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Side by Side Comparison of Chemical Compounds Generated by Aqueous Pretreatments of Maize Stover, Miscanthus and Sugarcane Bagasse

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Abstract In order to examine the potential for coproduct generation, we have characterised chemical compounds released by a range of alkaline and acidic aqueous pretreatments as well as the effect of these pretreatments on the saccharification ability of the lignocellulosic material. Comparative experiments were performed using three biomass types chosen for their potential as second-generation biofuel feedstocks: maize stover, miscanthus and sugarcane bagasse. The release of lignin from the feedstock correlated with the residual biomass saccharification potential, which was consistently higher after alkaline pretreament for all three feedstock types. Alkaline pretreatment released more complex mixtures of pentose and hexose sugars into the pretreatment liquor than did acid pretreatment. In addition, complex mixtures of aromatic and aliphatic compounds were released into pretreatment liquors under alkaline conditions, in a temperature-dependent manner, but far less so under acidic conditions. We show that the three feedstocks characterised interact with the pretreatment conditions in a specific manner to generate different ranges of products, highlighting the need to tailor pretreatments to both the starting feedstock and desired outcomes.

Keywords Biorefineries · Biomass chemicals · Pretreatment · Lignin · Phenolics · Pentoses · Maize · Miscanthus · Sugarcane bagasse

Leonardo D. Gómez and Ruben Vanholme contributed equally in this work.

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Introduction

The coproduction of value-added chemicals from biomass processing may best be achieved from the basis of understanding the range of products released from the feedstock under different processing conditions. This knowledge will allow the identification of processing conditions suitable for the release of both sugars for fermentation and value-added products. The noncrystalline nature of hemicelluloses makes them susceptible to extraction during pretreatments. Different thermochemical pretreatments will cause removal of the hemicellulose fraction to different extents. Various reports on the extent of this removal have been published and show that this varies between the species of biomass and conditions used [1, 2]. The insoluble polysaccharide fraction left after efficient separation of hemicelluloses is enriched in glucose, which on saccharification provides substrates similar to those used in

classic fermentation of starch- or sucrose-derived sugars, with lower content of problematic pentoses [3].

Lignin is a complex aromatic polymer that embeds the polysaccharide fraction in the biomass and provides the major constraint for hydrolytic deconstruction of polysaccharides. However, it represents up to 30 % of the biomass and has diverse existing and potential industrial applications [4].

The energy crops proposed as feedstock for biorefineries should have a high productivity and nutrient use efficiency to reach a favourable balance of emissions compared to oil-derived products. In addition, it is important that second-generation biofuel feedstocks do not negatively impact on global food security [5, 6]. Maize stover, sugarcane bagasse and miscanthus are all favoured as highly available or productive nonfood feedstocks for biomass biorefining and biofuels. Maize is a major crop grown for grain and gives rise to very large volumes of stem and leaf residues, on average 5.2 tons DM/ha [6], and constitute a potential source of biomass for biofuel production [7]. In the USA, maize stover constitutes a by-product of grain ethanol and food production and its integration in the biorefining process represents an addition to the value chain. Sugarcane bagasse is also an attractive feedstock for biorefineries (on average 11 tons/ha [6]) as it constitutes a by-product of the sugar and first-generation biofuel industries in tropical countries such as Brazil. In Brazil, a typical sugar processing plant produces on average 90 tons DM/ha of bagasse [8]. This bagasse has been partially incorporated into the sugarcane industry for energy generation by burning in power plants that generate heat and electricity for the sugar mills and export the excess into the grid. The utilisation of bagasse for cellulosic ethanol is an attractive possibility for increasing the sustainability around the sugarcane industry [8]. Miscanthus is a dedicated perennial energy crop and has been proposed as a model of a low input agricultural crop. It is at present used for burning in power plants but it is also proposed as a potential feedstock for cellulosic ethanol given the large yields and it's adaptability to marginal lands [9]. Yields of more than 40 ton DM/ha have been achieved for miscanthus in southern Europe and USA [6].

Whatever the biomass of choice, there is a need to use (thermochemical) pretreatments to potentiate the effective enzymatic hydrolysis of polysaccharides for fermentation [10]. Pretreatments have been considered for a long time as the key to take the biochemical conversion of biomass to levels which are compatible with industrial applications [11]. A great deal of research has been put into the development of different pretreatments over the last 10 years, and the literature is rich in diverse and creative approaches to increase the efficiency of pretreatments in terms of increased fuel production after fermentation ([12] and references therein). An interesting new approach is to evaluate pretreatments in terms of their potential for the cogeneration of value-added chemicals in addition to their ability to potentiate enzymatic hydrolysis [13].

In the present work, we characterise the sugars and lignin derivatives present in the pretreatment liquor of maize stover, miscanthus and sugarcane bagasse under a range of acidic and alkaline pretreatment conditions. This provides a detailed picture of the conditions necessary for favouring the production of different molecules and also allows a horizontal comparison of the compounds generated between species. In order to examine the impacts of temperature and acid or alkali concentration on pretreatment effectiveness, we first carried out experiments with a fixed concentration of NaOH or H_2SO_4 in a range of different temperatures and subsequently looked at varying chemical concentrations at low (20 °C) or high (180 °C) temperatures.

Material and Methods

Biomass Feedstock and Characterisation

Maize biomass was obtained from INRA France. M2 maize was grown at INRA Lusignan (France) under field conditions and harvested at R6 stage (silage) removing the cobs. The dry biomass was ground using a hammer mill and sieved to 1-mm particles. The *Miscanthus sinensis* accession MS-90-2 was grown under field conditions at the Wageningen University (The Netherlands), harvested at maturity and ground to 1-mm particles using a hammer mill. Sugarcane bagasse was kindly provided by the Cosan Group (Ibaté/SP, Brazil) after sucrose extraction and ground to 1-mm particles.

Cell wall composition was determined essentially as described in Torres et al. (22), which derives from Goering and Van Soest [14] Half a gram of biomass was weighed into a ANKOM filter bag (ANKOM Technology Corporation, Fairpoint, NY) and the following parameters were determined: neutral detergent fibre (NDF), acid detergent fibre (ADF) and permanganate lignin (pLig). All analyses were performed in duplicate and were carried out using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). Stem total cell wall, cellulose (Cel), hemicellulose (Hem) and permanganate lignin (pLig) contents were derived from detergent fibre parameters as follows: NDF corresponds to the total cell wall content; Cel was determined as the difference between acid detergent fibre (ADF) and permanganate lignin (pLig) and Hem was determined as the difference between NDF and ADF.

Pretreatment Conditions

Biomass (400 mg) was pretreated in 23-mL pressure bombs (Parr Instruments, Moline, IL), using 16 mL of pretreatment solution. Pretreatments were performed during 30 min to

determine the effect of increasing temperatures and 40 min in the presence of increasing chemical concentrations. Three technical replicates were used for each condition. After cooling to room temperature, the liquid and solid fraction were separated by centrifugation at 4,000*g* for 15 min. The liquid (pretreatment liquor fraction) was recovered for further analysis. The solids were washed three times in ethanol (5 mL for 10 min at 20 °C) before further processing.

Total Sugar Content in Pretreatment Liquors

Monosaccharide composition in the liquor fraction obtained from each pretreatment condition was determined by ion chromatography. Two mL of acidic liquor samples were initially neutralised with 1.33 mL barium hydroxide solution (150 mM), followed by barium carbonate powder. Alkaline liquor samples (2 mL) were neutralised with 0.4 mL of 2 N HCl. All samples were individually titrated to pH 7.0. Samples were adjusted to a final volume of 12 mL and centrifuged at 4,000*g*; 0.5 mL of the neutralised liquor fraction was transferred to microcentrifuge tube and vacuum dried. Hydrolysis using 500 μL of 2 M trifluoroacetic acid (TFA) was carried out at 100 °C for 4 h. After cooling to room temperature, samples were dried and twice washed with 200 μL of isopropanol. Hydrolysates were resuspended in 200 μL of water. Monosaccharide analysis of the TFA treated pretreatment liquor (1 mL) was performed by high performance anion-exchange chromatography (HPAEC) (Dionex IC 3000) on a Dionex Carbopac PA-20 column with integrated amperometry detection [15]. The separated monosaccharides were quantified using external calibration with an equimolar mixture of nine monosaccharide standards (arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose and xylose), which were subjected to TFA hydrolysis in parallel with the samples. The mass of sugars is expressed per mass of original biomass

Acetyl Bromide Soluble Lignin

The total lignin content was determined using the acetyl bromide method [16]. Note that because of the heterogeneity of lignin and its occurrence within a complex cell wall matrix, there is no single technique to accurately measure lignin amount [17]. Acetyl bromide soluble lignin (ABSL) levels have been reported to be influenced by lignin composition [18], by UV-absorbing soluble substances that are not efficiently removed during solvent washing of the cell wall material [19] and by xylan degradation [20]. Despite these possible interferences, ABSL is commonly used as a proxy for lignin amount. Plant materials obtained after each pretreatment condition were washed with ethanol (10 mL, 10 min at 20 °C) to remove soluble compounds. Pretreatment liquor samples were dried. Next, 3.5 mg of each sample was weighed into a 2-mL cap tube and 250 μL of freshly prepared acetyl bromide solution (25 %*v*/*v* acetyl bromide/glacial acetic acid) was added. Samples were incubated at 50 °C for 3 h. After cooling at room temperature, the hydrolysate was transferred to a 5-mL volumetric flask. One mL of 2 M NaOH was added to the 2-mL tube to generate the acetyl bromide excess before transfer to a volumetric flask; 175 μL of hydroxylamine-HCl was added to each sample which was vortexed vigorously. Finally, the volume was adjusted to 5 mL with glacial acetic acid and the absorbance was measured at 280 nm. The ABSL (%) was determined using the extinction coefficient 17.75 L g^{-1} cm^{-1} [16].

Furfural and Hydroxymethyl Furfural Determination

Liquor fractions obtained from each pretreatment condition were neutralised as described above and subjected to chromatography using a Luna® 5 μm C18(2) 100 Å LC Column 150×4.6 mm (Phenomenex, Cheshire, UK), together with a C18 4×2.0 mm ID guard column (Phenomenex, Cheshire, UK) to quantify furfural and HMF content. Analyses were carried out using a Surveyor HPLC (Thermo electron Corporation, Hemel Hempstead, UK), with an elution system of acetonitrile by reversed-phase in an isocratic gradient (5 % acetonitrile and 95 % deionised water) at 1 mL/min. The eluted furfuraldehydes were detected by UV absorbance at 284 nm using a Finnigan Surveyor PDA Plus detector. The furfurals were quantified by interpolation of a calibration curve within the range of 0.005–50 μg/mL in water.

Analysis of Phenolics in the Pretreatment Liquors

The neutralised lyophilized pretreatment liquors of water and H_2SO_4 pretreated samples were dissolved in 2 mL of water whereas neutralised pretreatment liquors of NaOH pretreated samples were dissolved in 10 mL of water. Solid phase extraction was performed on 2 mL of the dissolved samples using an Extract Clean 100 mg/1.5 mL C18-SPE column (Grace, Deerfield, IL, USA) and a vacuum manifold. The SPE column was preconditioned with 0.5 mL methanol followed by 1.5 mL of water). Two mL of the sample was applied on the column which was then rinsed with 300 μL of water. Flow-through and wash were discarded. The sample was then eluted with 1.10 mL of acetonitrile (ACN). The eluent was dried using a speedvac. Liquid-liquid extraction was performed with 80 μL/80 μL cyclohexane/water for maize stover samples and 100 μL/100 μL cyclohexane/water for miscanthus and sugarcane bagasse samples; 70 μL of the water phase was transferred to an ultra performance liquid chromatography (UPLC) vial. Of this, 15 μL of the lower water phase was injected on a Waters Acquity UPLC®. As such, a certain fraction of the original 16 mL pretreatment liquor was analysed. For the NaOH pretreated samples: a

1/213 fraction for sugarcane bagasse and miscanthus (i.e., the initial portion taken from the total pretreatment liquor volume (2 mL/16 mL), multiplied by the dilution factor because samples were too concentrated (2 mL/10 mL), multiplied by the portion of the liquid-liquid aqueous phase that is injected on the UPLC for analysis (15 mL/80 mL)) and 1/266 for maize stover; for the H_2SO_4 pretreated samples: 1/42.7 for sugarcane bagasse and miscanthus, 1/53.4 for maize stover. Later in the analysis, all peak areas were subjected to a correction factor inversely proportional to the fraction taken to enable comparing the feedstocks with a different analysed fraction. The UPLC system was equipped with a BEH C18 column (2.1×150 mm, 1.7 μM), coupled to a Synapt Q-Tof (Waters Corporation, Milford, MA, USA). A gradient of two buffers was used: buffer A (99/1/0.1, H_2O/ACN/formic acid, pH 3), buffer B (99/1/0.1, ACN/H_2O/formic acid, pH 3); 95 % A for 0.1 min decreased to 50 % A in 30 min (350 μL/min, column temperature 40 °C). The flow was diverted to the mass spectrometer equipped with an electrospray ionisation source and lockspray interface for accurate mass measurements. The mass spectrometer (MS) source parameters were capillary voltage, 1.5 kV; sampling cone, 40 V; extraction cone, 4 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow, 50 L/h and desolvation gas flow, 550 L/h. The collision energy for the trap and transfer cells were 6 and 4 V, respectively. For data acquisition, the dynamic range enhancement mode was activated. Full-scan data were recorded in negative centroid V-mode; the mass range between m/z 100 and 1,000, with a scan speed of 0.2 s/scan, with Masslynx software (Waters). Leucin-enkephalin (400 pg/μL solubilised in water/ACN 1:1 (vol:vol), with 0.1 % formic acid) was used for the lock mass calibration, with scanning every 10 s with a scan time of 0.5 s. From the resulting chromatograms, 13,936 peaks were integrated and aligned via Masslynx® software. For principal component analysis (PCA), this peaklist was further filtered for peaks that appeared at least once in all three replicates of the same conditions and for which the peak was at least in one sample above intensity 200. This resulted in 3,572 peaks for H_2SO_4 pretreated samples and 5,294 peaks for NaOH pretreated samples. PCA was performed in R software via the 'prcomp' command. As stated above, all peak areas were multiplied by a correction factor to enable comparing the feedstocks with a different analysed fraction.

Results

Compositional Analysis of the Three Biomass Feedstocks Studied

The biomass was grown and harvested to reflect a typical case study for the three species (see "Materials and Methods"). The composition of the three biomass feedstocks showed clear differences between the three species studied, and the individual composition is consistent with the range of values previously published for these feedstocks (Table 1) [21–24]. The total cell wall content in maize stover is lower than in the other biomasses, showing a higher proportion of soluble materials in the original biomass. Interestingly, the cellulose content in the materials is inversely correlated with the percentage of hemicellulose. This differential composition will have a clear effect in the type of compounds released during pretreatment.

Effect of Temperature in Alkaline and Acidic Pretreatments on Sugar Release into Pretreatment Liquors

There is a considerable diversity of biomass pretreatment conditions in the literature, in terms of nature and severity. The most common conditions, however, involve the use of dilute acid or alkali to disrupt the structure of the biomass to make the polysaccharides more accessible to the hydrolytic enzymes [10]. The strength of such pretreatment conditions is a function of the treatment time, the pH of the medium and the temperature [25]. In order to select a temperature to perform alkaline and acid pretreatments in maize stover, miscanthus and sugarcane bagasse, we subjected feedstock from the three materials during 30 min to increasing temperature conditions in the presence of 0.2 N of either NaOH or H_2SO_4. Such pretreatments often cause substantial quantities of sugars to be released from the biomass, particularly from the hemicellulose fraction, and these could be used in fermentation, anaerobic digestion or chemical applications to generate additional value.

Figure 1 (and Supplemental Tables 1 and 2) shows that the amount of sugars present in the pretreatment liquor (determined as sugars after TFA treatment) is highly dependent on the feedstock and condition used. In general, the pretreatment liquor of maize stover released more sugars than those of miscanthus or sugarcane bagasse, while the amount of sugars in the pretreatment liquor of miscanthus and sugarcane bagasse were in the same range. The lower proportion of total cell wall, as a percentage of dry matter, observed in maize (Table 1) could explain the larger amount of sugars released in the pretreatment liquors in this biomass. In addition, there was an increment in the amount of sugars in the liquor with increasing temperatures, an effect which was more pronounced for acidic pretreatments as compared to the alkaline pretreatments. For temperatures up to 110 °C, pretreatment liquor of alkaline conditions contained larger amounts of sugars compared to those of acidic conditions for all feedstocks used. However, at 180 °C, the amount of sugars in acid pretreated liquors was higher as compared to those in alkaline liquors for both miscanthus and sugarcane bagasse samples.

The monosaccharide composition of these liquors show that, in most of the conditions used, glucose or xylose were

Table 1 Cell wall composition of the three biomasses

Biomass	Total cell wall (% of dry matter)	Cellulose (% of cell wall)	Hemicellulose (% of cell wall)	Lignin (% of cell wall)
Maize stover	54.177 (±1.06)	38.518 (±2.397)	48.537 (±0.767)	12.945 (±1.813)
Miscanthus	87.559 (±0.777)	46.547 (±1.855)	33.737 (±0.599)	19.716 (±1.663)
Sugarcane bagasse	88.794 (±0.468)	51.802 (±2.155)	31.690 (±0.594)	16.509 (±2.342)

Values between brackets show standard deviation between determinations, n=4

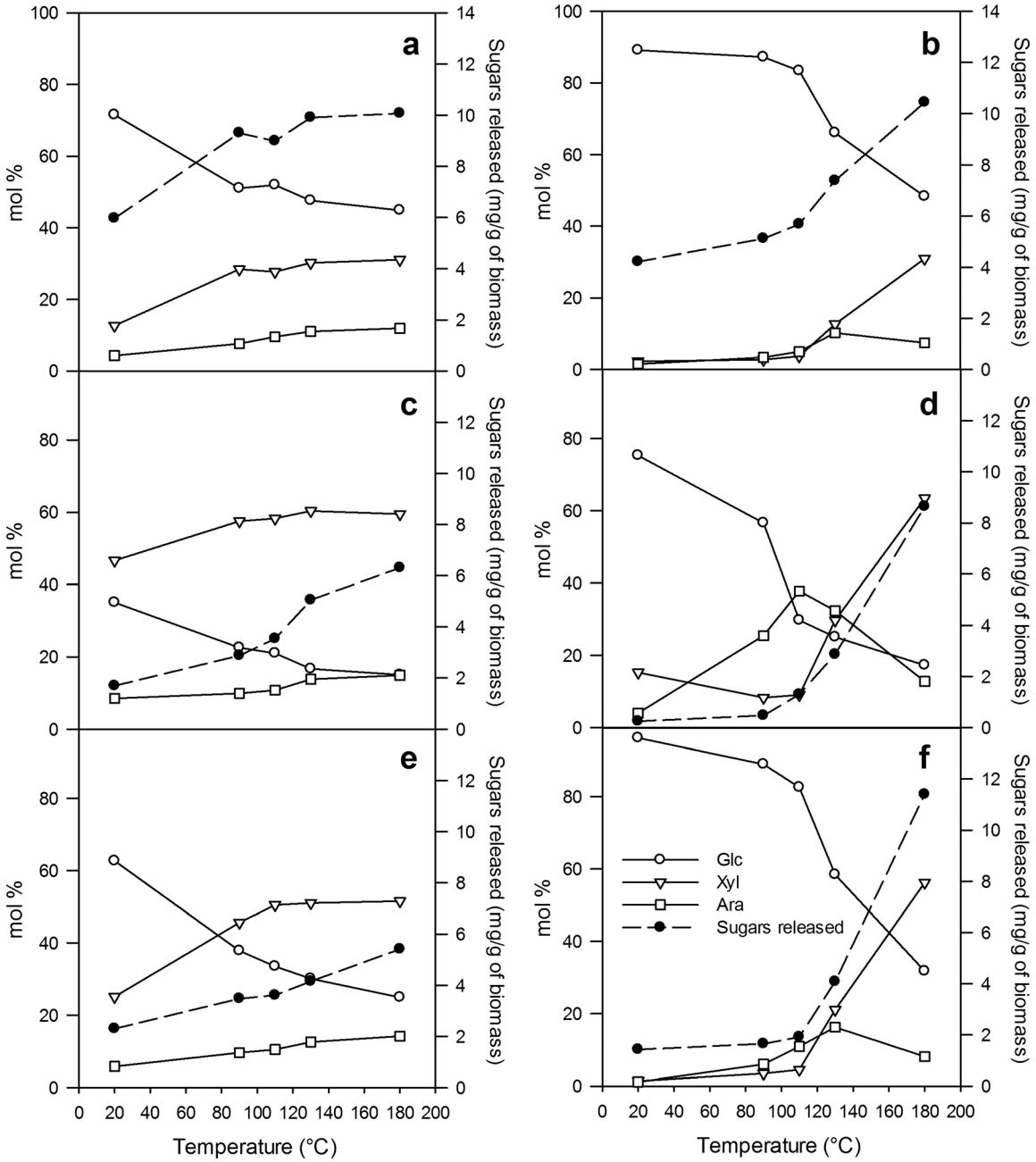

Fig. 1 Amount of sugars released and monosaccharide composition of the pretreatment liquor at different temperatures. Total sugars released after TFA treatment and mol% composition of the TFA-released monosaccharides of 0.2 N NaOH (**a**, **c**, **e**) or H_2SO_4 (**b**, **d**, **f**) from maize stover (**a**, **b**), miscanthus (**c**, **d**) or sugarcane bagasse (**e**, **f**) maintained at the indicated temperatures for 30 min. For standard deviations and full monosaccharide composition, see Supplemental Table 1 and 2. The experiments were carried out in triplicates

the most abundant monosaccharides, with maximal proportion of 89 and 62 % as compared to the total sum of the monosaccharides, respectively (Fig. 1 and Supplemental Tables 1 and 2). In relative abundance, these were followed by arabinose (up to 39 %), galactose (up to 10 %) and mannose (up to 6 %), whereas only traces of galacturonic acid and glucuronic acid were detected (below 2 %) in most of the samples (Supplemental Tables 1 and 2). However, the relative abundance of these sugars and sugar acids depended on the pH, temperature and feedstock. In general, alkaline conditions released a complex mixture of monosaccharides with a large representation of C5 sugars (Supplemental Tables 1 and 2). Acidic conditions, on the other hand, produced liquors with a higher proportion of glucose, particularly at low temperatures (Supplemental Tables 1 and 2). In addition, the relative abundance of glucose in the liquors decreased with increasing temperatures, mainly due to the relative increase of xylose, under both acid and alkaline conditions (Fig. 1). The relative abundance of arabinose showed a more complex profile: increasing with higher temperatures under alkaline conditions, but showed a maximum at intermediate temperatures under acidic conditions. In terms of feedstock, maize stover produced liquors with glucose as the predominant monosaccharide in both acid and alkaline conditions (Fig. 1). Miscanthus, in turn, presented a more complex monosaccharide profile in all pretreatments (Supplemental Tables 1 and 2). Under alkaline conditions, xylose was the predominant monosaccharide released from pretreated miscanthus (Fig. 1). In contrast, arabinose, galactose, galacturonic acid and glucuronic acid were particularly abundant in acid liquors at 110 and 130 °C (Supplemental Tables 1 and 2). Sugarcane bagasse pretreatment liquors showed a larger proportion of glucose than those of miscanthus under all pretreatments (Fig. 1). The xylose and arabinose found in pretreatment liquors most likely originated from hydrolysis of xylans, which are the major constituents of grass hemicelluloses and have a backbone chain of β-1,4-linked xyloses with mostly arabinose decorations [26]. The glucose could originate from the hydrolysis of glucose-containing matrix polysaccharides such as the noncrystalline portions of cellulose, β-1,3- and β-1,4-mixed linkage glucans and xyloglucans, although the latter are generally present in low quantities in grass cell walls [27]. In addition, some of the solubilised sugars detected could also originate from other sugar-containing compounds such as starch, glycosylated flavonoids and others. In general, the combined results show that alkaline pretreatment removes polysaccharides from the hemicellulosic fraction with an efficiency that increases with temperature (Supplemental Tables 1 and 2). Acidic pretreatment conditions at lower temperatures (20 to 90 °C) are less effective in removing the hemicelluloses than are alkaline pretreatments, but the effectiveness increases with the temperature. At the higher temperatures (180 °C), the acidic pretreatment becomes even more effective in removing the hemicelluloses than alkaline pretreatment, and this likely reflects acid hydrolysis of these polymers.

Effects of the Concentration of Acid and Alkaline on Sugar Release at Low and High Temperatures

Once the incremental effects of temperature on the sugar composition of the pretreatment liquor and saccharification hydrolysate of the three feedstocks were established, we performed a study of the effects on the biomass of increasing concentrations of acid and alkali pretreatment at a fixed low or high temperature. The conditions used were water and 0.2, 0.4 and 1 N of H_2SO_4 and NaOH applied at 20 and 180 °C to maize stover, miscanthus and sugarcane bagasse for 40 min.

Figures 2 and 3 show the effect of increasing chemical treatment concentration on amount of sugars present in the pretreatment liquor at 20 and 180 °C. The total amount of sugars released in the pretreatment liquor increased with increasing alkaline concentration at both 20 and 180 °C in each of the three feedstocks (Fig. 2). The effect of increasing acid concentration on the total amount of sugars released in the pretreatment liquor was more complex and depended on the temperature and biomass used (Fig. 3). With miscanthus and sugarcane bagasse as a feedstock, acid pretreatment appeared rather inefficient in releasing sugars from the biomass at 20 °C for all concentrations tested. Maize stover, in contrast, did release considerable amounts of sugars at 20 °C that are most likely derived from nonhemicellulosic sugar-containing compounds. At 180 °C, maize stover appeared to quickly reach a maximal level of sugar release under acidic pretreatment conditions; increasing acid concentration above 0.2 N did not release more sugars into the liquor. In the case of miscanthus and sugarcane bagasse, increasing acid concentration above 0.2 N had a positive effect on the total amount of sugar released at 180 °C. In terms of the monosaccharide composition of the pretreatment liquor, there was a relative increase in xylose and a relative decrease in glucose with increasing concentrations of NaOH for the three feedstocks at both 20 and 180 °C (Fig. 2). With a few exceptions, the monosaccharide composition of the pretreatment liquor was largely similar between the different concentrations of H_2SO_4 used, but clearly differed between the different temperatures tested (Fig. 3 and Supplementary Table 4). At 180 °C, the fraction of xylose in the acid pretreatment liquor of maize stover equalled the fraction of glucose in all concentrations tested. On the other hand, xylose was the predominant sugar in both miscanthus and sugarcane bagasse acid pretreatment liquors at 180 °C.

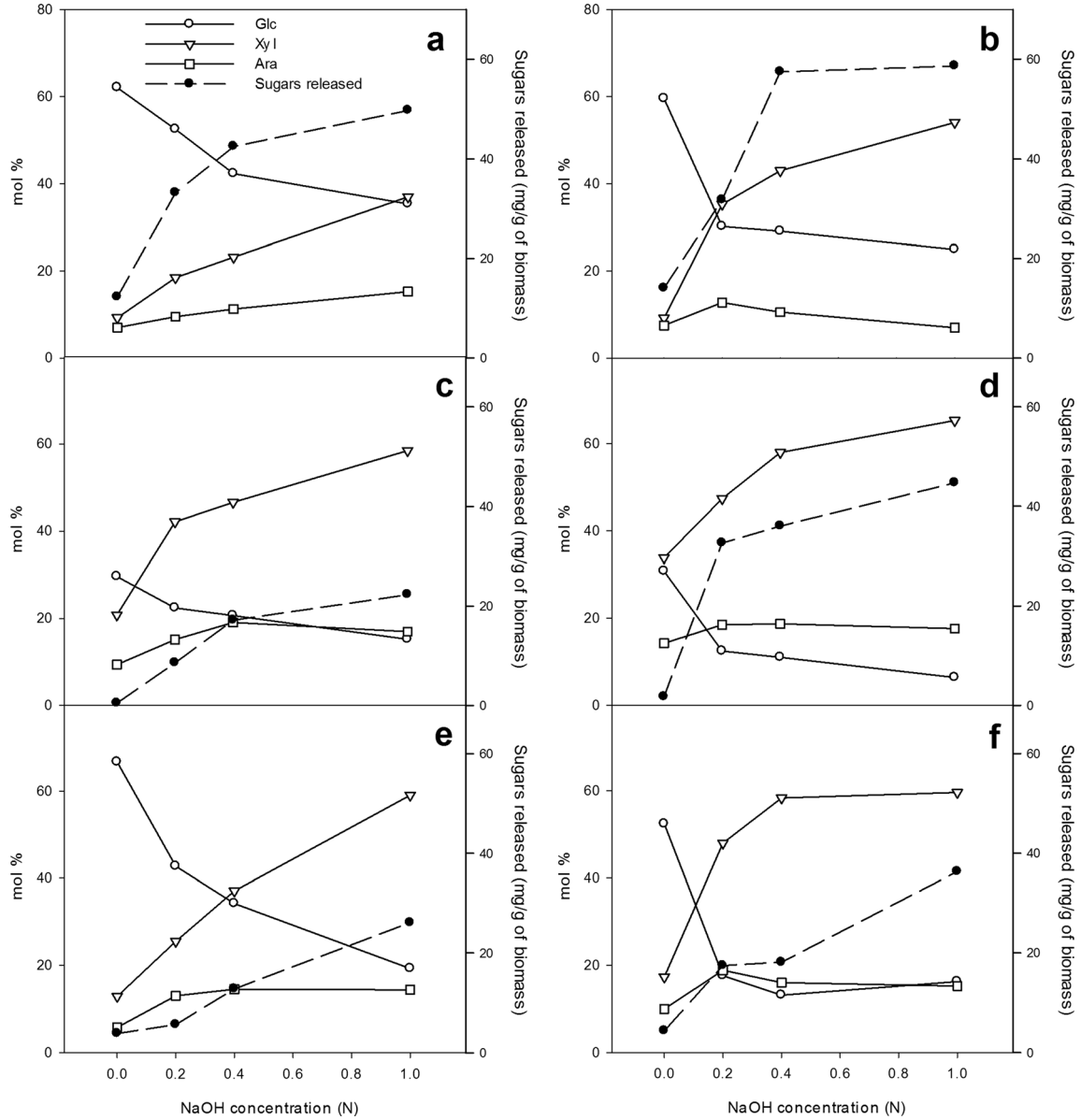

Fig. 2 Amount of sugars released and monosaccharide composition of the pretreatment liquor with increasing concentrations of NaOH. Total sugars released after TFA treatment and mol% composition of the TFA-released monosaccharides from maize stover (**a**, **b**), miscanthus (**c**, **d**) and sugarcane bagasse (**e, f**) using increasing concentrations of NaOH at 20 °C (**a**, **c**, **e**) and 180 °C (**b**, **d**, **f**) during 40 min. Sugars were determined by HPAEC after TFA treatment of the respective pretreatment liquors in all three repetitions of the experiment. For standard deviations and full monosaccharide composition, see Supplemental Table 3

Effects of the Pretreatments on the Amount of Lignin in the Feedstock Residue

Figure 4 shows the amount of lignin that remained in the pretreated insoluble biomass under the various pretreatment conditions. NaOH was most effective at removing lignin from each feedstock tested. However, the residual lignin in miscanthus and sugarcane bagasse residue pretreated with 0.2 N NaOH at 20 °C was in the same range as the residual lignin of the water pretreated samples. Miscanthus proved to be the most responsive to temperature and chemical treatment in terms of lignin release. Even at 180 °C, acidic pretreament was ineffective at reducing the lignin fraction of the feedstock residue, compared to alkaline. This is in accordance with the observation that acid pretreatment at temperatures above the glass transition temperature of lignin (~130 °C) is able to melt and redistribute lignin in the cell wall, but not to remove lignin from the biomass [28].

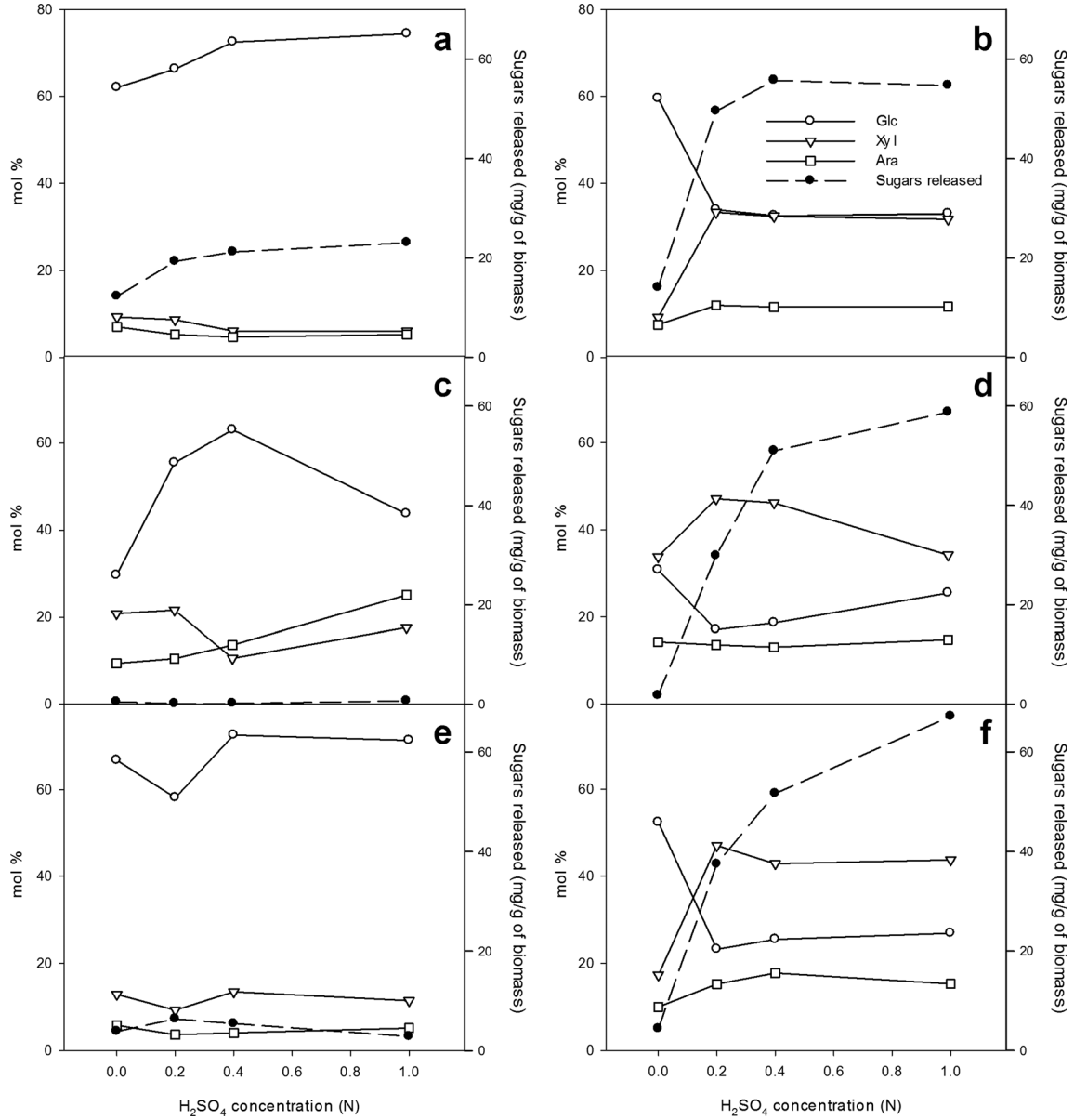

Fig. 3 Amount of sugars released and monosaccharide composition of the pretreatment liquor with increasing concentrations of H_2SO_4. Total sugars released after TFA treatment and mol% composition of the TFA-released monosaccharides from maize stover (**a**, **b**), miscanthus (**c, d**) and sugarcane bagasse (**e**, **f**) using increasing concentrations of H_2SO_4 at 20 °C (**a**, **c**, **e**) and 180 °C (**b**, **d**, **f**) during 40 min. Sugars were determined by HPAEC after TFA treatment of the respective pretreatment liquors in all three repetitions of the experiment. For standard deviations and full monosaccharide composition, see Supplemental Table 4

Effects of Pretreatments on the Release of Phenolics and Fatty Acids

To avoid column and detector saturation, a relatively small portion of the alkaline pretreatment liquors (corresponding to about 80 μL of the initial pretreatment liquor) was used for UHPLC-MS analysis. Because column and detector saturation was less of an issue for the pretreatment liquors of water and acid-treated samples, a larger portion of the sample (corresponding to 320 μL of the initial pretreatment liquor) was used for UHPLC-MS analysis in this case. Comparison of the total ion current of UHPLC-MS chromatograms of the pretreatment liquor of alkaline and acid-treated samples revealed that alkaline pretreated samples were indeed richer in molecules as compared to acid pretreated samples with the same molarity and temperature (Supplementary Fig. 1).

To make a general comparison between the pretreatment conditions (in terms of concentration, temperature and feedstock), all data were integrated and aligned after which PCA

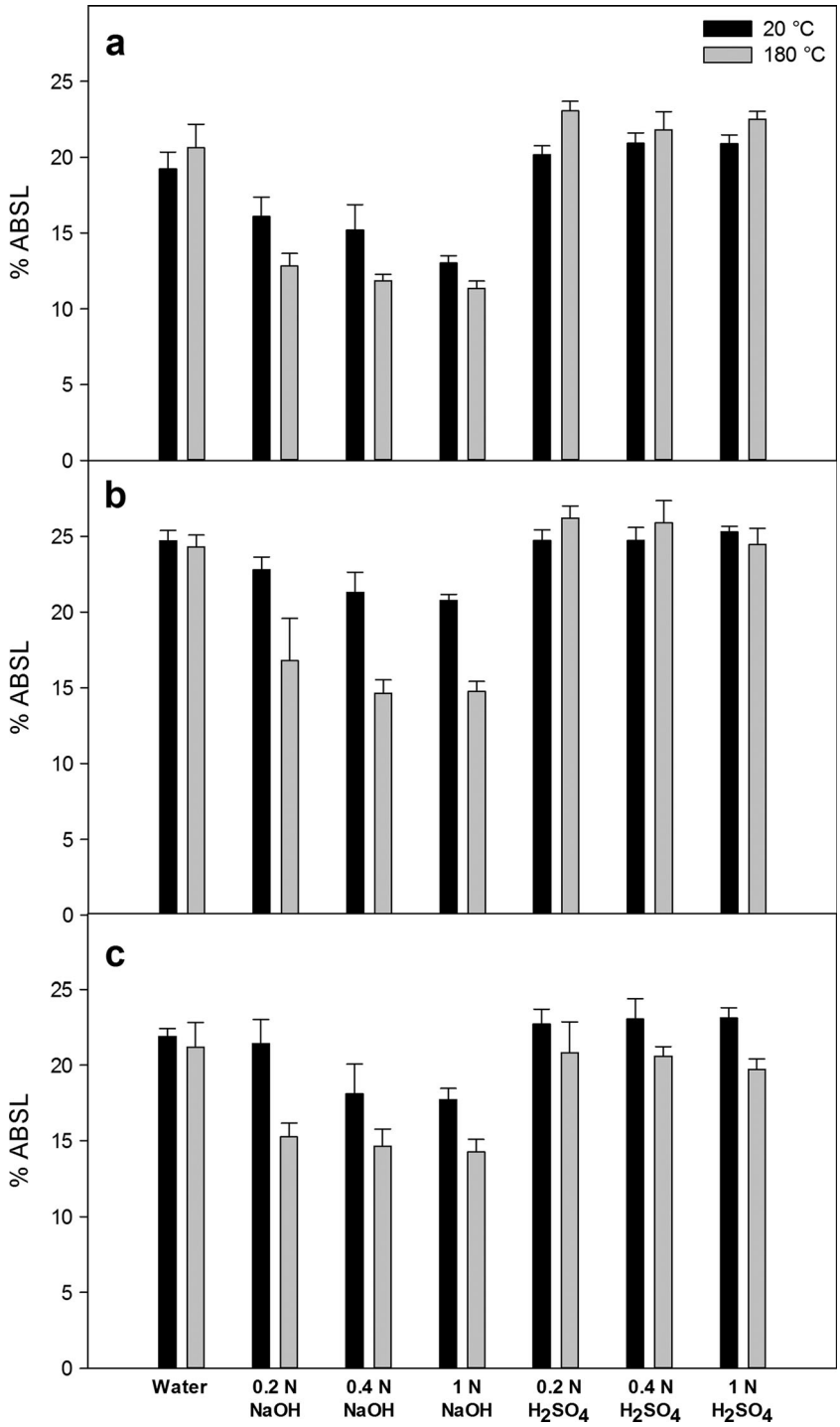

Fig. 4 Remnant lignin in feedstock residue after chemical pretreatment. Percentage of acetyl bromide soluble lignin (ABSL) as expressed to the total insoluble residue (*w*/*w*) after pretreatment for 40 min at 20 and 180 °C of maize stover (**a**), miscanthus (**b**) and sugarcane bagasse (**c**) at increasing concentrations of NaOH or H_2SO_4 at 20 and 180 °C

was performed (Supplementary Fig 2). Because of the large compositional differences between acid and alkaline samples and due to the different dilutions used for these two types of samples, PCA was performed on alkaline and acid pretreated samples separately. From the PCA plots, it can be deduced that the influence of temperature on the composition of water pretreated liquors was modest. In addition, it was clear that the composition of the pretreatment liquor with acid at 20 °C was not very different from water pretreatment, but the pretreatment liquor with acid at 180 °C was clearly different, indicating that the acid pretreatment needs higher temperatures to be effective in releasing compounds from the biomass compared to alkali treatments. In terms of the biomass, the PCA plots showed a high similarity between miscanthus and sugarcane bagasse, while maize stover samples were clearly different.

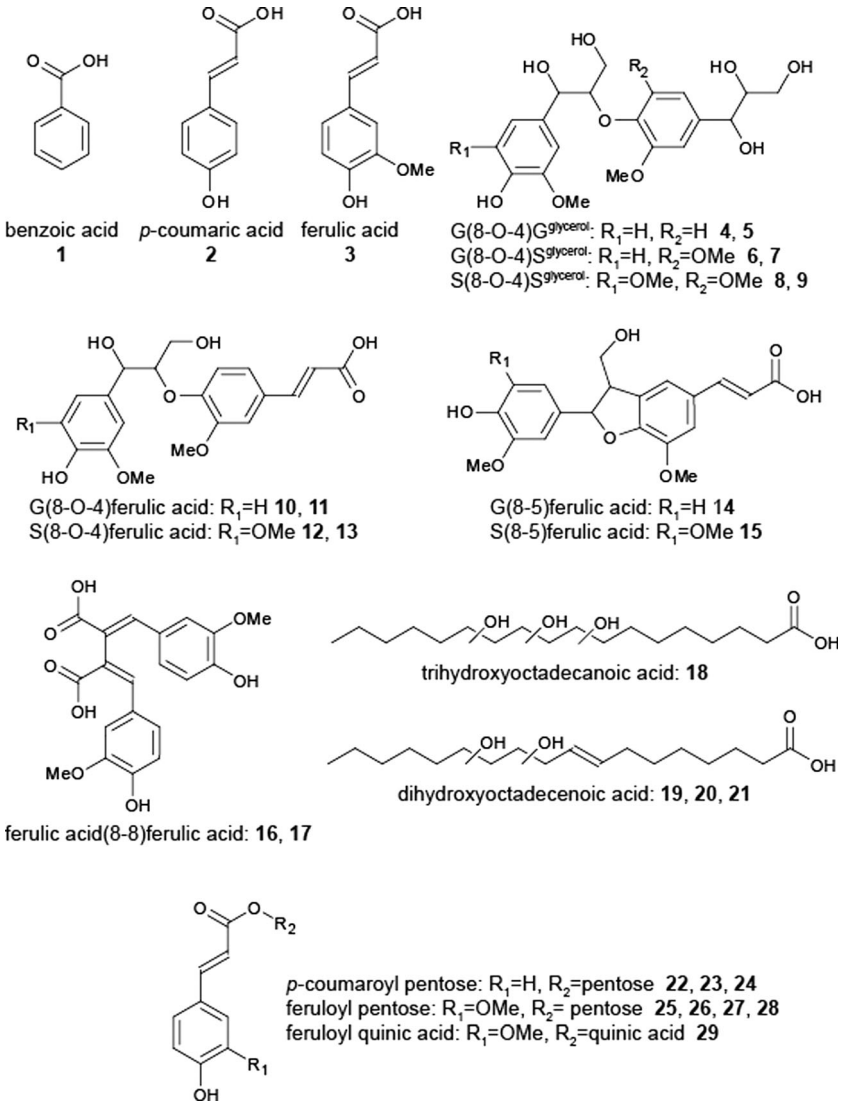

Fig. 5 Compounds structurally characterised in alkaline (*1–21*) and acid pretreatment liquors (*22–29*). See Supplemental Fig. 2 for their structural characterisation

Twenty-one compounds that were present in the alkaline pretreatment liquor could be structurally characterised based on their MS/MS fragmentation (Fig. 5, Supplemental Fig. 3). Their respective ion traces were manually integrated in each of the chromatograms (Table 2). *p*-Hydroxybenzaldehyde, *p*-coumaric acid and ferulic acid were among the highest accumulating compounds, based on MS traces. This class of 'aromatic monomers' was detected both at 20 and 180 °C and in all concentrations of NaOH used. In general, the concentration of *p*-coumaric acid increased both with increasing temperature and increasing NaOH concentration, whereas the concentration of *p*-hydroxybenzaldehyde was only influenced by temperature (and not by NaOH concentration) and the concentration of ferulic acid was only influenced by NaOH concentration (and not by temperature) (Table 2). In terms of feedstock, *p*-hydroxybenzaldehyde and *p*-coumaric acid had the highest concentrations in pretreatment liquors from sugarcane bagasse, followed by that of miscanthus and the lowest concentrations were found in pretreatment liquors of maize stover samples. Ferulic acid had also the highest concentrations in sugarcane bagasse liquors but had similar concentrations in miscanthus and maize stover samples. Interestingly, much smaller amounts of these free aromatic monomers were found in acid pretreated samples (not shown). In addition, a series of phenylpropanoid dimers present in the alkaline pretreatment liquor could be structurally characterised. These dimers can be classified in two classes: dimers with a glycerol aliphatic chain and ferulic acid containing dimers (Fig. 5, Table 2). The dimers with a glycerol aliphatic chain might arise from cleavage from beta aryl ethers in hot alkaline conditions [29–32]. The occurrence of these dimers in 20 °C samples hints that lignin degradation is occurring at these relatively low temperatures. However, it cannot be excluded that the dimers in 20 °C samples originate

Table 2 Abundance of the compounds characterised in alkaline pretreatment liquors

	Maize stover								Miscanthus								Sugarcane bagasse							
	Water		0.2 M NaOH		0.4 M NaOH		1 M NaOH		Water		0.2 M NaOH		0.4 M NaOH		1 M NaOH		Water		0.2 M NaOH		0.4 M NaOH		1 M NaOH	
Aromatic monomers	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C
1 *p*-hydroxybenzaldehyde	232	2486	6676	18368	12043	20825	13100	17315	39	5529	33318	71394	45682	72479	48139	70987	295	7217	67652	114765	76175	109028	75669	104939
2 *p*-coumaric acid	1865	6200	20541	39664	41984	55743	69022	78481	6	9446	60403	86250	98954	121878	209581	173383	6668	20777	70675	169709	119810	222879	245659	369714
3 ferulic acid	3037	1939	23819	26599	36647	34566	48658	46241	33	335	21671	21058	31556	28290	44645	43874	72	581	19252	37541	42517	43488	63507	64413
Dimers with glycerol aliphatic chain																								
4 G(8-O-4)Glycerol 1	5	14	22	382	35	829	34	1638	17	92	153	522	131	1589	173	4167	10	38	80	580	92	1381	100	2656
5 G(8-O-4)Glycerol 2	5	44	50	816	66	1604	52	3052	41	297	386	2174	435	4528	542	11432	29	98	167	1668	217	3175	269	5510
6 G(8-O-4)Sglycerol 1	117	108	493	918	571	1938	636	5647	92	169	705	974	935	3514	1211	10172	47	103	799	2419	1043	5720	1154	14754
7 G(8-O-4)Sglycerol 2	100	81	375	1511	409	2881	463	6618	54	144	453	2515	593	6909	831	19912	48	110	594	2444	709	6467	801	14947
8 S(8-O-4)Sglycerol 1	59	84	160	490	209	1022	273	3805	39	108	196	755	242	2137	338	8485	9	33	190	935	319	3017	315	9469
9 S(8-O-4)Sglycerol 2	58	101	188	1253	114	1723	224	4181	18	74	33	1237	58	2805	149	12244	4	30	60	804	91	2524	137	7727
Ferulic acid containing dimers																								
10 G(8-O-4)ferulic acid 1	136	137	6541	6559	7587	6461	9024	9494	361	159	19369	8215	21451	9190	25050	9032	188	208	10941	13445	13834	12398	15361	15378
11 G(8-O-4)ferulic acid 2	151	110	7038	6243	9587	6003	10568	5153	207	111	19436	9619	24514	11429	30389	5478	94	126	7733	10033	10931	12316	13641	9476
12 S(8-O-4)ferulic acid 1	87	42	961	6000	1342	4737	1545	5192	181	146	1322	6940	1560	6684	2294	6222	6	45	861	8737	1554	8774	1970	7134
13 S(8-O-4)ferulic acid 2	61	76	993	4829	1041	4522	1268	4935	175	159	1106	9062	1457	7589	2086	8065	11	59	1111	7532	1781	8296	2096	9286
14 G(8-5)ferulic acid	2	37	137	388	128	3356	123	1139	80	49	13837	13749	12409	12752	11903	12403	3	29	1743	4570	2132	4003	2968	3177
15 S(8-5)ferulic acid	16	5	6897	11540	9550	8099	6893	7437	118	93	2011	26398	2521	21996	3080	25700	6	79	898	12988	1395	13044	3171	13415
16 Ferulic acid(8-8)ferulic acid 1	3	8	1556	6050	3087	6984	5483	8355	2	2	904	2734	1798	3420	2855	4033	2	15	818	3538	1144	4388	2489	5130
17 Ferulic acid(8-8)ferulic acid 2	20	392	7488	17099	12006	22215	17651	30718	18	45	5686	11220	8007	18032	13989	26838	9	238	3410	22644	8283	29181	14562	42472
oxydized fatty acids																								
18 Trihydroxyoctadecanoic acid	458	621	665	10661	905	35804	2709	75882	5	523	1215	33590	2085	106461	11253	220672	174	692	627	10701	1012	55549	1589	192725
19 Dihydroxyoctadecanoic acid 1	194	212	362	7775	468	30016	1424	74002	1	18	37	3599	148	16010	991	36662	3	114	154	4851	289	17039	467	54986
20 Dihydroxyoctadecanoic acid 2	420	100	444	10085	563	32622	1620	52724	2	26	92	4665	173	16771	1802	31781	2	126	257	13994	344	32033	1877	63967
21 Dihydroxyoctadecanoic acid 3	142	179	114	3443	197	11580	217	9352	1	5	8	1516	58	7890	789	2223	1	40	268	4775	286	20949	411	28169

0% 0.5% 30% 100%

Color code. The percentage represents the relative peak area as expressed to that of the largest peak.

The values represent peak areas of the respective ions (*m/z* and retention time as in Supplemental Fig. 2), $n=3$. The colours represent relative peak areas with red and blue for the largest and smallest peak area, respectively, as indicated in the colour code

from compounds other than polymeric lignin. In this respect, it is important to note that a glycerol-type trimer, G(8-O-4)S(8-5)$G^{glycerol}$ has been found in xylem extracts of poplar [33]. S(8-O-4)ferulic acids and S(8-5)ferulic acid were found in the alkaline pretreatment liquors of all feedstocks tested. In general, these phenolic compounds were mainly present in the 180 °C pretreatment liquors and lower in the 20 °C liquors (Table 2). Interestingly, G(8-O-4)ferulic acids and G(8-5)ferulic acid showed not the same response as their S unit containing homologues. Strikingly, G(8-O-4)ferulic acids were mainly present in 20 °C pretreated liquors and were lower in the 180 °C liquors of miscanthus (Table 2). These differences in accumulation indicate that G- and S-derived compounds have different depolymerisation properties and/or that the released fragments have different re-polymerisation or degradation properties.

Ferulic acid(8-8)ferulic acid dimers were detected in all alkaline pretreatment liquors, and their concentration increased with increasing chemical load and temperature. The presence of ferulic acid dimers in alkaline extracts of grass cell wall material has previously been described [34].

A fourth class of compounds detected in alkaline pretreatment liquors were oxidised C18 fatty acids (Fig. 5, Table 2, Supplemental Fig. 3). These compounds might derive from the fatty acid moieties of phospholipids, triglycerides and waxes. Unsaturated C18 fatty acid moieties in these metabolites are relatively abundant in sugarcane and other grasses [35–37]. In alkaline conditions, the phospholipids, triglycerides and waxes will first be hydrolysed, and after which, the unsaturated fatty acid moiety can further be oxidised by O_2 via radical reactions [38]. Based on MS intensity, the trihydroxyoctadecanoic acid is a dominant peak in the samples pretreated with 1 N NaOH at 180 °C. In these samples, the peak area equalled or exceeded that of *p*-coumaric acid. Its abundance was positively correlated with the concentration of NaOH. In addition, relatively low amounts of oxidised fatty acids were detected in the samples pretreated at 20 °C. This observation is in line with the hypothesis that these compounds are derived from oxidation of unsaturated C18 fatty acids by O_2, a reaction that is enhanced at higher temperatures [38].

Also, the highest accumulating compounds (based on MS ion traces) in acid pretreatment liquors could be tentatively structurally characterised. All were *p*-coumarate and ferulate esters (Table 3, Supplemental Fig. 3). Their respective ion traces were manually integrated in each of the chromatograms of the acid pretreatment liquors, as these compounds could not be detected with certainty in alkaline pretreatment liquors (Table 3). Interestingly, both *p*-coumaroyl and feruloyl pentoses were found in any of the 180 °C acid pretreatment liquors but not in those of 20 °C. These compounds could

Table 3 Abundance of the compounds characterised in acid pretreatment liquors. The values represent peak areas of the respective ions (*m/z* and retention time as in Supplemental Fig. 2), *n*=3. The colours represent relative peak areas with red and blue for the largest and smallest peak area, respectively, as indicated in the colour code

		Maize stover								Miscanthus								Sugarcane bagasse							
		Water		0.2 M H_2SO_4		0.4 M H_2SO_4		1 M H_2SO_4		Water		0.2 M H_2SO_4		0.4 M H_2SO_4		1 M H_2SO_4		Water		0.2 M H_2SO_4		0.4 M H_2SO_4		1 M H_2SO_4	
	Cinnamoyl esters	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C	20 °C	180 °C
22	*p*-coumaroyl pentose 1	0	1	7	95	1	534	0	654	0	4	9	101	0	2484	0	4286	0	10	22	2165	16	10217	2	15140
23	*p*-coumaroyl pentose 2	0	6	0	39	0	187	0	155	0	0	2	24	0	730	0	1406	0	6	13	869	9	5311	7	7718
24	*p*-coumaroyl pentose 3	46	122	132	46	58	218	98	1771	0	10	2	14	0	716	0	2675	0	190	11	632	9	6629	4	25655
25	Feruloyl pentose 1	68	52	10	162	8	1005	5	1158	0	9	13	166	2	1697	3	1967	0	11	13	818	7	2927	1	4602
26	Feruloyl pentose 2	1	18	35	800	40	4936	31	5911	0	23	43	1039	14	10735	15	14096	0	45	64	3706	47	16846	17	28262
27	Feruloyl pentose 3	82	121	12	490	3	2337	0	2050	0	15	29	416	6	3062	4	3307	6	26	43	1710	16	5344	5	9740
28	Feruloyl pentose 4	69	102	8	215	7	2863	0	12428	0	122	8	182	0	8674	0	16872	26	735	32	2083	17	13993	9	37292
32	Feruloyl quinate	21958	15508	10005	1264	10484	5702	6382	12325	0	49	0	5	0	41	1	50	377	393	37	2	35	85	51	117

0% 0.5% 30% 100%

Color code. The percentage represents the relative peak area as expressed to that of the largest peak.

logically be derived from the degradation of hemicelluloses, as acid conditions at higher temperatures are able to degrade the glycosidic bonds in hemicellulose [39], but not the ester bond that link the *p*-coumarates and ferulates to hemicellulose. In addition, feruloyl quinate was found in the water extracts of the maize stover samples. Although there were some subtle differences in the concentrations of feruloyl quinate in the pretreatment liquor depending on the concentration of H_2SO_4 and temperature used, this compound remained largely unaffected during acid pretreatment.

Generation of Furfural and Hydroxymethyl Furfural During Pretreatment

Furfural and hydroxymethyl furfural (HMF) are dehydration products of pentose and hexose sugars, respectively, and are well-known inhibitors of fermentation, but could also serve as industrial platform chemicals [40, 41] and thus may be problematic or valuable depending on whether or not the pretreatment liquor is further processed. Significant amounts of either HMF or furfural were only detected using acid pretreatment at 180 °C (Fig. 6). The amounts of HMF and furfural in pretreatment liquors were feedstock dependent, with maize stover producing more HMF than either miscanthus or sugarcane bagasse; furfural was produced in higher quantities from miscanthus and sugarcane bagasse feedstock as compared to maize stover. Miscanthus pretreatment liquor contained relatively low amounts of HMF as compared to the amounts of furfural. The difference in HMF and furfural production reflects the monosaccharide composition released into the pretreatment liquor by the different feedstocks. Maize released a greater proportion of hexoses into the pretreatment liquor than miscanthus and sugarcane bagasse (Fig. 6) and hence also produced more HMF.

Discussion

Improving the profitability of lignocellulosic biofuel production is critical to its commercial development. In this paper, we have examined the types and quantities of products released from lignocellulosic feedstocks under a range of aqueous pretreatment conditions in order to identify conditions that both satisfy the need to potentiate enzymatic hydrolysis, as well as releasing potentially valuable coproducts. Alkaline pretreatments provide a range of potentially interesting chemical building blocks in the pretreatment liquors. In addition, the potential to use the sugars produced during alkaline pretreatments for fermentation is higher as less inhibitory substances such as HMF and furfural are produced as compared to acid pretreatments (Fig. 6). The amount of lignin remaining in the biomass is lower when NaOH is used during pretreatment (and not by use of H_2SO_4) and a temperature of 180 °C is more effective than 20 °C (Fig. 4). In addition, while at 20 °C there is a positive relation between chemical load and lignin removal, the chemical load does not seem to influence the remaining lignin at 180 °C (Fig. 4). The lower the temperature and the lower the chemical load during pretreatment that needs to be used to achieve an efficient saccharification, the more cost-effective the whole biorefinery process will be. Thus, according to our data, there is no need to go to higher temperatures than 130 °C and chemical concentration higher than 0.2 N when saccharification yield is the main interest.

In terms of added-value chemical compounds released during these pretreatment conditions, NaOH (0.2 N at 130 °C) releases similar amounts of sugars as compared to H_2SO_4, although there are some differences between the different feedstocks tested (Fig. 1 Supplementary Tables 1

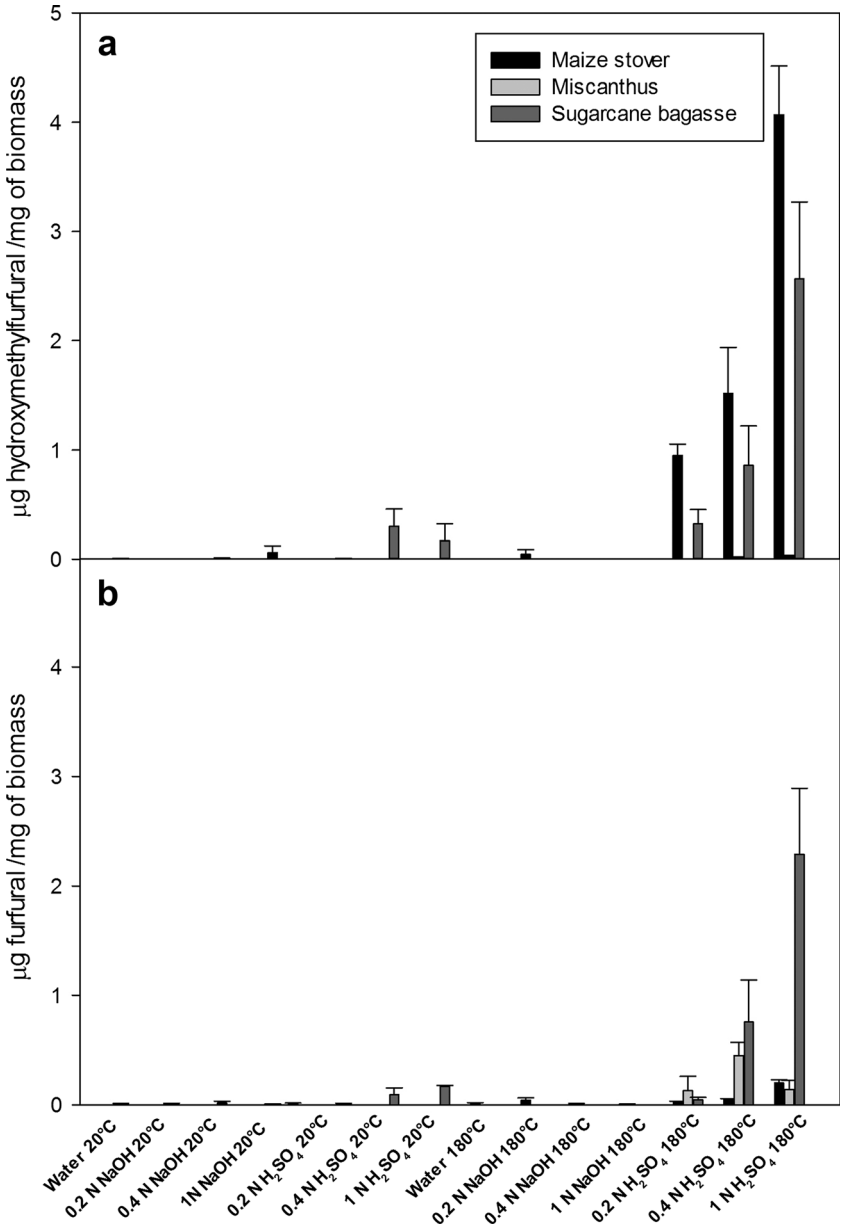

Fig. 6 Hydroxymethyl furfural (**a**) and furfural (**b**) production under increasing strength of chemical pretreatment at 20 and 180 °C during 40 min. The experiments were conducted in triplicates

and 2). In general, the pretreatment liquor is more enriched in pentose sugars (and the remaining cell wall material enriched in hexoses) when NaOH is used as compared to H_2SO_4. This might be favourable as these sugars could be used in fermentation with yeasts optimised to use pentose sugars [42] or pentose-fermenting organisms such as *Geobacillus* [43] or by filamentous fungi for enzyme production [44] or used in anaerobic digesters to produce methane for bioenergy applications. In addition to sugars, other potentially useful chemical building blocks present in the alkaline pretreatment liquors were characterised. A series of dimers with a glycerol aliphatic chain that are derived from lignin degradation were found after alkaline pretreatment at 180 °C and not at 20 °C (Tables 2 and 3). Similarly, ferulic acid containing dimers are more abundant at higher temperatures (with the exception of two G(8-O-4)ferulic acid dimers). However, the quantity of these products remained relatively low and it is questionable if they can be purified for further processing as technologies involving isolation of aromatic building blocks from biomass are still in their infancy [45]. However, recent developments in purification of phenolics via adsorption techniques look promising [46]. On the other hand, the mixture of these phenolic dimers might have a significant value in itself for the chemical industry.

In terms of yield, *p*-hydroxybenzaldehyde, *p*-coumaric acid and ferulic acid seem efficiently released by NaOH even at low temperatures and low chemical load (Table 2). The actual yield of these compounds was not determined in our study but

will be part of future research. Nevertheless, from a biorefinery perspective, these compounds are potentially of interest as there is a market for these products. For instance, ferulic acid is widely used in the food and cosmetic industries: It is used as the raw material for the production of vanillin and preservatives, as a cross-linking agent for the preparation of food gels and edible films and as an ingredient in sports foods and skin protection agents [47]. Ferulic acid can also be biocatalytically converted into other useful aromatic chemicals including vanillin, styrene and vinylguaiacol [48]. In addition, fatty acids were found in the alkaline pretreatment liquors (Fig. 5, Table 2). In theory, these fatty acids could be readily skimmed as sodium soap from the complex pretreatment liquor prior pH neutralisation, in a similar manner to that of tall oil being isolated during the kraft pulp process of coniferous wood [49]. The resulting soap has several potential applications, among which the esterification of the fatty acids with methanol or ethanol would produce fatty acid methyl or ethyl esters, respectively, which is biodiesel [49–51].

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