Unraveling substrate dynamics and identifying inhibitors in hydrolysates of lignocellulosic biomass by exometabolomics

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CHAPTER 3

Analysis of Oligosaccharides in Lignocellulosic Biomass Hydrolysates by High-Performance Anion-Exchange Chromatography Coupled with Mass Spectrometry (HPAEC–MS)

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Bioresource Technology **2013**, 133, 221-231

Abstract

The carbohydrate composition of lignocellulosic biomass hydrolysates is highly complex. High performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), a widely used method for carbohydrate analysis, provides limited chemical information on the detected peaks. To improve the detection and increase the chemical information of the carbohydrates, HPAEC was coupled with mass spectrometry (MS). Using a pooled hydrolysate sample, it was shown that HPAEC-MS can separate and detect many oligosaccharides in one experimental run based on retention time and mass. The method was validated on its linearity, reproducibility and response factors. The analysis of a group of different biomass hydrolysates revealed that remaining disaccharides was the bottleneck of the hydrolysis process. As an analytical tool, HPAEC-MS provides information for the improvement of hydrolysate pretreatment method and enzyme cocktail quality. Besides, the consumption ability of microbial host strains for various mono- and oligosaccharides in hydrolysates can be assessed.

Introduction

Lignocellulosic biomass is the largest feedstock form for 2nd generation biofuel production. Compared to fossil fuels, it is renewable, environmental friendly, while compared to starch and sugarcane, it is not competing with world food resources [1,2]. Generally, lignocellulosic biomass is composed of cellulose (35-50%), hemicellulose (30-35%) and lignin (10-25%), among which cellulose is the backbone structure, while hemicellulose and lignin are the binding networks [3]. To release the sugars in lignocellulosic biomass, a pretreatment step is required to break down the binding networks and expose cellulose. The exposed cellulose is then hydrolyzed by using either fungal enzyme cocktails or sulfuric acid [4]. The released monosaccharides, e.g. glucose and xylose, in the so-called hydrolysate, are the potential carbon sources for ethanol production through fermentation processes [5]. Due to the robust structure of lignocellulosic biomass and the inhibitory compounds formed during the pretreatment process, the hydrolysis efficiency on pretreated biomass is not satisfying [6,7]. This limits the increase of bioethanol yield. Therefore, to enhance hydrolysis efficiency of pretreated lignocellulosic biomass is of decisive importance for improving bioethanol yield.

In the last decade, efforts have been paid to improve the hydrolysis efficiency of fungal enzyme cocktails on lignocellulosic biomass. Genetic engineering of known cellulases production strains and screening for novel producers of lignocellulolytic enzymes are the two main paths [8,9]. The hydrolysis efficiency was mainly determined by measuring monosaccharides concentration in hydrolysates. However, more detailed knowledge on oligosaccharides composition in hydrolysates is crucial to further understand and improve the performance of fungal enzyme cocktails.

Knowing the general structure of lignocellulosic biomass, it can be predicted that biomass hydrolysates contain mono- and oligosaccharides composed of hexoses (C6), pentoses (C5) and the combination of C6 and C5 sugars. The C6 sugar units are mainly glucose, galactose and mannose, while the C5 sugar units are mostly xylose and arabinose [3,10]. These carbohydrates have also different degrees of polymerization (DP) and may carry sugar-acid residues, which makes the analysis of carbohydrate composition of biomass hydrolysates very challenging. High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is a widely used method for analyzing carbohydrates, especially for analyzing different monosaccharides and disaccharides for which reference compounds are present [11,12]. Though HPAEC is a powerful tool for separating carbohydrates, the PAD detector provides only limited chemical information on the detected

peaks. Such chemical information is needed for complex samples, such as biomass hydrolysates. Mass spectrometry (MS), with or without coupling to chromatography, can be a very informative technique for analyzing oligosaccharides [13,14]. Being highly selective and sensitive, MS provides detailed chemical information on the peaks, such as degree of polymerization and building blocks. Therefore, the coupling of HPAEC with MS holds high potential for separating and charactering complex mixture of carbohydrates. Several attempts have been made to couple HPAEC with MS [15-21]. And the performance of HPAEC-MS has been demonstrated for complex mixtures of galacto-oligosaccharides (GOS) [15], extracts of poplar leaves [22], vegetables [21], and bacterial cell hydrolysate [20]. Only one study with preliminary results could be found, in which HPAEC-MS was applied to biomass hydrolysates [19].

In this study, we demonstrate in more depth the performance of HPAEC-MS on a range of lignocellulosic biomass hydrolysates. HPAEC-MS could separate many of the carbohydrates and classify the peaks based on their degree of polymerization (DP) and building blocks, i.e. pentoses, hexoses, and aldonic acids, in one experimental run. The method was validated and the relative quantity of different classes of carbohydrates could be assessed using relative response factors. A series of biomass hydrolysates, generated from various biomass types and by different pretreatment-hydrolysis methods, together with their fermentation samples, were successfully analyzed with HPAEC-MS.

Materials and Methods

HPAEC-MS

Commercially available reference compounds of carbohydrates were purchased from either Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands) or Megazyme International Ltd. (Wicklow, Ireland). Internal standards sucralose, ribose-$^{13}C_5$ and glucose-d7 were purchased from Sigma-Aldrich. All standard solutions were prepared in MilliQ water.

HPAEC-MS was performed on a Thermo Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA) equipped with a Carbopac PA1 column (250 x 2.0 mm i.d., Dionex, Sunnyvale, CA) operated at 35°C. Elution was performed with a flow of 215 µl/min and the injection volume was 3 µl. The following eluents were used: 100 mM NaOH (A) and 100 mM NaOH + 500 mM NaOAc (B). The following gradient was used: 0-5 min, isocratic 100% A, 5-78 min, linear gradient from 100 to 74% A followed by a washing step with 100%

B for 6 min and re-equilibration for 10 min at 100% A. Prior to MS detection, an ASRS300-2 mm suppressor (Dionex) was used as an in-line desalter to convert the eluate into an MS-compatible solution. The membrane was continuously regenerated with acid generated by electrolysis of water. Milli-Q water was fed from an air-pressurized bottle into the regenerant chamber at a flow rate of 3.33 ml/min. A regenerant current of 150 mA was applied. Mass detection was carried out on a Thermo LTQ LT-1000 mass detector using electrospray ionization in the positive ion mode (ESI spray voltage, 3.5 kV; heated capillary temperature, 275°C; sheath gas, 30; auxiliary gas, 5; full scan range, m/z 125-1000; number of microscans, 3; maximum injection time, 200 ms.). The mass detector was tuned using a calibration solution of lactose. For all different classes of carbohydrates the main ion detection corresponded to $[M+Na]^+$.

HPAEC-MS/MS was performed on the same system and using identical conditions as described above. MS^n-experiments were performed on pre-selected peaks using wide band activation and based on dependent scan settings with collision energy of 30% (Xcalibur software V2.0, Thermo Electron Corporation).

Partial validation of HPAEC-MS

Partial validation of HPAEC-MS for oligosaccharides analysis was performed on three aspects, linearity, reproducibility and relative response factors. Linearity was determined by constructing calibration curves of reference compounds. The reference compounds were dissolved in MilliQ water with concentrations ranging from 1 to 100 µg/ml. From the constructed calibration curves, the coefficient of determination (R^2) was calculated (Table 1). Reproducibility was determined by preparing a pooled sample using 21 different hydrolysates (see section 2.3 and Table 4), followed by analyzing this sample in triplicate on three different days, and calculating the coefficient of variation (CV) of these nine HPAEC-MS runs (Table 2). Relative response factors were calculated by comparing the calibration curves of the reference compounds with glucose-d7 (Table 3).

Biomass hydrolysate samples preparation

Sugar cane bagasse is a kind gift from Zilor, Brazil, and corn stover is from the University of Cape Town, South Africa. Wheat straw, barley straw and willow wood are purchased from Oostwaardshoeve, the Netherlands, and oak sawdust is from ESCO, the Netherlands, a wood-flooring supplier. All biomass is pre-dried at 80°C for 5 hours when received, and stored at room temperature in air-tight bags. Prior to pretreatment, biomass (except oak saw

dust) was ground to pieces with average length of 3 mm, and was dried again at 80°C for minimum 16 hours.

Four pretreatment methods were used to prepare biomass hydrolysates, namely dilute acid (2% H_2SO_4), mild alkaline (3% $Ca(OH)_2$), alkaline/peracetic acid, and concentrated acid (72% H_2SO_4). The biomass pretreated with the first three methods was hydrolyzed with Accellerase 1500 (Genencor), while the concentrated acid pretreated biomass was hydrolyzed in acid (40% and 15% H_2SO_4). The detailed steps of these methods are described in Zha *et al*. [23]. Before HPAEC-MS analysis, all hydrolysates were filtrated through 0.22 µm filter, and internal standards were added (c=20 µg/ml). Each sample was then diluted ten times with MilliQ water, and 3 µl of the diluted sample was injected.

Table 1 Linearity of the HPAEC-MS analyzing results on carbohydrate standards, expressed as coefficient of determination (R^2).

Compounds	R^2 (n=8)
Glucose	0.9950
Xylose	0.9900
Galactose	0.9900
Arabinose	0.9870
Mannose	0.9990
Ribose	1.0000
Cellobiose	1.0000
Xylobiose	0.9890
Arabinobiose	0.9980
Mannobiose	0.9990
Cellotriose	0.9970
Xylotriose	0.9930
Mannotriose	0.9940
Xylotetraose	0.9970

Table 2 Reproducibility of the HPAEC-MS analyzing results on selected carbohydrates in pooled hydrolysate sample, expressed as coefficient of variation (CV). (GlcA: glucuronic acid, GalA: galacturonic acid)

Selected compounds	CV % (n=9)
DP1 C6	6
DP1 C5	5
DP2 C6	4
DP2 C5	5
DP2 C5C6	5
DP3 C6	6
DP3 C5	8
DP3 C5C6C6	9
DP3 C5C5C6	6
DP4 C5	9
Galactonic acid	13
Gluconic acid	16
GalA	7
Galactonic acid/ gluconic acid-C6	12
GlcA/GalA-C5	9

Table 3 Response factors of carbohydrates belong to several different classes in HPAEC-MS results.

Compounds	Classes	Peak areas	Response factor with regard to glucose-d7
Glucose	DP1 C6	6804392	1.0
Galactose (Gal)	DP1 C6	6897888	1.0
Arabinose (Ara)	DP1 C5	6321841	0.9
Mannobiose	DP2 C6	12161242	1.8
Cellobiose	DP2 C6	11111423	1.6
Xylobiose	DP2 C5	14217711	2.1
Arabinobiose	DP2 C5	10074466	1.5
3-Gal-Ara	DP2 C5C6	8614681	1.3
Xylotriose	DP3 C5	13503524	2.0
galactonic acid	C6 acid	3809735	0.6
gluconic acid	C6 acid	3809735	0.6
GalA (galacturonic acid)	C6 acid	2644657	0.4
GlcA (glucuronic acid)	C6 acid	1882777	0.3
Lactobionic acid	C6C6 acid	3911437	0.6

Bagasse hydrolysate fermentation samples preparation

Bagasse hydrolysates, prepared with diluted acid and concentrated acid method, were fermented in 2 l New Brunswick fermentors, with working volume of 1 l. The hydrolysates were stored at 4°C before being used as fermentation media. The detailed fermentation set-up and process are described in Zha *et al*. [24]. The strains used were *Saccharomyces cerevisiae* CEN.PK 113-7D (CBS 8340) and *Pichia anomala* TNO11-29 (CBS 132101), for which the loading was 0.1 g and 0.2 g cell dry weight per 1 l hydrolysate, respectively. During the whole batch fermentation process, temperature was kept at 30°C, pH at 5.0 by adding 2 M KOH or 1 M H_2SO_4, dissolved oxygen at 0 by flushing 0.5 l/min N_2 continuously. Fermentation samples were taken every 120 min during the fermentation process. The samples were directly cooled to 4°C, centrifuged to remove yeast cells, and stored at -20°C. For each fermentation, three samples were selected to be analyzed with HPAEC-MS. These three samples were taken immediately after inoculation, mid of growth phase, and in the stationary phase. Before HPAEC-MS analysis, the samples were treated as described above.

Results and Discussion

HPAEC-MS method development

The technical challenge of coupling HPAEC with MS is the desalting of the mobile phases prior to MS detection using a Na^+- H^+ exchange suppressor [16-18]. For the analysis of biomass hydrolysates, HPAEC-MS has only been described once [19].

Here we describe an extensive study on the application of HPAEC-MS in analyzing a variety of different biomass hydrolysates (Table 4). Figure 1A shows the complex base peak HPAEC-MS chromatogram of a pooled hydrolysate sample, similar to PAD detection. The molecular mass information provided by mass spectrometry (MS) can be used to distinguish hexose (C5) and pentose (C6) sugars, as well as their degree of polymerization (DP) by extracting *m/z* related ions (Figure 1B). Thus MS helps to separate the coeluting peaks in the base peak chromatogram, and identify carbohydrates according to their DP and subunit types (hexose/pentose). This approach enables a deeper understanding of carbohydrates composition in biomass hydrolysates, and allows focusing on the compound of interests for further study.

The number of peaks in the chromatograms increases with the increase of DP until DP3 (Figure 1B), suggesting an increased complexity of the carbohydrates remaining in the hydrolysates. With DP4 and higher DP's (data not shown), the number of peaks and the peak areas decrease, indicating that the pooled hydrolysate sample mainly consisted of mono-, di- and trisaccharides. It should be noted that the HPAEC-MS method was tuned with lactose, a DP2 hexose, and thus the highest ionization efficiency is observed for disaccharides. Analysis of reference carbohydrates indeed showed a decrease in response with increase of DP (data not shown).

As shown in Figure 1B, HPAEC-MS is also able to detect uronic acids, such as glucuronic acid (GlcA) and galacturonic acid (GalA), aldonic acids, such as gluconic acid and galactonic acid, and their oligomers. These results demonstrate that both neutral and acidic mono- and oligosaccharides can be separated and detected, by extracting their characteristic *m/z* values, in a single experimental run with HPAEC-MS.

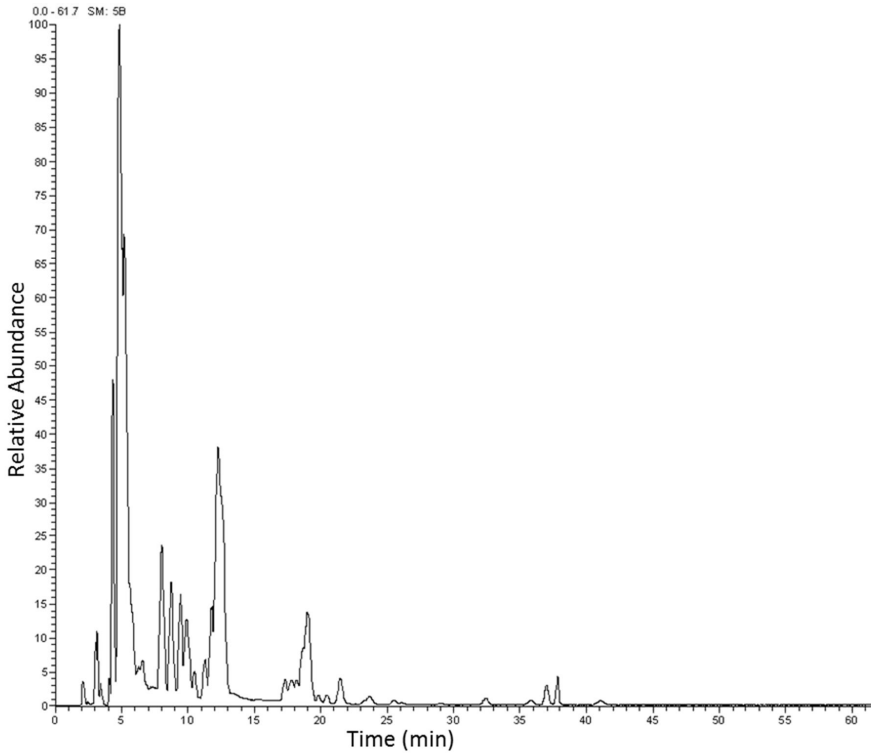
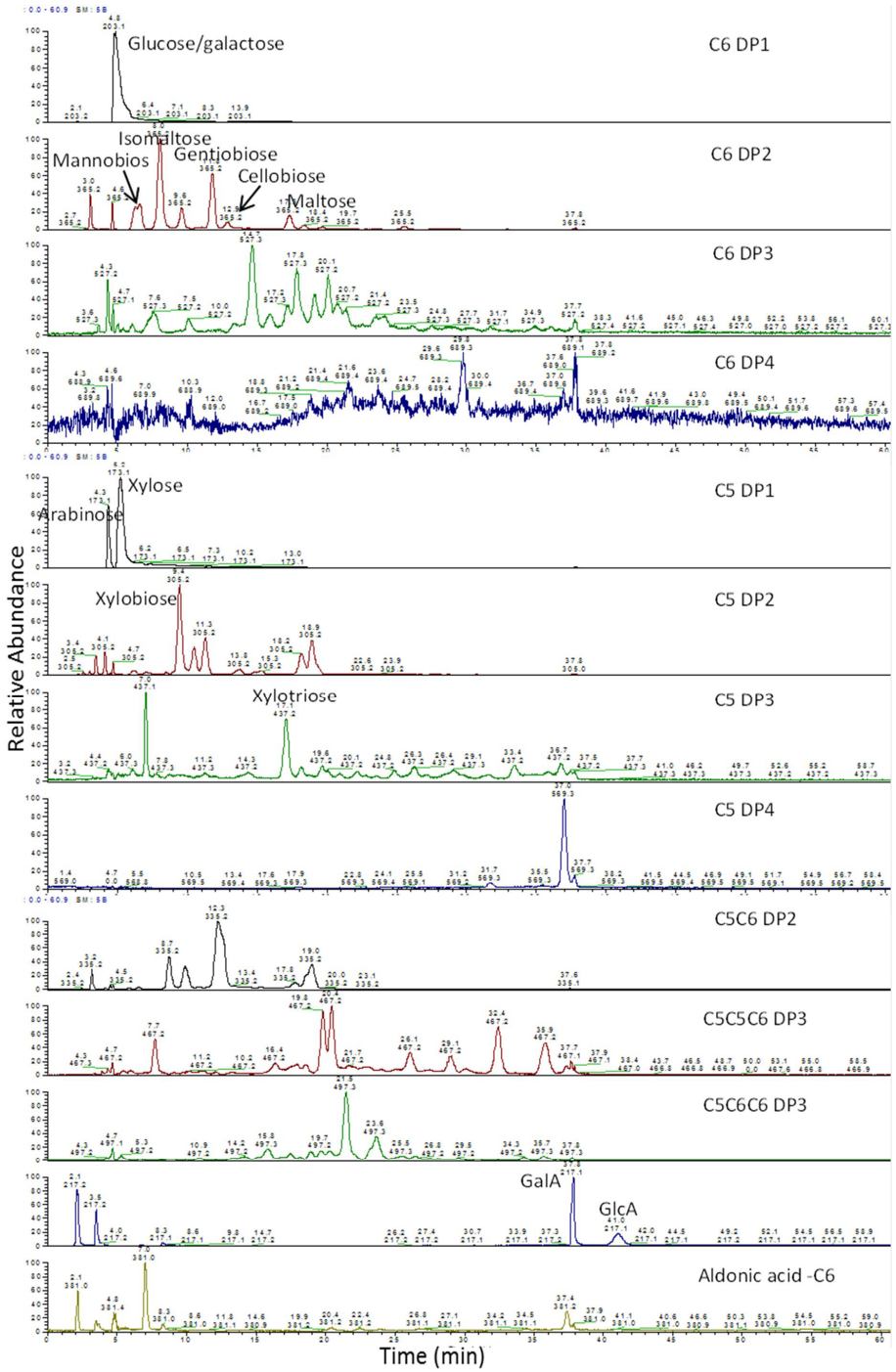


Figure 1A HPAEC–MS base peak chromatogram of the pooled hydrolysate sample.

Figure 1B (on the next page) HPAEC–MS extracted ion chromatograms ($[M+Na]^+$) of the pooled hydrolysate sample. C6: hexose, C5: pentose, GlcA: glucuronic acid, GalA: galacturonic acid, DP: degree of polymerization; extracted ion chromatograms corresponding to C6 DP1 (*m/z* 203.1), C6 DP2 (*m/z* 365.2), C6 DP3 (*m/z* 527.1), C6 DP4 (*m/z* 689.2), C5 DP1 (*m/z* 173.1), C5 DP2 (*m/z* 305.2), C5 DP3 (*m/z* 437.3), C5 DP4 (*m/z* 569.5), C5C6 DP2 (*m/z* 335.1), C5C5C6 DP3 (*m/z* 467.1), C5C6C6 DP3 (*m/z* 497.3), GalA/GlcA (217.1), and aldonic acid-C6 (381.0). Labeled peaks were identified by authentic standards.


Glucose/galactose
C6 DP1
Isomaltose
Mannobios
Gentiobiose
Cellobiose
Maltose
C6 DP2
C6 DP3
C6 DP4
Xylose
Arabinose
C5 DP1
Xylobiose
C5 DP2
Xylotriose
C5 DP3
C5 DP4
C5C6 DP2
C5C5C6 DP3
C5C6C6 DP3
GalA
GlcA
Aldonic acid -C6
Relative Abundance
Time (min)

Identification with HPAEC-MS/MS

An additional advantage of the coupling of HPAEC with MS is the possibility of on-line tandem mass spectrometry (MS/MS) of individual peaks. This can be used as the first step in identifying unknown carbohydrates. Although NMR is the ultimate technique for full identification of unknown compounds [15], HPEAC-MS/MS can give information on the identity of peaks quite easily. NMR will be required to further identify the detailed structure of some novel unknown compounds. HPAEC-MS provides information on the building blocks of the oligosaccharide, i.e. number of pentoses and hexoses. Figure 2A and 2B show MS/MS spectra obtained for two peaks observed with HPAEC-MS in the quality control sample with *m/z* 335. It can be clearly seen that one of the fragments, *m/z* 203, corresponds to the loss of 132 Da, which can be explained by the loss of a pentose unit. In addition, the fragment with *m/z* 203 corresponds to a hexose unit. Therefore, it can be concluded that the peaks with *m/z* 335 correspond to pentose-hexose (C5C6) disaccharides. Moreover, fragments corresponding to the loss of 60, 90 and 120 Da are visible for both C5C6 disaccharides. These losses are characteristic cross-ring cleavages, and it has been reported for hexose disaccharides that different ratios of these fragments correspond to specific linkages [13]. Although almost no reference compounds are available for C5C6 disaccharides, the MS/MS spectra in figure 2A and 2B show that for the two C5C6 disaccharides, different ratios can be observed for these fragments, indicating differences in linkages between these compounds. Figure 2C and 2D show similar MS/MS spectra of peaks with *m/z* 467 and 497. Based on the MS/MS fragments, these peaks can easily be identified as a C5C5C6 trimer (*m/z* 467) and a C5C6C6 trimer (*m/z* 497). Also here MS/MS spectra provide (partial) linkage information. Further research and especially data from relevant reference compounds is necessary to make more use of the MS/MS data for identification. The analysis of C5C6 oligosaccharides in biomass hydrolysates has not been reported before with HPAEC-MS, although their presence has been shown using other techniques [25,26]. These studies clearly showed the complexity of biomass hydrolysates and thus the added value of using MS as a detection method in combination with HPAEC.

HPAEC-MS method validation

It was shown above that HPAEC-MS is capable of separating and detecting complex mixtures of oligosaccharides. To apply the method in practice, it is very important to evaluate the performance of the method. As HPAEC-MS will probably be used in screening experiments where oligosaccharide patterns are compared in a quantitative manner, the method was validated on three aspects: linearity, reproducibility, and relative response

factors of various carbohydrates. The pooled hydrolysate sample was used for testing reproducibility, while purchased reference compounds were used as standards for studying linearity and response factors.

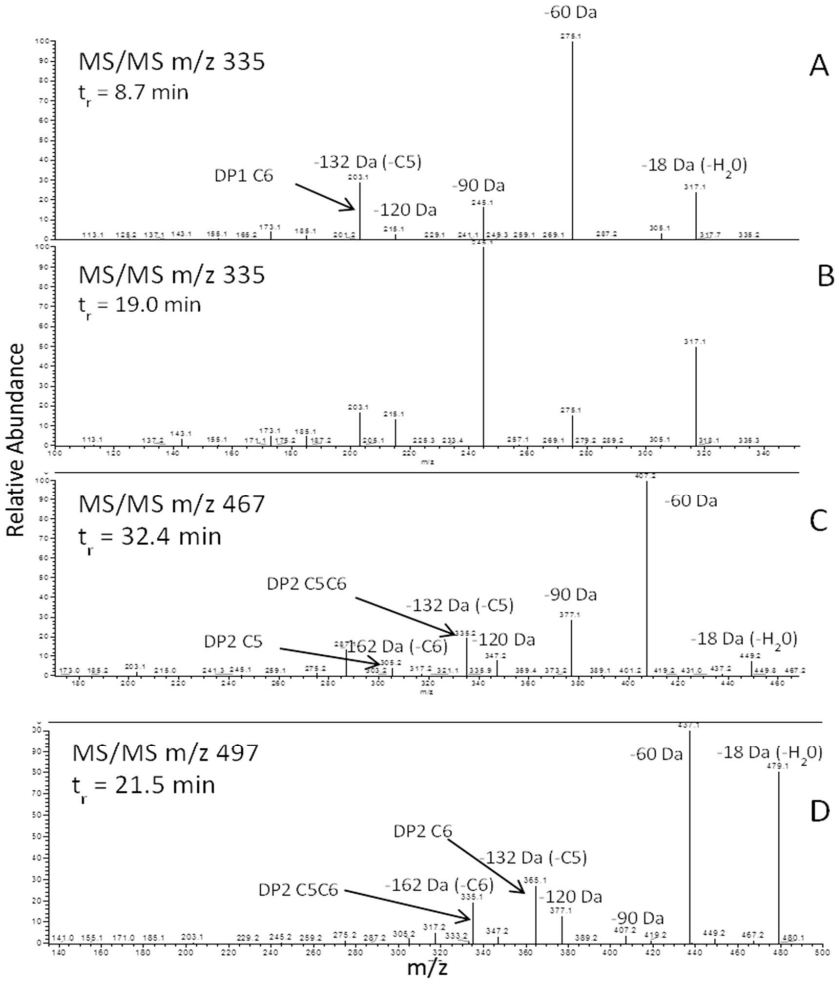


Figure 2 MS/MS spectra of peaks observed in the pooled hydrolysate sample with HPAEC–MS; (A) *m/z* 335, tr = 8.7 min, (B) *m/z* 335, tr = 19.0 min, (C) *m/z* 467, tr = 32.4 min, (D) *m/z* 497, tr = 21.5 min.

Linearity

The linearity of HPAEC-MS was studied with purchased reference compounds, as shown in Table 1. The testing standards were individual carbohydrates with eight different concentrations, ranging from 1 to 100 µg/ml. Based on the measurement results, calibration curves were generated, with which coefficient of determination (R^2) was calculated for each compound (Table 1). With all the compounds tested, the response of HPAEC-MS was linear in the range of 1-100 µg/ml, and can thus be used for quantification, although the influence of sample matrix needs to be considered with complex samples, such as biomass hydrolysates.

Reproducibility

To test the reproducibility of HPAEC-MS on biomass hydrolysates, the pooled sample was analyzed in triplicates on three different days, which resulted in nine individual measurement results. Ten carbohydrates of DP1-4 and five acidic sugars were selected for calculating their relative peak areas using the nine measurement results. These relative peak areas were in turn used for coefficient of variation (CV) calculation (Table 2). As far as the selected carbohydrates are concerned, CV values increased with increasing of DP, but were all below 10%. Compared to carbohydrates, the CV values of acidic sugars were somewhat higher, up to 16% (Table 2). These results indicate that HPAEC-MS can measure carbohydrates with reasonably high reproducibility.

Relative response factor

Typically, biomass hydrolysates contain many different carbohydrates of which many are unknown. Identification of each individual unknown carbohydrate is time-consuming and practically impossible. However, for optimizing experimental conditions, it is helpful to have estimates of concentrations of these carbohydrates. One possibility is to use so-called relative response factor, i.e. relative peak area at a certain concentration with respect to a reference compound. Therefore, the relative response factors of several carbohydrates and acidic sugars were calculated from their calibration curve, and compared with glucose-d7. As shown in Table 3, the peak areas of DP1's were almost equal to that of glucose-d7, while the relative response factors of DP2 and DP3 were ~1.7 and 2.0. Acidic sugars had response factors of ~0.5. The high response factors observed for DP2 and DP3 may due to the fact that lactose, a DP2 hexose, was used for tuning MS. These relative response factors can be used for (semi)-quantification of identified peaks, for which there are no reference compounds, or unidentified peaks when the building blocks and degree of polymerization are known.

HPAEC-MS method application

Biomass hydrolysate samples

Generally, lignocellulosic biomass hydrolysates are prepared from a diverse range of biomass types and with various methods. Different biomass are used in different areas of the world and the method chosen for biomass hydrolysate preparation differs [27]. Despite the high variety in biomass hydrolysate preparation, a main goal is to achieve high hydrolysis efficiency. This is mainly done by improving pretreatment processes and enzyme cocktail quality [8]. Analysis of biomass hydrolysate samples with HPAEC-MS provides important information on their carbohydrate composition, resulting in new targets for improving hydrolysis efficiency. To study the influence of biomass type and hydrolysate preparation method on the carbohydrate composition of biomass hydrolysates, 21 different biomass hydrolysates were generated in our lab from six types of biomass: wheat straw (WS), barley straw (BS), corn stover (CS), bagasse (Bag), oak saw dust (Oak) and willow wood chips (Willow) (Table 4). The methods used for generating these hydrolysates were (1) dilute acid (DA), (2) mild alkaline (MA), (3) alkaline/peracetic acid (PAA), and (4) concentrated acid (CA) [23]. The hydrolysates prepared with DA, MA and PAA method were enzymatically hydrolyzed, while the hydrolysates prepared with CA method were hydrolyzed with H_2SO_4.

These 21 hydrolysates were analyzed with HPAEC-MS, and the results show that their overall carbohydrate compositions are comparable. The base peaks and extracted ion chromatograms (DP1-3) of wheat straw-dilute acid (WS-DA) and wheat straw-concentrated acid (WS-CA) hydrolysates are shown here as examples (Figure 3). As expected, the highest peak was C6 DP1, glucose/galactose, while the second highest peak was C5 DP1, xylose and arabinose. Next to these peaks, several disaccharides were detected, among which were not only C6-C6 and C5-C5, but also hybrid disaccharides C6-C5 (Figure 3). DP3's and DP4's were also observed, but with significantly lower amounts. In most hydrolysates, DP4's only count for less than 1% of the detected carbohydrates (Table 4). Remarkably, the peak areas of C6-C5 disaccharides were larger in most hydrolysates compared to C6-C6 or C5-C5, suggesting the degradation of C6-C5 disaccharides is the most difficult among the three DP2 forms. Besides, of all the C6-C6 disaccharides, cellobiose only counted for 1-3% (Table 4). This result indicates that the majority of the remaining C6-C6 disaccharides detected in these hydrolysates were not the degradation products of cellulose. It can be concluded that the most abundant C6-C6 disaccharides were isomaltose, gentiobiose, and mannobiose, which are not the degradation products of hemicellulose either (Figure 1A). Therefore, it is suspected that some of the remaining DP2's are formed through transglycosylation. It is known that enzymes possess hydrolytic activity, such as β-glycosidases and β-xylosidases,

also catalyze transglycosylation reactions [28], but these reactions were not widely reported in hydrolysis processes of pretreated lignocellulosic biomass. During enzymatic hydrolysis processes, when high concentration of monomer hexoses and pentoses are present together with hydrolytic enzymes, it is highly possible that these enzymes also catalyze the transglycosylation reactions.

Table 4 Biomass hydrolysates used for HPAEC-MS analysis, and the percentage of monosaccharides and oligosaccharides to detected carbohydrates, calculated with equation: $\frac{\text{DPX}}{\text{detected carbohydrates}}(\%)=\frac{\text{sum of DPX peak area}/\text{DPX response factor}}{\text{total peak area}}$; response factors used: 1.0 (X=1), 1.65 (X=2), 2.0 (X=3 & 4). Hydrolysates prepared with different methods have specific distribution of disaccharide forms: C5C5, C5C6, C6C6. Grey marked area indicates the highest percentage of a disaccharide form among hydrolysates prepared with the four methods: CA, DA, MA, PAA. Bag: bagasse, BS: barley straw, CS: corn stover, Oak: oak saw dust, Willow: willow wood chips, WS: wheat straw; CA: concentrated acid, DA: dilute acid, MA: mild alkaline, PAA: peracetic acid; DC: detected carbohydrates.

	DP1/ DC	Glucose/ DP1	DP2/ DC	Cellobiose/ DP2	C5C5/ DP2	C5C6/ DP2	C6C6/ DP2	DP3/ DC	DP4/ DC
Bag-CA	53%	57%	38%	3%	13%	45%	42%	3%	0%
BS-CA	47%	62%	40%	3%	13%	45%	42%	3%	0%
CS-CA	56%	51%	35%	3%	15%	45%	40%	2%	0%
Oak-CA	53%	69%	40%	3%	11%	50%	38%	3%	0%
Willow-CA	54%	62%	34%	3%	9%	39%	52%	3%	0%
WS-CA	55%	57%	35%	3%	14%	46%	40%	2%	0%
Bag-DA	70%	57%	23%	2%	20%	45%	35%	2%	0%
BS-DA	45%	62%	35%	2%	45%	37%	18%	13%	1%
CS-DA	69%	44%	23%	1%	45%	35%	19%	3%	0%
Willow-DA	63%	51%	24%	1%	34%	35%	31%	3%	0%
WS-DA	61%	52%	27%	2%	30%	41%	29%	4%	0%
BS-MA	51%	60%	40%	0%	24%	63%	14%	7%	1%
CS-MA	54%	50%	37%	0%	25%	63%	12%	7%	0%
Oak-MA	52%	66%	43%	0%	15%	58%	27%	4%	0%
Willow-MA	55%	66%	39%	0%	23%	67%	10%	6%	0%
Bag-PAA	53%	63%	37%	1%	15%	71%	15%	6%	2%
BS-PAA	55%	70%	35%	3%	14%	65%	21%	5%	4%
CS-PAA	62%	60%	31%	1%	14%	64%	22%	3%	2%
Oak-PAA	54%	72%	41%	2%	8%	55%	37%	4%	0%
Willow-PAA	54%	59%	39%	1%	15%	61%	24%	4%	0%
WS-PAA	48%	62%	37%	1%	16%	61%	22%	6%	6%

Like in WS-CA and WS-DA hydrolysates (Figure 3), disaccharides were the most common form of residual oligosaccharides in all 21 hydrolysates (Table 4). This observation indicates that disaccharides are the bottleneck of biomass hydrolysis, caused mainly by incomplete hydrolysis, independent from biomass type and pretreatment method. However, the number and areas of the disaccharides in different hydrolysates varied, this is demonstrated with four hydrolysates, namely WS-DA, WS-CA, bagasse-dilute acid (Bag-DA), and bagasse-concentrated acid (Bag-CA) (Figure 4). It can be seen that the disaccharide pattern of WS-DA is similar to Bag-DA, while rather different from WS-CA. Also, the disaccharide pattern of WS-CA is more comparable to Bag-CA than to WS-DA. This is most probably due to WS-CA and Bag-CA were hydrolyzed with acid, while WS-DA and Bag-DA were hydrolyzed enzymatically.

Based on the observation that disaccharides were the most common form of residual oligosaccharides in all 21 hydrolysates, we used the following equation to indicate hydrolysis efficiency:

$$\frac{\text{DP2}}{\text{detected carbohydrates}} = \frac{\text{sum of DP2 peak area} \,/\, \text{DP2 response factor}}{\text{total peak area}}$$

A larger *DP2/detected carbohydrates* value corresponds to a lower hydrolysis efficiency. It should be mentioned that not all carbohydrates present in a hydrolysate sample can be detected with HPAEC-MS method, so the *detected carbohydrates* in the equation is not the same as the total sugar amount in the hydrolysate. Therefore, the hydrolysis efficiency assessed with this equation is only a relative value, which cannot be used to compare with the hydrolysis efficiency determined with other analytical methods.

The *DP2/detected carbohydrates* values of the 21 analyzed hydrolysates were calculated (Table 4), the used *DP2 response factor* value was 1.65, which is the average value of all the disaccharide standards tested (Table 3). As shown in table 4, the hydrolysates prepared with dilute acid (DA) method had the highest C5C5 percentage, while the mild alkaline (MA) and peracetic acid (PAA) prepared hydrolysates had relatively higher percentage of C5C6. Different from enzymatically hydrolyzed hydrolysates, the most abundant disaccharide form in concentrated acid (CA) hydrolysates was C6C6. This indicates that the residual disaccharides composition in a hydrolysate was mainly influenced by hydrolysate preparation method, while biomass type played only a minor role.

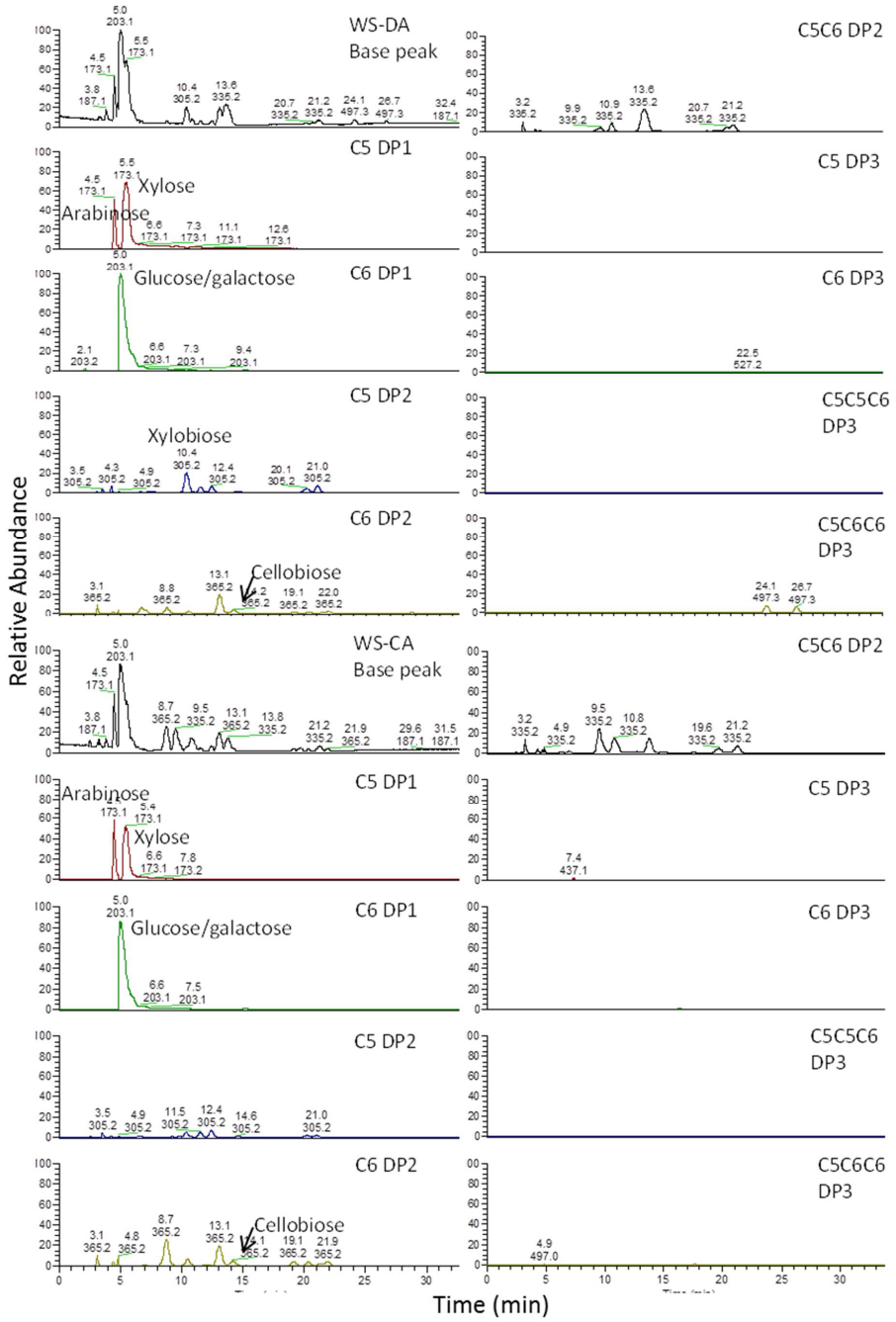


Figure 3 HPAEC–MS base peak and fixed scale extracted ion chromatograms of wheat straw–diluted acid (WS–DA) (top) and wheat straw–concentrated acid (WS–CA) (bottom) hydrolysates.

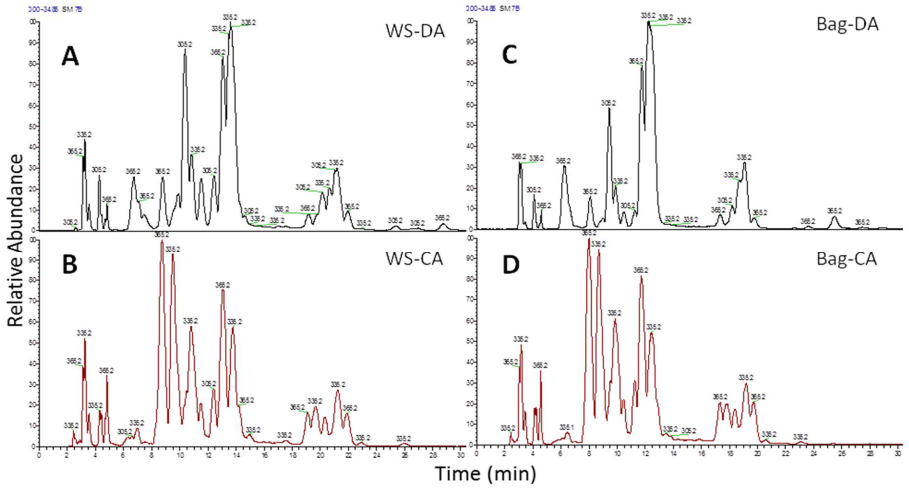


Figure 4 HPAEC–MS extracted ion disaccharide chromatograms of wheat straw (WS) and bagasse (Bag) hydrolysate prepared with dilute acid (DA) and concentrated acid (CA), corresponding to C6C6 (*m/z* 365), C5C5 (*m/z* 305) and C5C6 (*m/z* 335).

Furthermore, in most hydrolysates, disaccharides count for as high as 25-40% of the total carbohydrates detected. The low hydrolysis efficiency is possibility due to the high biomass dry weight percentage used in the hydrolysis mixture (~23%), which lead to high lignin content and high inhibitory compounds concentration. It was shown that the presence of lignin reduces the accessibility of cellulose enzyme, and soluble lignin can negatively influence the enzymatic hydrolysis efficiency [6]. Besides lignin, compounds that are formed or released during biomass pretreatment process also has inhibitory effect. These compounds were mainly phenols, furans, and small carboxylic acids, such as formic acid, furfural, vanillin and syringaldehyde [6,7].

To industrialize bioethanol production using lignocellulosic biomass as feedstock, high biomass dry weight percentage in hydrolysis mixture is essential, as it leads to high glucose concentration in hydrolysate and high ethanol yields in bioreactor. Therefore, the results suggest that one important aspect of improving enzyme cocktails is to enhance residual disaccharides hydrolysis efficiency at relatively high biomass dry weight percentage.

Bagasse hydrolysates fermentation samples

To study the dynamics of carbohydrates in biomass hydrolysates during a fermentation process, three ethanol production fermentations were carried out using bagasse-dilute acid (Bag-DA) and bagasse-concentrated acid (Bag-CA) hydrolysate. Bag-DA was fermented with

S.cerevisiae CEN.PK 113-7D, while Bag-CA was fermented with *S.cerevisiae* CEN.PK 113-7D and *P.anomala* TNO11-29. For each fermentation, three time samples were analyzed with HPAEC-MS method, which represented the beginning, the mid-point of growth phase and the stationary phase of the fermentation.

Among all the carbohydrates detected, 17 of them were chosen to analyze their trends during the fermentation process, by comparing the peak areas of each selected carbohydrate at the three fermentation time points. The 17 chosen carbohydrates are the largest peaks of DP1-3 and a sugar acid, glucuronic acid (GlcA). Table 5 shows the retention time, the putative identification, the degree of polymerization, and the peak areas after correcting with internal standards of these 17 peaks. As expected, glucose was consumed in all three fermentations, as it is the most preferred carbon source for the two yeast strains used [29,30]. Disaccharides and trisaccharides kept constant during all three fermentation processes, except maltose, cellobiose and gentiobiose. Maltose decreased in all three fermentations, while cellobiose and gentiobiose reduced only during the Bag-DA fermentation process. Maltose is thought to be consumed by both strains during the fermentation processes. It is known from literature that *S. cerevisiae* produces saccharolytic enzymes with limited activity, but due to lack of transporters, the only disaccharide the strain used as carbon source here is maltose [31,32]. The consumption of maltose was also reported in *P. anomala* strains [33]. Different from maltose, cellobiose and gentiobiose were suspected to be degraded by the residual enzymes in Bag-DA hydrolysate, since the reduction of these two disaccharides was not observed in Bag-CA hydrolysate, into which no enzymes was added.

The observation that cellobiose and gentiobiose were the only oligosaccharides degraded in the Bag-DA fermentation can be attributed to the property of the enzyme cocktails used. Accellerase 1500 mainly contains glucanase, hemi-cellulase and beta-glucosidase, suggesting that the main hydrolysis substrates are cellulose and hexose oligosaccharides. Enzymes that are responsible for hydrolyzing other oligosaccharides, such as isomaltose, mannobiose, and hybrid disaccharides, were hardly present in the enzyme cocktails used. As discussed in the previous session, hybrid disaccharides were the most common form of residual carbohydrates in hydrolysates, so it could be beneficial to include enzymes like α-D-xylosidase, β-1,4-D-galactosidase, α-L-arabinofuranosidase, and β-1,4-D-mannosidase in the enzyme cocktails [7,10,34]. These enzymes will help degrade the hybrid and α-linkage disaccharides during the hydrolysis process and/or in the fermentor later on.

It can also be seen from Table 5 that xylose was not consumed. It is reasonable since *S.cerevisiae* is not a pentose fermenting yeast [35] and the *P.anomala* strain used only

consumes xylose under aerobic conditions [36]. In this study, the peak areas of xylose even somewhat increased during all 3 fermentations, suggesting the release of xylose residues from more complex carbohydrates, apparently not detected in our HPAEC-MS analysis.

Through analyzing fermentation samples by using HPAEC-MS, the dynamics of major carbohydrates in biomass hydrolysates were monitored. This showed that HPAEC-MS is capable of providing information on the carbon sources that a specific fermenting microbe utilizes in a complex medium. A similar approach can also be applied to help indirectly analyze and identify the production of specific fungal or bacterial enzymes, with either newly isolated or genetically modified strains.

Table 5 Internal standard corrected peak areas of the selected 17 carbohydrates in fermentation samples (the decreased peak areas are marked in bold). Bag: bagasse, DA: dilute acid, CA: concentrated acid; S.c: *S. cerevisiae*, P.a: *P.anomala*; GlcA: glucuronic acid.

			Bag-DA S.c			**Bag-CA S.c**			**Bag-CA P.a**		
	RT	**putative identification**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
C5 DP1	5.2	xylose	493.2	547.7	618.4	386.6	403.2	486.4	287.1	314.8	400.0
C5 DP2 a	9.4	xylobiose	57.8	57.3	60.6	18.7	19.5	20.7	14.9	15.1	17.7
C5 DP2 b	10.4		6.2	6.7	6.1	20.2	21.3	21.8	15.6	15.7	18.8
C5 DP2 c	11.3		9.2	10.3	9.9	33.1	34.6	35.8	24.7	25.3	30.6
C5 DP3	17.1	xylotriose	1.5	1.8	2.2	---	---	---	---	---	---
C5 DP3	7.0		---	---	---	4.0	4.3	4.3	3.1	3.2	3.2
C6 DP1	4.8	glucose	**775.7**	**215.9**	**24.1**	**527.2**	**351.1**	**69.5**	**407.9**	**180.5**	**15.4**
C6 DP2 a	6.2	mannobiose	46.4	46.1	46.2	3.1	3.5	3.3	2.3	2.2	2.8
C6 DP2 b	8.0	isomaltose	14.8	14.1	10.0	117.5	125.4	126.4	95.1	96.0	120.7
C6 DP2 c	11.8	gentobiose	**79.5**	**33.8**	**0.0**	92.7	99.4	85.4	76.2	76.0	88.6
C6 DP2 d	12.9	cellobiose	**9.5**	**3.8**	**3.2**	22.1	26.0	23.3	19.6	17.0	21.4
C6 DP2 e	19.7	maltose	**5.0**	**1.0**	**0.0**	**31.8**	**17.3**	**2.1**	**25.3**	**16.3**	**0.0**
C6 DP3	20.1		5.6	5.8	5.7	---	---	---	---	---	---
C6 DP3	14.7		---	---	---	7.0	6.8	7.3	5.3	5.4	6.4
C5C6 a	12.3		211.6	222.7	218.5	80.6	83.0	82.3	60.5	63.3	81.3
C5C6 b	19.0		65.4	66.2	58.7	46.6	40.8	35.4	36.7	40.5	30.7
C6 acid	41.0	GlcA	10.1	9.7	10.1	9.3	9.2	8.6	6.0	6.2	7.7

Conclusion

To better understand complex carbohydrate samples, like lignocellulosic biomass hydrolysates, HPAEC coupled with MS was used. The HPAEC-MS method successfully separated many oligosaccharides in biomass hydrolysates and classified them based on their DP's in one experimental run. The method requires little sample work-up and provides chemical information, such as degree of polymerization and building blocks, of the detected carbohydrates. The application of HPAEC-MS was demonstrated by analyzing 21 different biomass hydrolysates. Results show that disaccharides, including C6C6, C5C5, C5C6, are the main remaining soluble sugar forms in these hydrolysates, which compositions are mainly influenced by the hydrolysate pretreatment method.

Acknowledgement

This project was (co)financed by the Netherlands Metabolomics Centre (NMC) which is part of the Netherlands Genomics Initiative / Netherlands Organization for Scientific Research.

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