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Simultaneous analysis of C1 and C4 oxidized oligosaccharides, the products of lytic polysaccharide monooxygenases acting on cellulose

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ABSTRACT

Lytic polysaccharide monooxygenases play a pivotal role in enzymatic deconstruction of plant cell wall material due to their ability to catalyze oxidative cleavage of glycosidic bonds. LPMOs may release different products, often in small amounts, with various oxidation patterns (C1 or C4) and with varying stabilities, making accurate analysis of product profiles a major challenge. So far, HPAEC has been the method of choice but it has limitations with respect to analysis of C4-oxidized products. Here, we compare various HPLC methods and present procedures that allow efficient separation of intact C1- and C4-oxidized products. We demonstrate that both PGC and HILIC (in WAX-mode) can separate C1- and C4-oxidized products and that PGC gives superior chromatographic performance. In contrast to HPAEC, these methods are directly compatible with mass spectroscopy and charged aerosol detection (CAD), which enables online peak validation and quantification (LOD in the low ng range that enables reliable quantitation at realistic assay conditions for LPMOs). While the novel methods show lower resolution than HPAEC, this is compensated by easy peak identification, allowing, for example, discrimination between chromatographically highly similar native and C4-oxidized cello-oligomers. HPAEC-MS studies revealed chemical oxidation of C4-geminal diol products, which implies that peaks commonly believed to be C4-oxidized cello-oligomers, in fact are on-column generated derivatives. Non-destructive separation of C4-oxidized cello-oligosaccharides on the PGC column allowed us, for the first time, to isolate C4-oxidized standards. HPAEC fractionation of a purified C4-oxidized tetramer revealed that on-column decomposition leads to formation of the native trimer, which may explain why product mixtures generated by C4-oxidizing LPMOs seem to be rich in native oligosaccharides when analyzed by HPAEC. The findings and methods described here will aid in future studies in the emerging LPMO field.

1. Introduction

Although oxidation was suggested as an important enzyme reaction in fungal degradation of cellulose already in 1974 [1], it has only recently been shown that enzymes known today as lytic polysaccharide monooxygenases (LPMOs) are key enzymes involved in degradation of recalcitrant polysaccharides such as cellulose and chitin [2,3]. LPMOs are abundant in Nature and are currently classified as auxiliary activity families AA9, AA10, AA11 and AA13 in the CAZy database [4–6]. Some LPMOs generate products oxidized in C1, meaning that their products are lactones that spontaneously convert to aldonic acids [7–11]). Other LPMOs are strict C4 oxidizers, meaning that their products are 4-keto-sugars, which will spontaneously convert to their corresponding hydrates/gemdiols [7,12] in aqueous conditions. Finally, other LPMOs show mixed activities, producing both C1-oxidized and C4-oxidized sugars [13–15]. These varying specificities have been mapped on the LPMO phylogentic tree [15,16] and seem to be correlated with conspicuous structural features of the catalytic copper site [14,17]. The abundance of LPMO-encoding genes indicates that more substrate specificities exist [3] and, indeed, recently, LPMO activity on hemicelluloses and starch has been demonstrated [6,13,18,19]. These developments will continue to expand the need for analytics within the field of oxidized complex plant saccharides.

LPMO activity leads to complex product mixtures. The complexity of products is related to variation in the degree of polymerization (DP) and to the occurrence of both native and oxidized products, of which the latter can occur in several forms. As an example, Fig. 1 shows variants of cellobiose that could emerge upon exposing cellulose to cellulases and one or more LPMOs. Chromatographic analysis of these products is challenging for example due to the minor structural difference between native and C4-oxidized oligosaccharides (Fig. 1, structure 3 and 1, respectively). Importantly, in particular for LPMOs with dual oxidative regioselectivity, chromatographic separation and proper detection of C1- and C4-oxidized products is highly desirable, since the two product types have identical masses, complicating their identification by commonly used MALDI-ToF mass spectrometry. Because of the pH dependency of the lactone-aldonic acid equilibrium, the formation of double adducts by aldonic acids and the predominance of the keto-form of C4-oxidized products, it is possible to detect mixed C1/C4 LPMO activity by MALDI-ToF MS (see Fig. S1 in Forsberg et al., [14] for an ex-

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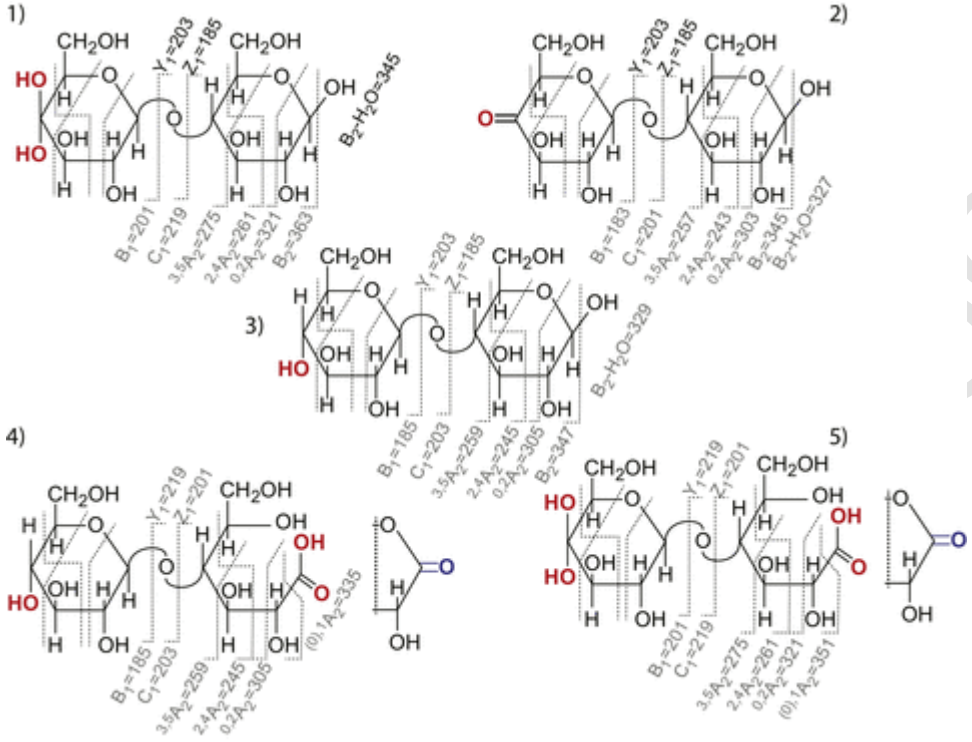

Fig. 1. Variants of cellobiose emerging during degradation of cellulose with an LPMO-containing enzyme cocktail. Variable functional groups are coloured red and major fragmentation ions (*m*/*z*, sodium adducts) are indicated. The figure shows C4-gemdiol (1), C4-Keto (2), native (3), aldonic acid (4) and double oxidized (5). The β-1,5-lactone which exists in low amounts in equilibrium with aldonic acid is depicted in blue as a truncated sugar rings (4 and 5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ample), but this is far from straightforward and product quantification is not an option.

The chromatographic analysis of aldonic acid products is well established [20], and especially High- performance anion-exchange chromatography (HPAEC) operated at high pH has proven an excellent method for the separation and quantification of native and C1-oxidized cello-oligosaccharides. HPAEC has also been used diagnostically for detection of C4-oxidized products [7,12].

Keto-saccharides are prone to tautomerization and chemical modification at alkaline conditions [12], which potentially limits the use of HPAEC for their analysis. A major disadvantage of the HPAEC approach is its lack of compatibility with MS-based detection. Due to the presence of high salt concentrations and high pH, MS-analysis of HPAEC samples requires extensive ion removal and neutralization before they can enter the MS. Online anion suppression can be done [21,22], but requires complex experimental set-ups that may still not decrease the ionic strength sufficiently, leading to a loss of sensitivity and other complications. Generally, operating an MS instrument with high concentrations of non-volatile salts requires outstanding robustness. To verify the potential modification of C4-oxidized sugars during HPAEC we carried out HPAEC-MS experiments in the present study, which confirmed that on-column decomposition occurs, showing that the HPAEC approach has limitations when it comes to analyzing such oxidized products.

We have previously shown that hydrophilic interaction liquid chromatography (HILIC) can be used for analysis of aldonic acids, albeit with limited MS-compatibility due to high ionic strengths of the required eluent. However, some of the recently developed HILIC columns also include a weak anion exchange (WAX) feature and this could potentially improve separation [23] even with MS-compatible eluents. In addition to HILIC, we have also demonstrated good separation of aldonic acids with porous graphitized carbon (PGC) chromatography [20] and selective separation behavior for carbohydrates with different spatial hydroxyl-group organizations has also been demonstrated [24]. Notably, the PGC approach comes with excellent MS-compatibility. In this study, we have explored if HILIC-WAX and PGC based methods can be developed further to also allow identification of C4-oxidized LPMO products.

Since LPMOs generate small amounts of oxidized products, sensitive analytical methods simultaneously detecting both types of oxidation products are required. We present and explore the potentials of two novel, sensitive and selective chromatographic methods (HILIC-WAX and PGC) for the simultaneous analysis of C1- and C4-oxidized cello-oligosaccharides, under conditions that promote stability of the C4-oxidized products. We used the supreme features of LC–MS to accomplish the challenging discrimination between native and C4-oxidized products. The two methods presented also enabled identification of double oxidized products that occur in some product mixtures. Finally, the PGC method allowed purification of C4-oxidized cello-oligosaccharide, which allowed for detailed studies of the chromatographic behavior of these compounds and yielded useful standards.

2. Materials and methods

2.1. HPAEC-PAD

The conditions for HPAEC-PAD were identical to those described in Westereng et al., 2013 [20]. The analysis was conducted using an ICS3000 system from Dionex (Sunnyvale, California U.S) set up with PAD with a disposable electrochemical gold electrode. Two μL samples were injected on a CarboPac PA1 2 × 250 mm analytical

column with a CarboPac PA1 2 × 50 mm guard column kept at 30 °C. The solutes were eluted at 0.25 mL/min with initial conditions set to 0.1 M NaOH (100% eluent A), followed by applying a linear gradient towards an increasing proportion of eluent B (1 M NaOAc in 0.1 M NaOH). The gradient reached 10% B 10 min after injection, and 30% B after 25 min; this was followed by a 5 min exponential gradient (Dionex curve 6) to 100% B. Column reconditioning was achieved by running initial conditions for 9 min.

2.2. HPAEC-MS

The HPAEC-PAD setup described above was also coupled to an ion trap mass spectrometer, essentially in the same way as described by Coulier and coworkers [22]. Immediately after the column, the flow was split 1:1, directing one part to the PAD and the other part to an anion suppressor (Dionex ASRS 300, 2 mm). The suppressor was continuously regenerated with water and the ion suppressor current was set sufficiently high to neutralize the eluent (adjusted at every start up before connection to the MS). After the suppressor, the flow was sent to the ESI-interface of a Velos Pro LTQ linear iontrap (Thermo Scientific, San Jose, CA USA) operated in positive mode with an ionization voltage of 3.5 kV, auxiliary and sheath gas settings of 30 and 5 respectively (arbitrary units) and with capillary and source temperatures of 275 °C and 250 °C, respectively. The scan range was set to *m*/*z* 150–2000 and MS2 was performed with CID or HCD fragmentation with helium as the collision gas. Data handling was done with Xcalibur 2.2 SP1.48 (ThermoScientific). Due to ion suppression effects at high sodium acetate concentrations, the MS-acquisition was stopped and the flow redirected to waste when eluent B reached 30% (300 mM NaOAc). The two detectors, PAD and MS were operated in parallel rather than in series to avoid MS-artefacts that could be caused by (unintended) product oxidation occurring on the PAD.

2.3. HPLC–MS

Liquid chromatography (HILIC or PGC; see below) was performed on a UHPLC (Ultimate 3000RS, Dionex, Sunnyvale, CA, USA) coupled to a linear iontrap LTQ Velos Pro with ESI-interface (Thermo Scientific, San Jose, CA USA). The eluent was splitted 1:10 and the resulting flow from the LC to the MS was in all cases 0.04 mL/min. The ESI was operated in positive mode at 4 kV spray current, with a sheath gas flow of 30 and an auxiliary gas flow of 5 (arbitrary units). The capillary temperature was 250 °C. Lithium doping was performed post column with a secondary pump delivering a flow of 0.01 mL/min 0.5 mM lithium acetate in 50% acetonitrile via a t-split immediately before the ESI-source. For lithium doping, a flow rate equal to that coming from the LC (0.04 mL/min) was used. Chromatographic conditions for each type of column are described below.

2.4. HILIC

A weak anion exchange HILIC column (GlycanPac AXH-1, 2.1 × 250 mm; 1.9 μm Thermo Electron Corporation, San José, USA) was operated at 0.4 mL/min and ambient temperature with eluents A (H_2O), B (acetonitrile) and C (100 mM ammonium formate, pH 4). The samples were mixed with acetonitrile to a final concentration of 70% (v/v) prior to injection. 2 μL sample was injected and the analytes eluted by the following method: Isocratic 0–7 min with 19% A, 80% B and 1% C. From 7–13 min linear gradient to 49% A, 50% B and 1% C which was kept isocratic from 13 to 19 min. After 19 min the column was reconditioned with starting conditions for 10 min. The native and C4-oxidized products elute during the initial isocratic part of the run, and hence a short method could be developed for those species in particular. The C1-oxidized products require gradient conditions to elute and in order to improve the peak shape of these, a pH gradient was introduced using channel D (10 mM formic acid, pH 3) during the gradient part of the run. Due to column stability issues at lower pH, pH 3 was the lowest possible. In total the pH gradient run conditions were 0–5 min isocratic 19%A, 80%B and 1%C. Linear gradient from 5 to 7 min to 80%B, 1%C and 19%D. Further linear gradient from 7 to 13 min to 50%B, 1%C and 49%D. These conditions were kept isocratically until 19 min and hereafter immediately returned to starting conditions followed by 10 min equilibration.

2.5. PGC

For PGC chromatography, a PGC guard column (Hypercarb; 2.1 × 10 mm; 3 μm) was followed by an analytical PGC column (Hypercarb; 2.1 × 150 mm; 3 μm). Both were from Thermo Electron Corporation, San José, USA. The column was operated at 0.4 mL/min and 70 °C using the gradient described in Westereng et al., [20]. In short: 0–1 min, 100% eluent A (10 mM NH_4-acetate, pH 8.0); 1–15 min, linear gradient to 27.5% eluent B (acetonitrile); 15–28 min, linear gradient to 60% B; 28–35 min, isocratic at 60% B. The column was reconditioned by applying 100% eluent A for 9 min. Preparative chromatography (Hypercarb; 10 × 150 mm; 5 μm) of C4-oxidized standards was done using the conditions described previously for separation of celloaldonic acids [20].

2.6. PGC quantification by charged aerosol detection (CAD)

PGC was used in combination with CAD for quantitative purposes. The CAD detector was an ESA Corona Ultra from ESA Inc., Dionex, Sunnyvale, USA. The CAD detector is a universal detector, where the detector response is independent of analyte and may be calibrated with external standards. Due to incompatibility between alkaline mobile phases and the principle in CAD, pH was lowered to 6.5 in eluent A in cases where CAD was applied. In addition, the effect of varying the ionic strength for CAD detection was also tested between 5 and 20 mM NH_4-acetate.

2.7. NMR spectroscopy

NMR spectra were recorded at 298 K (24.85 °C) on a BRUKER Avance 600 spectrometer equipped with a 5 mm cryogenic CP-TCI z-gradient probe. The NMR data were processed and analyzed with Bruker TopSpin 2.1 and TopSpin 3.0 software. pH titration of cellobionic acid (GlcGlc1A) was performed on two samples containing ∼1.6 mg cellobionic acid in 600 μL 10%/90% D_2O/H_2O and 3 μL 5% (w/v) 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (Aldrich, Milwaukee, WI, USA), where the latter was used as internal standard for the chemical shift. The first sample was titrated from pH 1.28 by adding sub-μL amounts of 1 M NaOH while the second sample was titrated from pH 8.45 and down in a similar manner using 1 M HCl. This approach ensures minimal changes in dilution and ionic strength of the samples. The pH of the samples was measured with a mini pH electrode. For each titration step a 1D proton and a ^{13}C heteronuclear single-quantum coherence (HSQC) spectrum were recorded.

2.8. Samples

Samples containing oxidized cello-oligosaccharides were obtained as indicated below. After the enzyme reactions the samples were centrifuged to remove possible insoluble materials before analysis.

1) *Sc*LPMO10C (1 μM), ascorbic acid (1 mM), phosphoric acid swollen cellulose (PASC, 1% w/v), sodium acetate (10 mM, pH 6.0), 50 °C, 1000 rpm, 12 h. *Sc*LPMO10C, also known as CelS2, is a strictly C1 oxidizing AA10-type LPMO [14].
2) *Nc*LPMO9C (1 μM), ascorbic acid (1 mM), phosphoric acid swollen cellulose (PASC, 1% w/v), sodium acetate (10 mM, pH 6.0), 50°C, 1000 rpm, 12 h. *Nc*LPMO9C is a strictly C4 oxidizing AA9-type LPMO [12,25].
3) *Nc*LPMO9C (1 μM), ascorbic acid (1 mM), Glc5, sodium acetate (10 mM, pH 6.0), 50 °C, 1000 rpm, 12 h
4) One part of 3) was subsequently treated by adding 1 μM CDH (*Myrococcum thermophilum* produced according to [26]) to form double oxidized products.
5) Aliqouts from 1, 2 and 4 were combined in even amounts by volume to obtain a mixture of all possible oxidized products.
6) GlcGlc1A used for pH titration was obtained as described in [20].
7) C4-oxidized oligosaccharides for purification were generated by a C4-specific LPMO (NCU02240 from *N. crassa*, cloned as described in Borisova et al., [17] and purified using a purification scheme developed by Kittl et al., [25], with slight modifications, as described in the Supplementary information) in a reaction consisting of 40 μg enzyme, 5 mL 1% w/v PASC, 3.6 mM ascorbic acid in 10 mM Tris/HCl pH 8.0, which was incubated at 50 °C, 1000 rpm, for 12 h. Native oligosaccharides in the product mixture were removed by treatment with a β-glucosidase (105 min at 50 °C, using *An*Cel3A from *A. niger* purified from Novozym 188, produced by Novozymes Denmark, based on the method described by Sipos et al., [27]). The products of this procedure were assessed by MALDI-ToF.

3. Results and discussion

3.1. HPAEC

HPAEC is a common method of choice when it comes to analyzing carbohydrates and it is well suited for analysis of aldonic acids [20]. To further analyze the performance of this method, the HPAEC, with ion suppression set-up, was coupled to a pulsed amperometric detector (PAD) and ESI–MS in a setup similar to [22]. Fig. 2A shows that this set-up enabled direct annotation of aldonic acids up to DP7 generated by a C1-oxidzing LPMO. On the other hand, for C4-oxidized products the corresponding masses could not be detected with MS (Fig. 2B). Peaks eluting in the mid-section of the chromatogram (20–30 min), which are known to be correlated with C4-oxidation [7,12,15,28], displayed a loss of *m*/*z* 2 compared to the theoretical mass of the gemdiols, as exemplified by the clear *m*/*z* 379 and *m*/*z* 541 signals for the C4-oxidized dimer and trimer, respectively. This mass loss may occur due to additional chemical oxidation that is promoted by the alkaline conditions on the column (insert in Fig. 2D). Comparison of the MS2 fragmentation of a control compound from sample 3 (Glc4gemGlc, Fig. 2C) and its chemically oxidized analogue (Fig. 2D) showed that the oxidation occurred at the non-reducing (i.e. the LPMO-oxidized) sugar, since the mass loss of *m*/*z* 2 was seen for B and C-ions only, while Y and Z-ions were the same for both compounds. These observations imply that although HPAEC can be used as a diagnostic tool for detecting C4-oxidation, the diagnostic peaks correspond to derivatives and not to the original C4-oxidized compounds.

Notably, an HPAEC chromatogram of a pure C4-oxidized tetramer (obtained as described below) showed a peak corresponding to the native trimer in addition to the triplet peaks (at 25–30 min retention time) that are diagnostic for the decomposed C4-oxidized product (Fig. S1). The fraction containing this putative native trimer was desalted and subjected to PGC, which confirmed that the C4-oxidized tetramer indeed had been partially decomposed to a native trimer (Fig. S1). This remarkable observation provides an explanation for the apparent generation of native cello-oligomers by C4-oxidizing LPMOs, which is observed when analyzing samples by HPAEC.

In addition to C1- and C4-oxidized cello-oligosaccharides, LPMOs with dual oxidative regioselectivity produce C1/C4 double-oxidized species that elute late in the HPAEC chromatograms. These compounds elute at an ionic strength that is too high to allow sufficient ion removal by the ion suppressor and can therefore not be analyzed by HPAEC-MS.

3.2. Determination of pK_a for cellobionic acid

Due to the limitations of the HPAEC method, there is a need for complementary methods for simultaneous analysis of all possible products. To facilitate the identification of suitable conditions for efficient separation of C1-oxidized oligosaccharides, we determined the pK_a of cellobionic acid (GlcGlc1A, sample 6 in Materials and methods). The pH titration of GlcGlc1A was obtained by recording ^{13}C HSQC spectra at different pH values ranging from 1.28- to 8.45. The data was analyzed by plotting the chemical shifts of the proton and the carbon adjacent to the carboxyl group as a function of pH (Fig. 3). The resulting titration curves for the Glc1A unit showed sigmoidal transitions. By fitting the experimental data to the Henderson-Hasselbach equation, the pK_a of the carboxyl group was determined to be 3.51 ± 0.06. This value corresponds well with the pK_a value of D-gluconic acid (pK_a = 3.7 [29]), suggesting that the DP of the aldonic acid has little effect on the pK_a value. Hence the pK_a value of longer C1-oxidized oligosaccharides is likely to be similar to that of cellobionic acid. The determination of the p*K*a value is important because it guides the choice of pH in the mobile phase for optimizing elution of the aldonic acids, and is particularly important for methods not based on anion exchange.

3.3. PGC

PGC has proven as a good chromatographical approach for aldonic acids and native cello-oligosaccharides at slightly alkaline conditions (NH_4OAc, pH 8.0) [20]. Under these conditions the aldonic acids are charged (pK_a = 3.5; see above) and thus behave quite differently compared to native oligosaccharides. Here we used the previously established chromatographic protocol [20] to analyze a sample containing a mixture of native cello-oligomers, aldonic acids, C4-gemdiols and double oxidized species (sample 5; Materials and methods). The PGC stationary phase is known for its strong affinity for longer oligosaccharides and it has been shown that neutral cello-oligosaccharides with DP > 5 do not elute from the column, even at high elution strength [20]. It was thus not unexpected to only see products with DP 2–4 in the PGC chromatogram (Fig. 4). MS detection showed that the analytes elute in clusters based on DP, with acidic products eluting first and the neutral variants eluting later in the same cluster. This elution pattern is in agreement with the properties of the PGC column, which separates analytes based on their po-

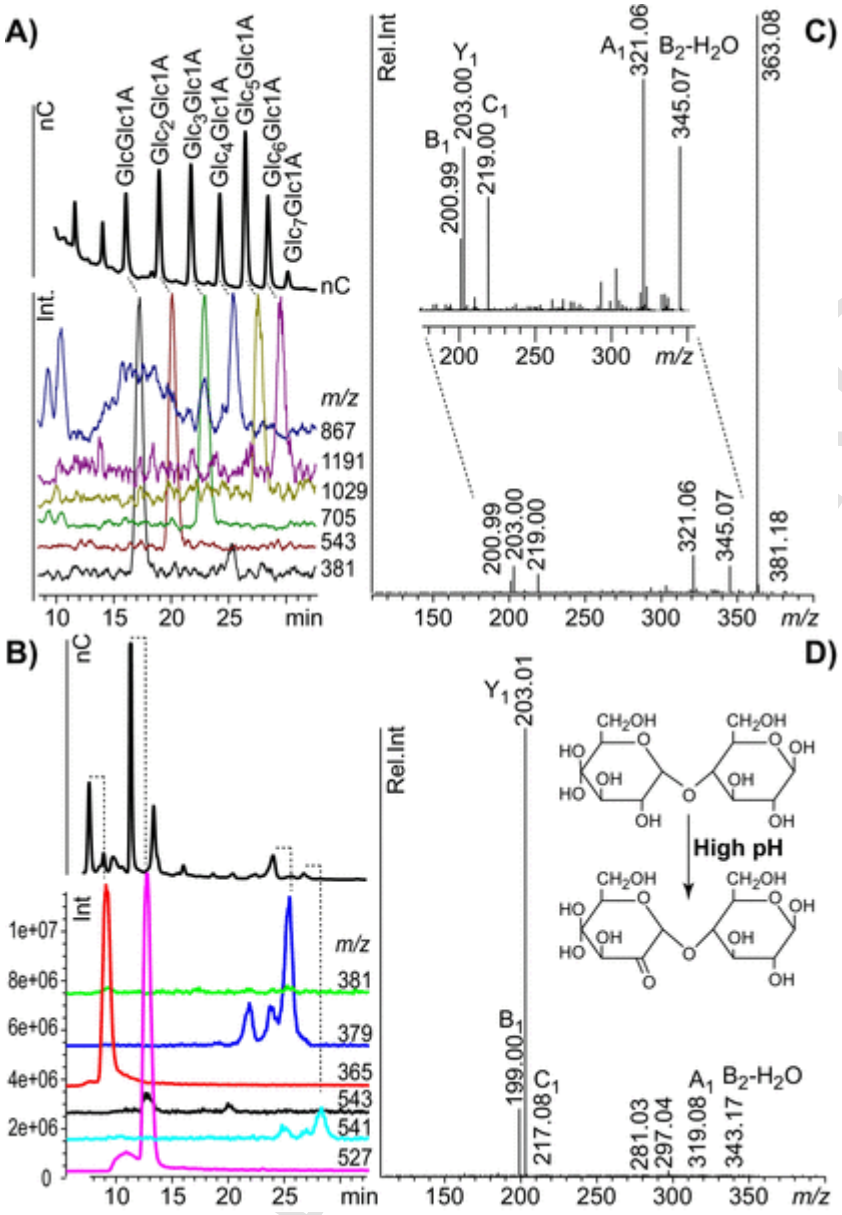

Fig. 2. HPAEC-MS analysis of LPMO products. (A) HPAEC chromatograms of C1-oxidized cellooligosaccharides (sample 1; Materials and Methods) released from PASC upon treatment with *Sc*LPMO10C (also known as CelS2, [8]); The figure shows the PAD trace (upper black chromatogram) and HPAEC-MS Extracted Ion Chromatogram (EIC) traces for sodium adducts (note that there is a 1.3 min delay relative to the PAD trace, which is due to the dead volume of the suppressor unit and connecting tubing). The EIC signals were scaled for optimal viewing. (B) HPAEC chromatogram of C4-oxidized cellooligosaccharides (sample 3; Materials and Methods) released from Glc_5 upon treatment with strictly C4-oxidizing *Nc*LPMO9C [12]. The figure shows the PAD trace (upper chromatogram, black) and HPAEC-MS Extracted Ion Chromatogram (EIC) traces for sodium adducts (same signal delay as in A). All EIC traces except 365 and 527 are magnified 10×. The early major peaks are the sodium adducts of the native dimer and trimer, at 365 and 527, respectively. The later peaks, generally annotated as C4-oxidized sugars in the literature, are in fact decomposition products of the C4-oxidized dimer (*m*/*z* 379, expected 381) and the C4-oxidized trimer (*m*/*z* 541, expected 543) with an additional oxidation leading to a mass loss of 2 Da. MS traces *m*/*z* 543 and *m*/*z* 381 represent K-adducts of the native trimer and dimer, respectively. (C) Direct infusion MS^2 of Glc4gemGlc obtained in the reaction of treating Glc_5 with *Nc*LPMO9C (sample 3; Materials and Methods). (D) MS^2 of the *m*/*z* 379 peak in (B). Expected *m*/*z* values for fragmentation ions are shown in Fig. 1. Comparison of panels C and D shows that non-reducing end fragments in D show a mass loss of 2 Da compared to C. The insert in panel D shows a possible structure of a chemically oxidized tautomer of C4-oxidized dimer; it should be noted that the second oxidation may occur at any of the carbons containing a hydroxyl.

larity and molecular planarity. The structural differences between native oligomers and corresponding C4-gemdiols as well as between aldonic acids and corresponding double oxidized species are miniscule. For both pairs, the only difference is the presence of a single hydroxyl group at an sp^3 hybridized carbon at the non-reducing end. Interestingly, the resolution increases with increasing DP (Fig. 4), leading to baseline separation of the native and the C4-oxidized tetramer. While the PGC approach gives excellent separation of aldonic acids and gemdiols of the same DP, the gemdiols are hardly separated from native oligomers, meaning that MS detection is essential (note that an alternative approach would be to remove the native oligomers by β-glucosidase treatment prior to chromatography; see below).

Since the mobile phase contains ammonium, the expected adducts would be ammonium next to commonly observed sodium and potassium. Sodium and potassium adducts lead to overlapping *m*/*z* values

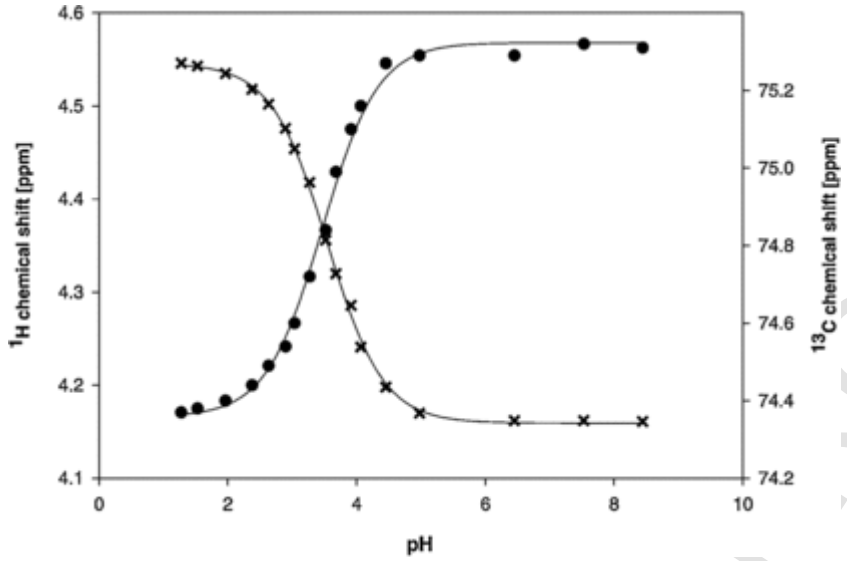

Fig. 3. Determination of the pK_a of cellobionic acid by NMR. The plot shows the chemical shifts for the proton (dots) and the carbon (crosses) adjacent to the carboxyl group as a function of pH.

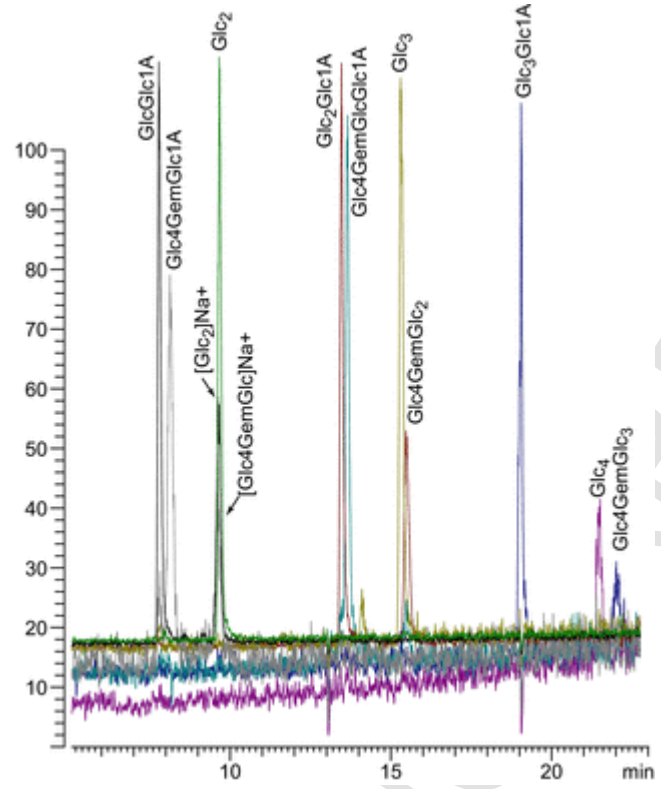

Fig. 4. Overlayed Extracted Ion Chromatogram (EIC) traces (12 individual traces, lithium adducts if not otherwise indicated) for PGC separation of sample 5 (see Section 2), containing various possible native and oxidized cello-oligomer species. The sample did not contain double oxidized tetramer and because oligosaccharides longer than DP4 does not elute, the visualisation is cut after the last eluting tetramer. Note that the native and gemdiol dimer species are partially observed as their sodium adducts due to the dimers strong preference for sodium [30].

for native and oxidized species due to the mass difference between the two metals being equal to the mass of an oxygen atom. This limitation was eliminated by post-column addition of Li-acetate, which promoted single adduct formation (Fig. 4). However, when using moderate lithium doping as in Fig. 4, considerable amounts of sodium adducts were still observed. More intense lithium doping, by increasing the flow rate of the post column addition of 0.5 mM Li-acetate from 25% to 100% of the flow rate coming from the LC, leads to further suppression of sodium adducts (Fig. S2). However, this latter approach leads to increased ion suppression and thereby reduces sensitivity. Lithium was selected for metal-doping due to its superior properties in MSn fragmentations [30].

3.4. Preparation of C4-oxidized cello-oligosaccharide standards

Since the gemdiols were stable under the conditions used for PGC, the PGC method was used for semi-preparative purification of C4-oxidized oligosaccharides (in milligram scale, Fig. S3 + Fig. S4). To do so, product mixtures of sample 7 (see Section 2) were treated with β-glucosidase, which hydrolyze native and C1-oxidized cello-oligosaccharides, while leaving the C4-oxidized compounds intact. This selectivity is due to the fact that the β-glucosidase acts on the non-reducing end of the cello-oligosaccharides and is apparently inhibited by a gemdiol group at the C4-position. After the selective removal of the native cello-oligosaccharides, we were able to isolate, for the first time, pure C4-oxidized cello-oligosaccharides using a semi-preparative PGC column (Hypercarb, 10 × 150 mm, 5 μm) (Fig. S4). These oligosaccharides were used for validation purposes during the method development, as described above. Furthermore, the purified material was quantified by CAD after generating standard curves from known concentrations of the corresponding native oligosaccharides and subsequently used to estimate the sensitivity of the PGC method.

CAD detection is a universal detection principle and can be used for product quantification but has poor sensitivity at elevated pH. Therefore, the effects of reducing the eluent pH from 8 to 6.5 were studied. In accordance with the notion that also at pH 6.5, the pK_a of the aldonic acids is much lower than the eluent pH, the chromatograms obtained at this lower pH showed separation comparable to that seen in Fig. 4 (see Fig. S5). For pH values higher than the pK_a, the separating parameter is governed by ion-pairing effects of the ammonium acetate. Increasing ionic strength causes increased retention of the C1-oxidized products and, thus, better separation from the na-

tive and C4-oxidized products (Fig. S5). The experiments showed that ammonium acetate concentrations ≥ 10 mM are desirable (Fig. S5).

Since retention of the C4-oxidized products is not affected by pH or ion pairing (Fig. S5), quantification of these species in particular could be improved by lowering the ionic strength as this reduces background noise. Hence, by lowering the ionic strength down to only 1 μM NaCl, low ng amounts of C4-oxidized products could be detected, i.e. amounts that are typically released during LPMO reaction in a research laboratory.

In conclusion, the PGC methodology affords reliable detection, validation and quantification of all oligomer species that may emerge from treating cellulose with LPMOs. In cases where ultrahigh sensitivity is required, internal standards either as heavy isotope labeled oligosaccharides or by standard addition [31] could be used for quantification by MS.

3.5. HILIC

In an alternative approach, we tested the combined features of HILIC and weak anion exchange, as available in the GlycanPac AXH-1 column, to separate the analytes in question. Both native and C4-oxidized oligosaccharides eluted at the initial isocratic conditions with 80% acetonitrile (from 0 to 7 min, Fig. 5), whereas the more strongly retained aldonic acids eluted during and after the application of a 6 min gradient from 80%-50% acetonitrile followed by isocratic conditions at 50% acetonitrile (all eluents contained ammonium formate, pH 4). Under these conditions, the aldonic acids eluted in broad peaks with suboptimal resolution. A longer, 12 min gradient provided better peak separation, but at the same time increased peak broadening (data not shown). Peak broadening could be due to the equilibrium between the aldonic acid and its corresponding β-1,5-lactone, since the pH of the mobile phase (pH 4.0) was close to the pK_a (3.5) of the aldonic acids (see above). Introducing formic acid (pH 3) in the first half of the gradient (after 5 min isocratic run) improved peak shapes significantly (Fig. 5 left panel insert). Due to column stability issues, it was not possible to use even lower pH values.

No separation was observed between C4-oxidized oligosaccharides and their native counterparts (Fig. 5 right panel). The C4-oxidized products eluted in broad peaks, possibly due to the equilibrium between the keto and the gemdiol form, which may have been affected by the high concentration of organic solvent (acetonitrile). Because HILIC is always operated at high organic solvent concentrations it may hence not be a suitable principle for analysis of C4-oxidized products.

4. Conclusions

The combination of PGC with MS-detection provided a valuable analytical strategy for simultaneous analysis of LPMO generated C1 and C4-oxidized products under non-destructive conditions and even partially resolve C4-oxidized products and native oligomers. The simultaneous analysis by PGC is mainly governed by the behavior of the aldonic acids and is determined by pH and ion pairing. CAD detection was also successfully implemented, and PGC-CAD provides a suitable dynamic range (LOD in the low ng range), enabling reliable quantitation at realistic assay conditions for LPMOs. An alternative to CAD would be MS based quantification with internal standards of the analytes using the standard addition method (where you at the same time have control of adduct formation). Notably, the purifi-

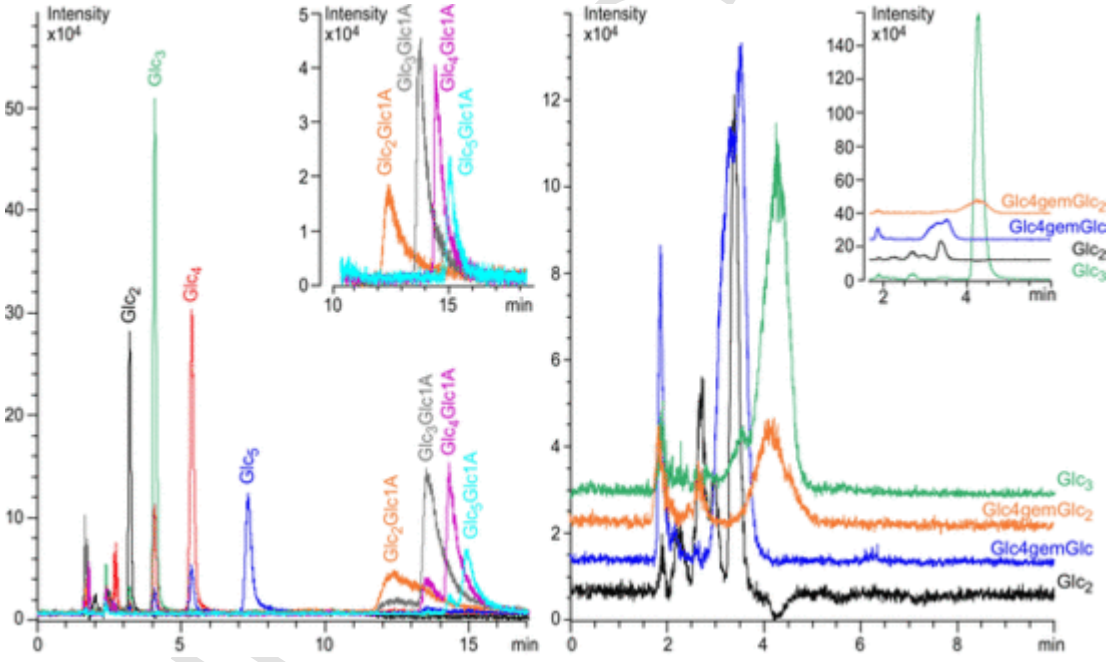

Fig. 5. Left panel: Overlayed EIC of a *Sc*LPMO10C sample (sample 1, see Materials and Methods) containing native cello- and aldonic acid oligosaccharides analyzed on HILIC-WAX (ammonium adducts except Glc_2 which is depicted as the sodium adduct due to its strong preference for sodium [30]). The aldonic acid peaks show peak broadening, with peak widths as follows: Glc_2Glc1A, 4.4 min; Glc_3Glc1A, 2.8 min; Glc_4Glc1A, 2.0 min; Glc_5Glc1A, 1.4 min. This situation can be improved by applying a pH gradient as described in Materials and methods (left panel insert), with peak widths being reduced to: Glc_2Glc1A, 2.5 min; Glc_3Glc1A, 1.5 min; Glc_4Glc1A, 1.4 min; Glc_5Glc1A, 1.1 min. Native oligos are not affected by pH. Right panel: Overlaid EIC for a sample containing native cellobiose and cellotriose and C4-oxidized dimer and trimer (sample 3, see Section 2). All traces reflect sodium adducts. The chromatograms show a lack of resolution between native oligomers and their C4-oxidized counterparts. The right panel insert shows traces for the ammonium adducts and that the signal for the ammonium adduct of Glc_3 is approximately 10-fold higher than the signal for the corresponding sodium adduct. This demonstrates the complexity resulting from multiple adduct formation and shows that quantitative interpretation of the EIC traces should be avoided.

cation of authenticated standards of C4-oxidized cello-oligosaccharides has not been reported before.

HPAEC is, and will remain, the method of choice in many applications, even in studies involving C4-oxidizing LPMOs. However, as shown here, when C4-oxidized sugars are present in the samples, chromatograms get undesirably complex due to the chemical instability of the C4-oxidized oligosaccharides at the alkaline conditions used. This instability results in on-column decomposition yielding native oligosaccharides being one DP shorter than the original C4-oxidized oligosaccharides, and also results in chemical oxidation of the gemdiol. Since HPAEC-PAD provides good resolution and very good sensitivity compared to detectors other than mass spectrometry, we would argue that, HPAEC is still a good screening method for LPMO catalyzed reactions, despite the instability of C4-oxidized products. In HPAEC-PAD, quantification of the chemically stable native and C1-oxidized products, for which authenticated standards exist, is straightforward but it is challenging for the C4-oxidized cello-oligosaccharides due to their on-column decomposition. Absolute quantification of C4-oxidized products thus requires the PGC-based method described here. We conclude that for analysis of LPMO product mixtures, PGC is more promising than HILIC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.03.064.

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