 hours prior to enzymatic hydrolysis, deactivation of especially β-glucosidase activities is observed.

Fermentation

In order to evaluate the effect of 4-HBA on yeast fermentation of glucose to ethanol, 4-HBA was added to a final concentration of 1 g/L to a hydrolysis liquid from enzymatic hydrolysis of pretreated EFB. As with the enzymatic hydrolysis no inhibitory effect was observed (S1, Table 2).This result is in line with previously reported studies, where it was found that 4-HBA did not have an inhibitory effect on ethanol production [23,24].

4. Conclusions

In industrial scale biomass processing, a key requisite to optimize product yield, is to know how the structural elements react during processing, to avoid formation of potentially undesired compounds that may obstruct high product yields.

In the present work an unknown major compound, characteristic to oil palm empty fruit bunches (EFB), was consistently observed after hydrothermal pretreatment. The compound was purified, isolated and identified to be 4-hydroxybenzoic acid (4-HBA) with LC-DAD-ESI-MS/MS. 4-HBA was found to be liberated continuously during pretreatment of EFB biomass. Its origin was investigated and a hypothesis about a route to formation of 4-HBA *via* degradation of rhamnose to 5-methylfuran-2-carbaldehyde followed by reaction with formic acid was tested. 5-methylfuran-2-carbaldehyde is actually a degradation product from rhamnose analogues to

glucose degradation to HMF. Results of an experimental pretreatment showed that the carboxylation with formic acid does not take place. Methylfuran-2-carbaldehyde may nevertheless be an important degradation compound from biomasses rich in deoxysugars such as rhamnose or fucose.
In accord with the fact that 4-hydroxybenzoic acid is a structural sub-element in lignin, it was concluded that the 4-HBA liberated during hydrothermal pretreatment of EFB originates from the lignin in the EFB feedstock. At the relevant concentrations 4-HBA was not inhibitory to the enzymatic cellulose hydrolysis nor the yeast fermentation.
The liberation of 4-hydroxybenzoic acid during hydrothermal pretreatment of EFB may open up for new possibilities to obtain 4-hydroxybenzoic acid from biomass streams.

Acknowledgements

Thanks to Chrysoula Mirtsou-Xanthopoulou, DONG Energy for assisting with some of the experimental work.
Thanks to Tomas Fernqvist, RISØ, Technical University of Denmark for assisting with the mini pretreatment experiments.
This work was supported by the Danish National Advanced Technology Foundation via the Technology Platform “Biomass for the 21st century – B21st”.

References

1. S. H. Mood, A. H. Golfeshan, M. Tabatabaei, G. S. Jouzani, G. H. Najafi, M. Gholami, M. Ardjmand, Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment, Renewable Sustainable Energy Rev. 27 (2013) 77-93.
2. M. Pedersen, A. S. Meyer, Lignocellulose pretreatment severity - relating pH to biomatrix opening, New Biotechnol. 27 (2010) 739-750.
3. J. Larsen, M. O. Haven, L. Thirup, Inbicon makes lignocellulosic ethanol a commercial reality, Biomass Bioenergy. 46 (2012) 36-45.
4. D. M. Alonso, S. G. Wettstein, J. A. Dumesic, Gamma-valerolactone, a sustainable platform molecule derived from lignocellulosic biomass, Green Chem. 15 (2013) 584-595.
5. P. Gallezot, Conversion of biomass to selected chemical products, Chem. Soc. Rev. 41 (2012) 1538-1558.
6. H. Rasmussen, H. R. Sørensen, A. S. Meyer, Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms, Carbohydr. Res. 385 (2014) 45-57.
7. J. C. Kurnia, S. V. Jangam, S. Akhtar, A. P. Sasmito, A. S. Mujumdar, Advances in biofuel production from oil palm and palm oil processing wastes: A review, Biofuel Res. J. 9 (2016) 332-346.
8. H. Jeon, K.-E. Kang, J.-S. Jeong, G. Gong, J.-W. Choi, H. Abimanyu, B. S. Ahn, D.-J. Suh, G.-W. Choi, Production of anhydrous ethanol using oil palm empty fruit bunch in a pilot plant, Biomass Bioenergy. 67 (2014) 99-107.
9. O. Hassan, T. P. Ling, M. Y. Maskat, R. Md. Illias, K. Badri, J. Jahim, N. M. Mahadi, Optimization of pretreatments for the hydrolysis of oil palm empty fruit bunch fiber (EFBF) using enzyme mixtures, Biomass Bioenergy. 56 (2013) 137-146.
10. A. S. Baharuddin, A. Sulaiman, D. H. Kim, M. N. Mokhtar, M. A. Hassan, M. Wakisaka, Y. Shirai, H. Nishida, Selective component degradation of oil palm empty fruit bunches (OPEFB) using high-pressure steam, Biomass Bioenergy. 55 (2013) 268-275.
11. L. Rosgaard, S. Pedersen, J. Langston, D. Akerhielm, J. R. Cherry, A. S. Meyer, Evaluation of minimal Trichoderma reesei cellulase mixtures on differently pretreated barley straw substrates Biotechnol. Prog. 23 (2007) 1270–1276.
12. A. B. Bjerre, A. B. Olesen, T. Fernqvist, A Plöger, A. S. Schmidt, Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose, Biotechnol. Bioeng. 49 (1996) 568–577.
13. M. Pedersen, A. S. Meyer, Influence of Substrate Particle Size and Wet Oxidation on Physical Surface Structures and Enzymatic Hydrolysis of Wheat Straw, Biotechnol. Prog. 25 (2009) 399–408.

14. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. 2006, NREL - Biomass Program.
15. B. Du, L. N. Sharma, C. Becker, S. Chen, R. A. Mowery, G. P. van Walsum, C. K. Chambliss, Effect of Varying Feedstock-Pretreatment Chemistry Combinations on the Formation and Accumulation of Potentially Inhibitory Degradation Products in Biomass Hydrolysates, Biotechnol. Bioeng. 107 (2010) 430-440.
16. F. Lu, S. D. Karlen, M. Regner, H. Kim, S. A. Ralph, R. Sun, K. Kuroda, M. A. Augustin, R. Mawson, H. Sabarez, T. Singh, G. Jimenez-Monteon, S. Zakaria, S. Hill, Philip J. Harris, W. Boerjan, C. G. Wilkerson, S. D. Mansfield, J. Ralph, Naturally p-Hydroxybenzoylated Lignins in Palms, Bioenerg. Res. 8 (2015) 934-952.
17. Y. Neo, A. Ariffin, C. Tan, Y. Tan, Phenolic acid analysis and antioxidant activity assessment of oil palm (E. guineensis) fruit extracts, Food Chem. 122 (2010) 353–359.
18. R. Sun, J. M. Fang, L. Mott, J. Bolton, Fractional isolation and characterization of polysaccharides from oil palm trunk and empty fruit bunch fibres, Holzforschung, 53 (1999) 253–260.
19. U. Holopainen-Mantila, K. Marjamaa, Z. Merali, A. Käsper, P. Bot, A. Jääskeläinen, K. Waldron, K. Kruus, T. Tamminen, Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers, Bioresour. Technol. 138 (2013) 156–162.
20. Y. Zheng, Y. Cheng, C. Yu, R. Zhang, B. M. Jenkins, J. S. VanderGheynst, Improving the efficiency of enzyme utilization for sugar beet pulp hydrolysis, Bioprocess Biosyst. Eng. 9 (2012) 1531-1539.
21. M. C. Edwards, J. Doran-Peterson, Pectin-rich biomass as feedstock for fuel ethanol production, Appl. Microbiol. Biotechnol. 95 (2012) 565–575.
22. E. Ximenes, Y. Kim, N. Mosier, B. Dien, M. Ladisch, Deactivation of cellulases by phenols, Enzyme Microb. Technol. 48 (2011) 54–60.
23. Y. Zha, J. A. Westerhuis, B. Muilwijk, K. M. Overkamp, B. M. Nijmeijer, L. Coulier, A. K. Smilde, P. J. Punt, Identifying inhibitory compounds in lignocellulosic biomass hydrolysates using an exometabolomics approach, BMC Biotechnol. 14 (2014) 14-22.
24. A. Dumitrache, H. Akinosho, M. Rodriguez, X. Meng, C. G. Yoo, J. Natzke, N. L. Engle, R. W. Sykes, T. J. Tschaplinski, W. Muchero, A.J. Ragauskas, B. H. Davison,S. D. Brown, Consolidated bioprocessing of Populus using Clostridium (Ruminiclostridium) thermocellum: a case study on the impact of lignin composition and structure, Biotechnol. Biofuels, 9 (2016) 31.

Supplementary material

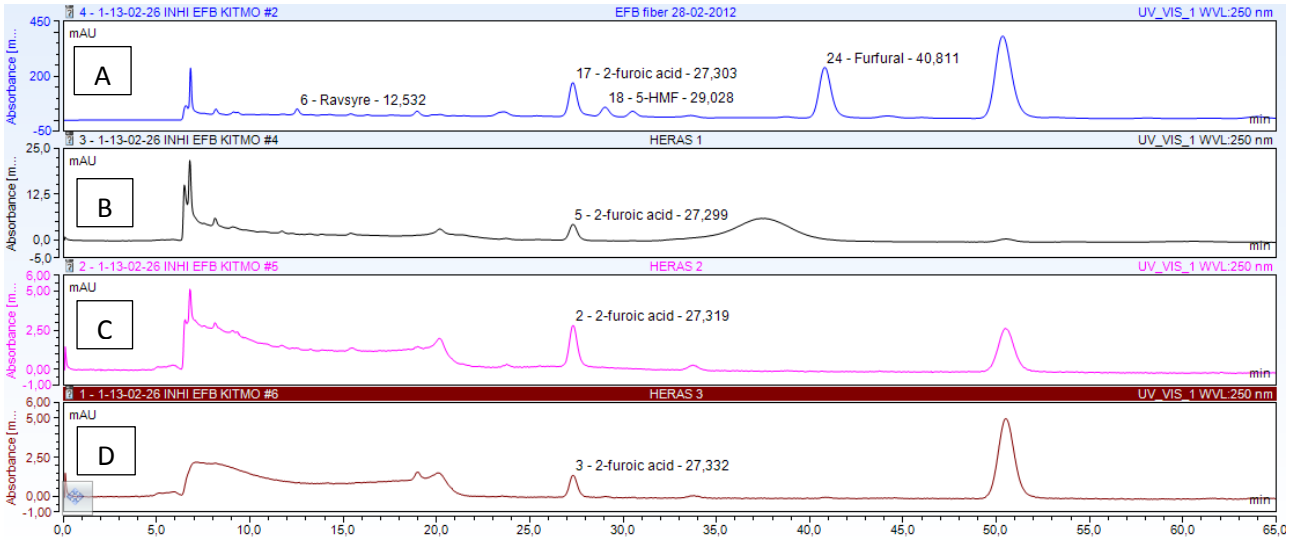
4-Hydroxybenzoic acid from hydrothermal pretreatment of oil palm empty fruit bunches - its origin and influence on biomass conversion

Helena Rasmussen,[a] Kit H. Mogensen,[a] Martin D. Jeppesen,[a] Hanne R. Sørensen[a] and Anne S. Meyer[b*]

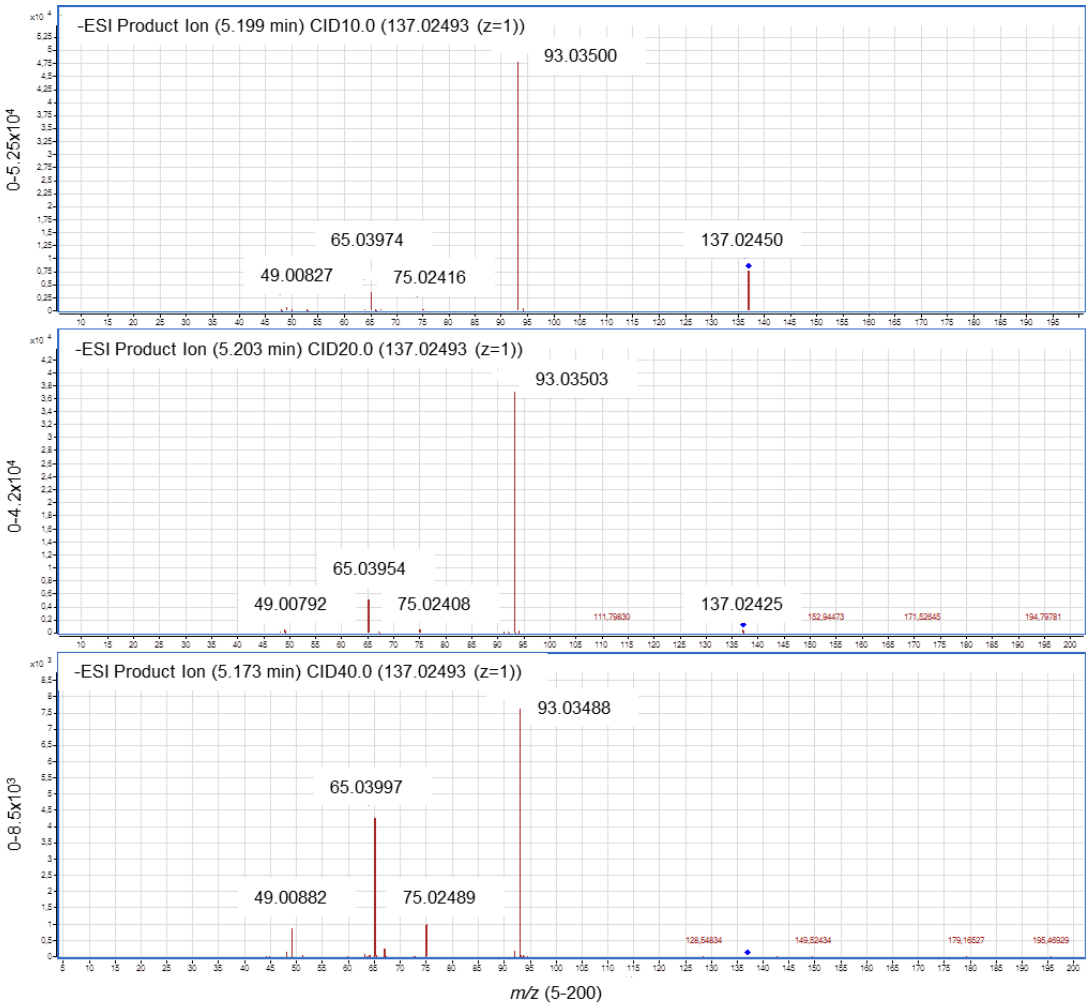
[a] DONG Energy, Kraftværksvej 53, DK-7000 Fredericia, Denmark

[b] Center for BioProcess Engineering, Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Lyngby, Denmark.

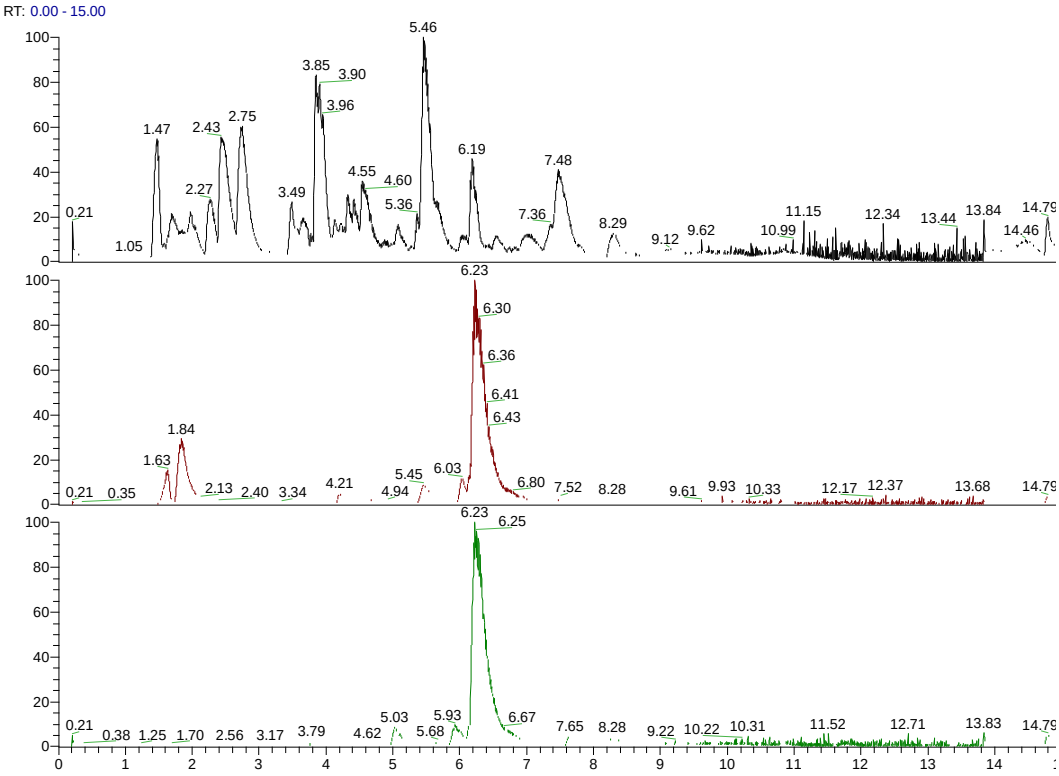
*Corresponding author: e-mail: am@kt.dtu.dk; Tel: (+45) 45 25 2800



S1 Figure 48. HPLC analysis of the fractions after preparative chromatographic purification. A: Sample before purification (unknown compound rt 50.5 minutes). B: Fraction 1. C: Fraction 2. D: Fraction 3.



S1 Figure 49. Fragmentation of the 137.02465 ion at different collision energies, negative ionisation. Top: 10 eV, Middle: 20 eV, Bottom: 40 eV



S1 Figure 50. Base peak chromatograms, positive ionisation. Top: LfHP of EFB in pilot scale. Middle: Rhamnose and formic acid reacted at pretreatment conditions. Bottom: 5-methylfuran-2-carbaldehyde standard.

S1 Table 15. Fragmentation patterns of 4-hydroxybenzoic acid (4-HBA) and the unknown compound concluded to be the same.

Fragmentation intensities 4-HBA/ unknown compound	m/z 49	m/z 65	m/z 75	m/z 93	m/z 137
10 eV	0/0	3/8	0/0	100/100	11/16
20 eV	0/0	7/14	0/0	100/100	0/1
40 eV	2/10	56/55	8/13	100/100	0/0

S1 Table 16. Yields after 30 hours of fermentation in the presence of 4-hydroxybenzoic acid. Ethanol yield is based on gram ethanol produced per gram glucose from enzyme hydrolysed pretreated EFB. [a] Similar (two tailed T-test, 95 % significance level) and [b] different from [a] (two tailed T-test, 95 % significance level)

4-hydroxybenzoic acid (g/L)	Ethanol concentration (g/L)	Ethanol yield (g/g)
0	29.75 ± 0.31[a]	0.40
1	31.05 ± 0.86[a]	0.41
10	0.50 ± 0.06[b]	0.01