Electronic Supplementary Material (ESI) for Analytical Methods.


# Electronic Supplementary Information

## MALDI-MS Method Optimization

### (I) Cationization Agents

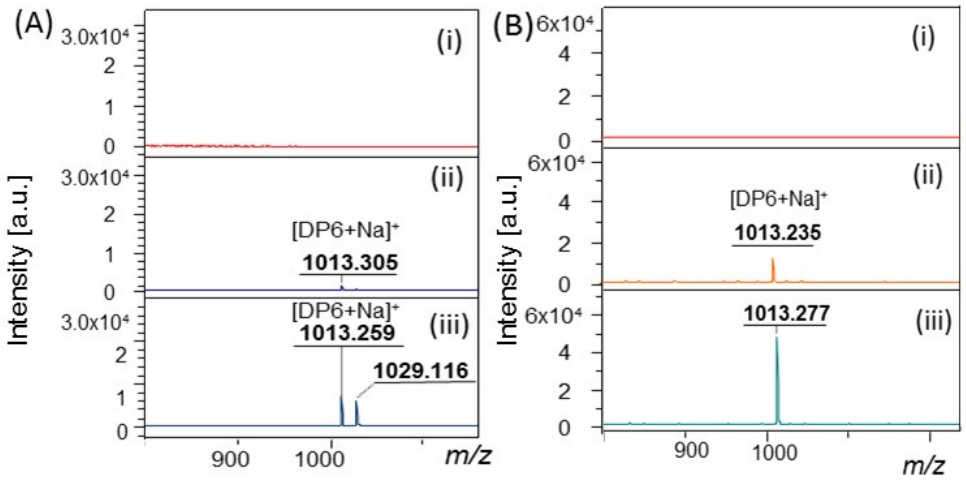


Figure S1. MALDI-TOF MS of cellohexaose (DP6) (ii) 0,25 mg/mL and (iii) 1 mg/mL in the absence (A) and presence (B) of sodium chloride as a cationization agent. The blank analysis (DHB matrix alone) is shown in (i). The *m/z* values correspond to native oligosaccharides in sodiated (1013 = $[DP6+Na]^+$) and potassiated (1029 = $[DP6+K]^+$) forms as shown in Table 1.

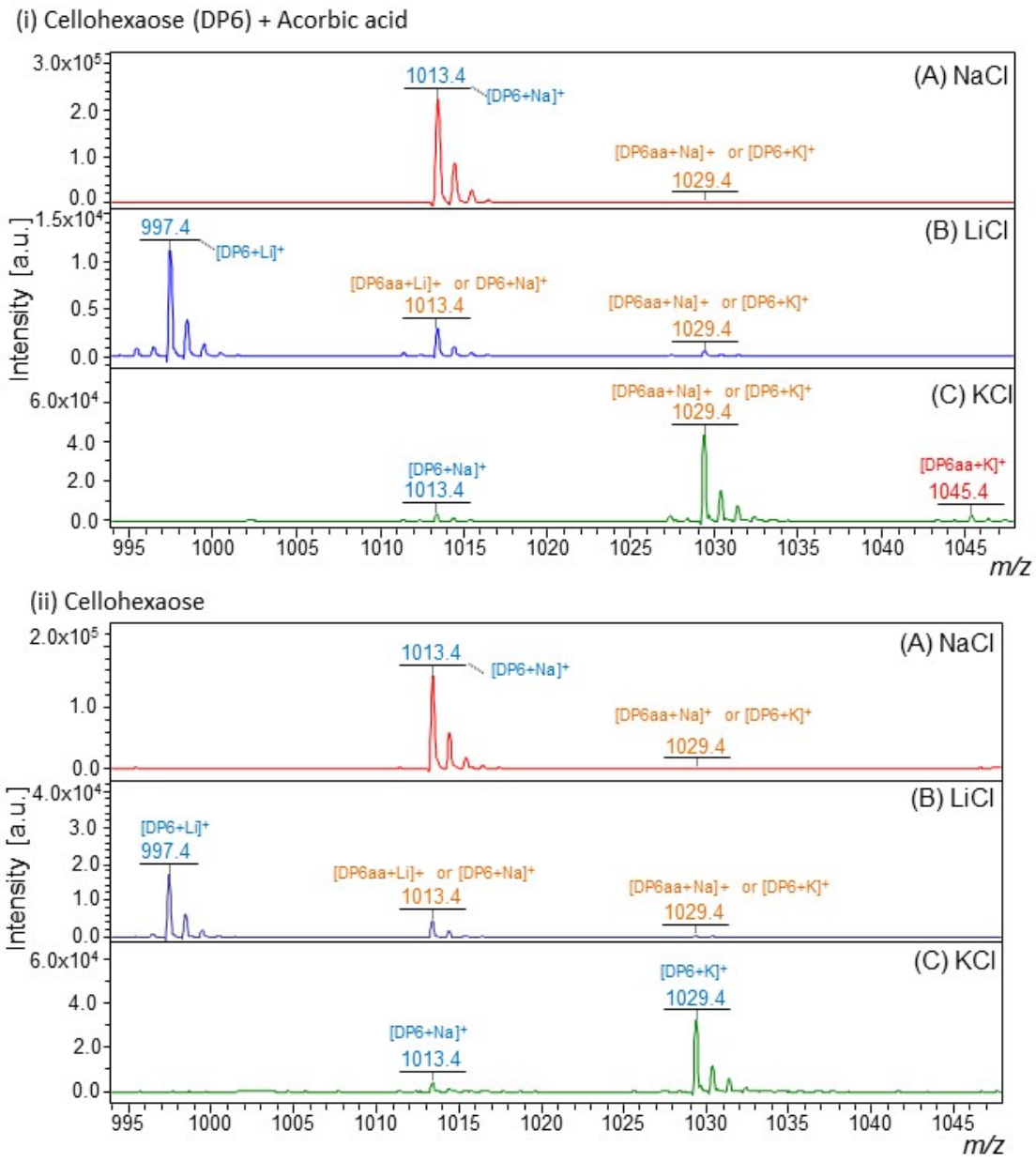


**Figure S2.** MALDI-TOF MS analysis of the cellohexaose analytical standard (DP6) in the presence (i) and in the absence (ii) of ascorbic acid (used as electron donor in the reaction with LPMO), having as cationizing agent sodium (**A**), lithium **(B**) and potassium chloride (**C**). Native oligosaccharides are labeled in blue letters, oxidized oligosaccharides are labeled in red letters and the "overlapping" *m/z* values (native or oxidized oligosaccharide) are labelled in orange letters. When doping the samples with sodium chloride (**A**) it is possible to detect ions related to sodium cellohexaose (*m/z* 1013; $[DP6+Na]^+$ in blue) and a "overlapping" *m/z* values (*m/z* 1029; $[DP6+K]^+$ or $[DP6_{AA}+Na]^+$, in orange). The doping with lithium chloride (**B**) does not reveal the identity of overlapping masses of common species, since the *m/z* 997 ion refers to cellohexaose with lithium adduct $[DP6+Li]^+$, while the ion *m/z* 1013 may refer either to a lithium adduct of oxidized oligosaccharide ($[DP6_{AA}+Li]^+$) or to a native oligosaccharide with sodium adduct ($[DP6+Na]^+$). The ion *m/z* 1029 in turn can indicate ions with the same interpretation duality, because lithium adducts are not predominant in this type of ionization by MALDI-TOF MS. However, the doping with potassium chloride (**C**) provides relevant information, since the detection of an ion with 16 Da in relation to the *m/z* 1029 (in i) reveals that it refers to a potassium adduct of oxidized oligosaccharide $[DP6_{AA}+K]^+$ in aldonic acid form (AA). This suggests that the oxidized oligosaccharides are because of the action of this reducing agent (non-enzymatic agent). In addition, it emphasizes the importance of the use of controls in the absence of enzyme to certify the enzymatic activity. It is noteworthy that the doping (cationization) of the sample with different salts allowed defining the identity of overlapping *m/z* values, and the data of each salt were complementary between them.

## (II) Buffers

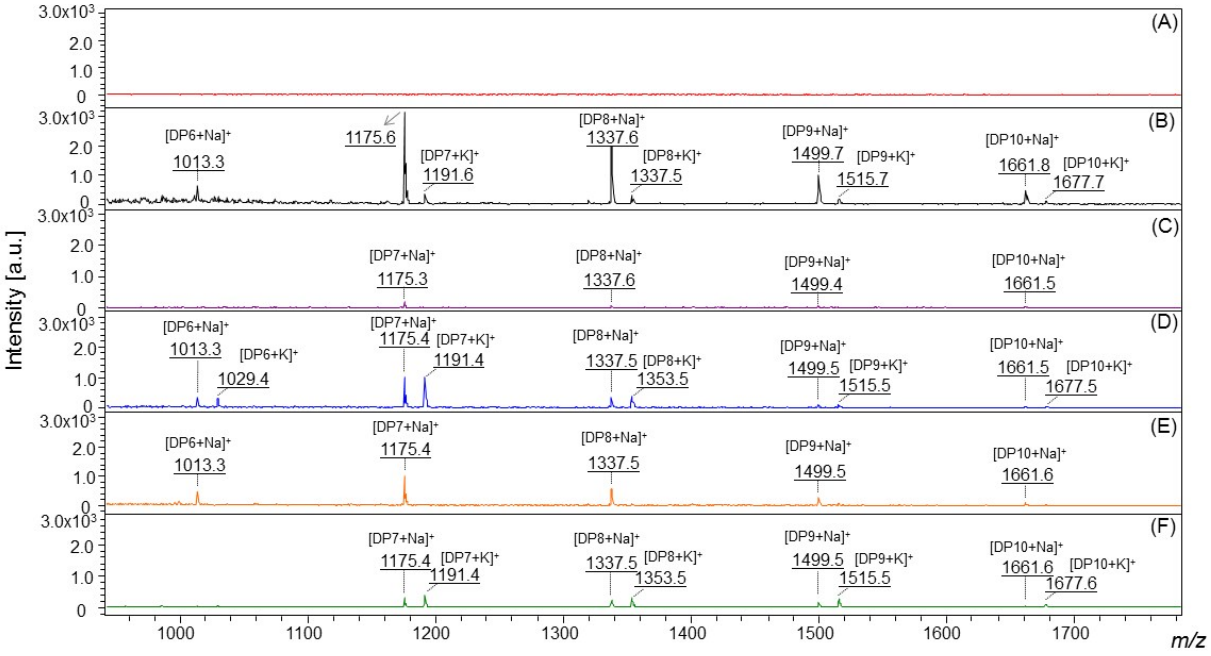


**Figure S3.** MALDI-TOF MS analyses of a mixture of cello-oligosaccharides in the presence of different buffers or water. In the first spectrum (A), DHB matrix (in red) is shown as a blank control. The other spectra show the profile of cello-oligosaccharides when incubated with water (B) and buffers of ammonium acetate (C), potassium phosphate (D), sodium phosphate (E) and phosphate saline (F). *m/z* values of DP6-DP10 (native oligosaccharides) are observed in the form of sodium $[DPn+Na]^+$ and potassium $[DPn+K]^+$ adducts, as described in Table 1. When using potassium phosphate (**Figure S3-D**) and phosphate-saline (**Figure S3-F**) buffers, species with "overlapping" *m/z* values (native cello-oligosaccharides with potassium adduct $[DPn+K]^+$ or oxidized oligosaccharides with sodium adducts $[DPn_{AA}+Na]^+$) were detected. These ions were also detected after incubation with water instead of buffer (**Figure S3-B**), but only with low intensity. Considering that these ions were detected in the absence of an oxidizing agent (enzymatic or non-enzymatic), they most likely refer to native oligosaccharides detected in potassiated form and not to oxidized oligosaccharides (that have the same *m/z* value as previously shown in **Figure S1**). Even though the addition of sodium chloride as a cationization agent reduces the intensity of these overlapping *m/z* values, the fact that these buffers contain potassium in their composition provided the high intensity of these ions. Therefore, considering the possibility of using other buffers, it is suggested not to use buffers containing potassium salts in LPMO activity analyses. When using ammonium acetate (**Figure S3-C**) and sodium phosphate (**Figure S3-E**) buffers, ions referring to native oligosaccharides were detected only as sodium adducts. Furthermore, the intensity of the native oligosaccharide signals was higher in the sodium phosphate buffer (**Figure S3-E**). In addition, this buffer allowed the detection of more oligosaccharides (DP6 to DP10) as compared to the ammonium acetate buffer (**Figure S3-C**), which did not allow the detection of DP6.

## (III) Matrix Application

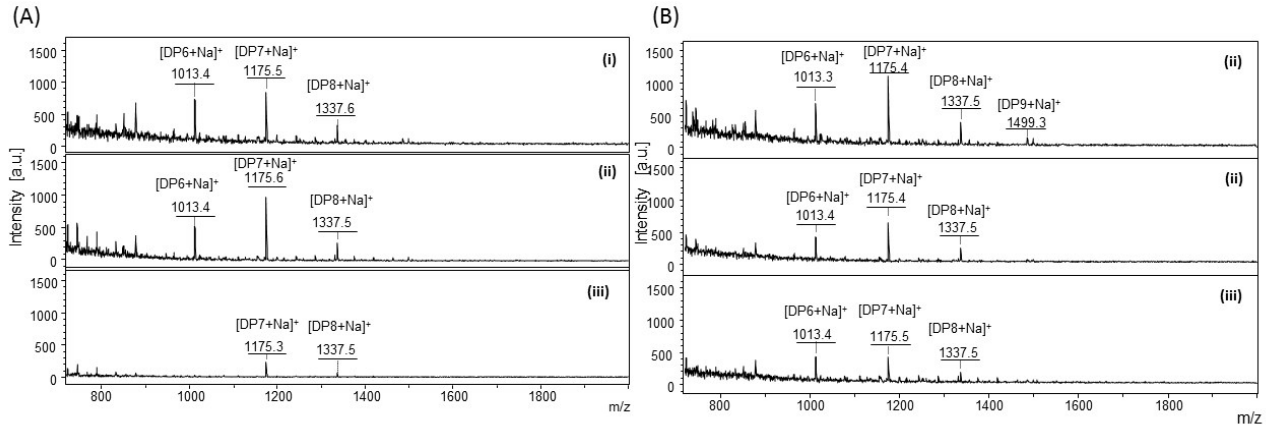


**Figure S4.** MALDI-TOF MS of a mixture of native cello-oligosaccharides analyzed by two sample preparation methods: (A) "two-layer" and (B) "dried droplet" in different ratios of sample-NaCl-matrix (i) 1:1:2, (ii) 1:1:4 and (iii) 1:1:6.

## (IV) Matrix Concentration

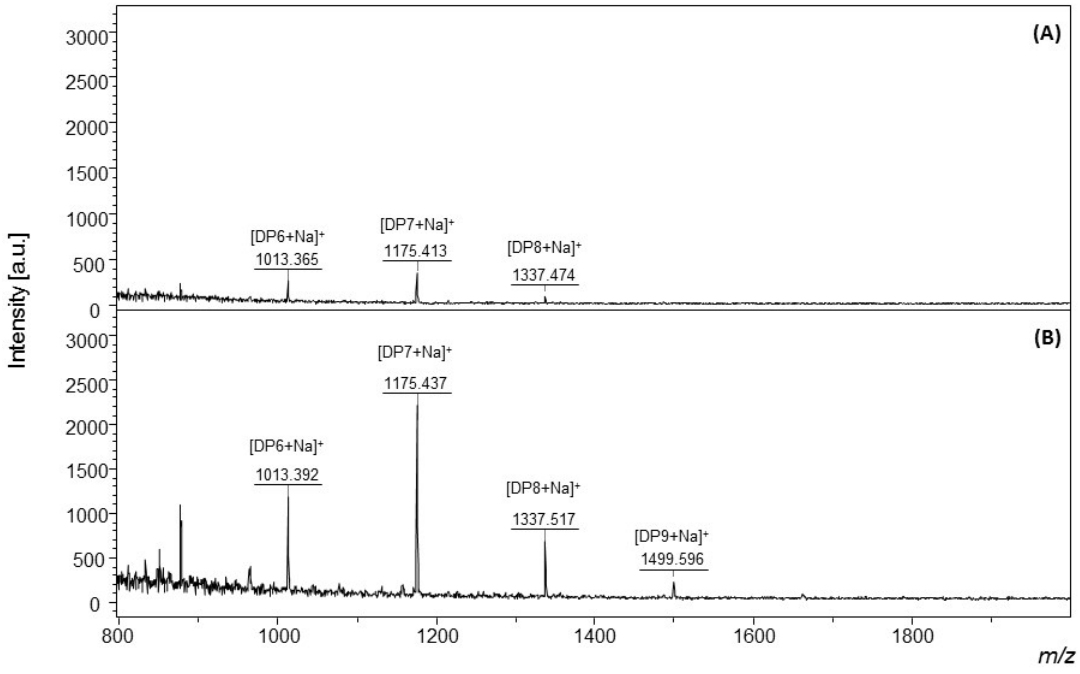


**Figure S5.** MALDI-TOF MS of a mixture of native cello-oligosaccharides analyzed by two DHB matrix concentration (A) 20 mg/mL and (B) 9 mg/mL. In this experiment, the sample was applied by dried droplet method (the best method as shown in Figure S4) with the sample-NaCl-matrix ratios of 1:1:4.

## (V) Sample-NaCl-Matrix Proportion

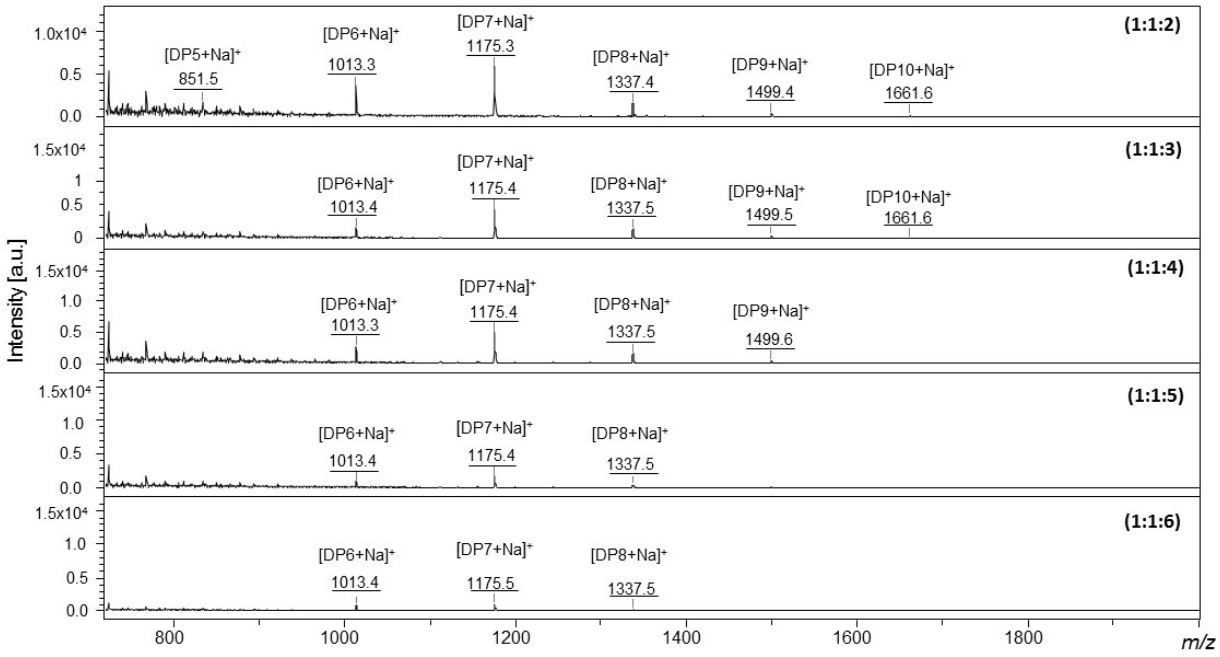


**Figure S6.** MALDI-TOF MS of a mixture of native cello-oligosaccharides analyzed by different sample-NaCl-matrix ratio: (A) 1:1:2, (B) 1:1:3, (C) 1:1:4, (D) 1:1:5 and (E) 1:1:6. In this experiment, the sample was applied by dried droplet method (the best method as shown in Figure S4) using DHB matrix in 9 mg/mL (the best concentration as shown in Figure S5).

## UHPLC-ESI-MS analyses

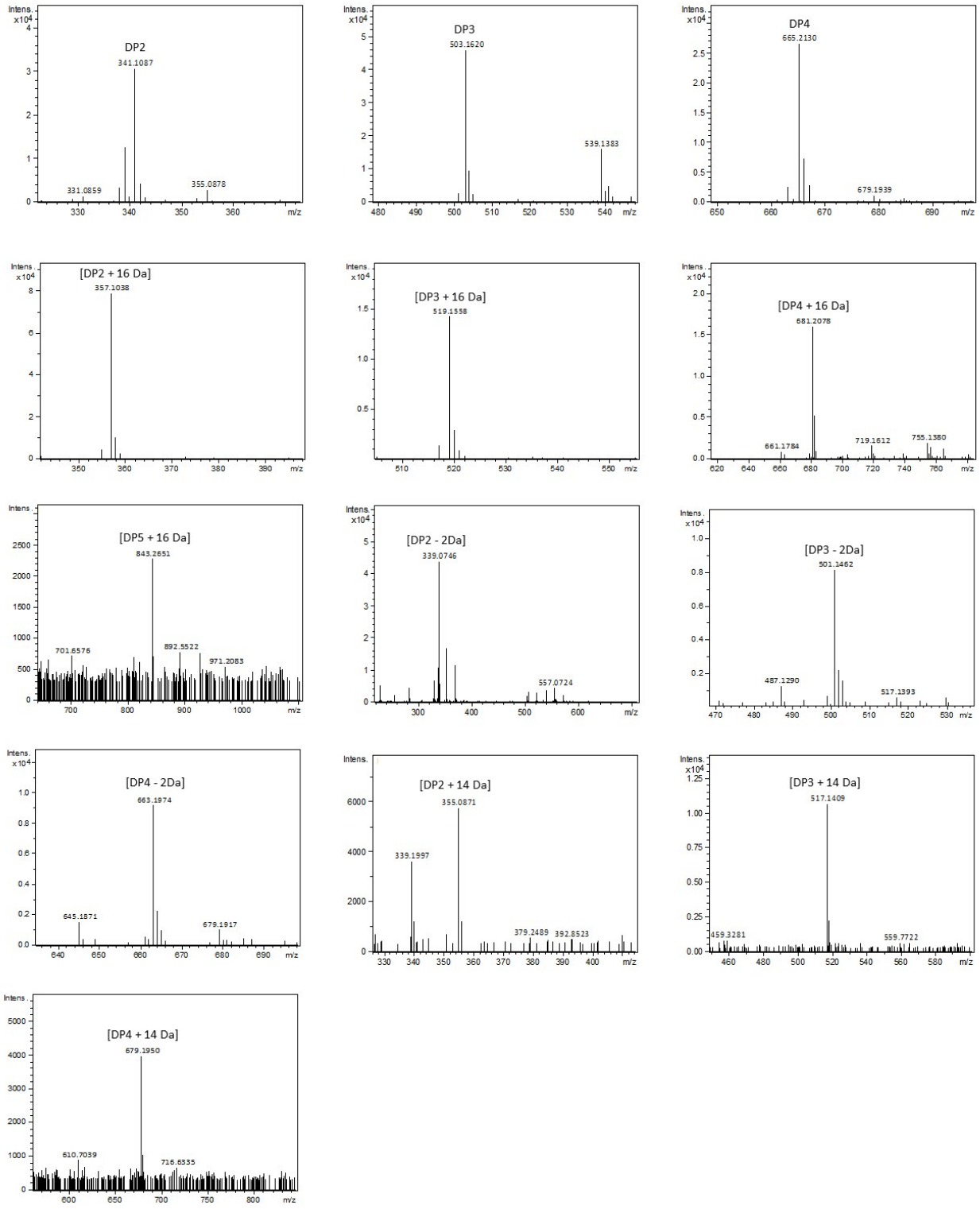


**Figure S7.** Mass spectra for oligosaccharides detected in the UHPLC-ESI-MS analyses.

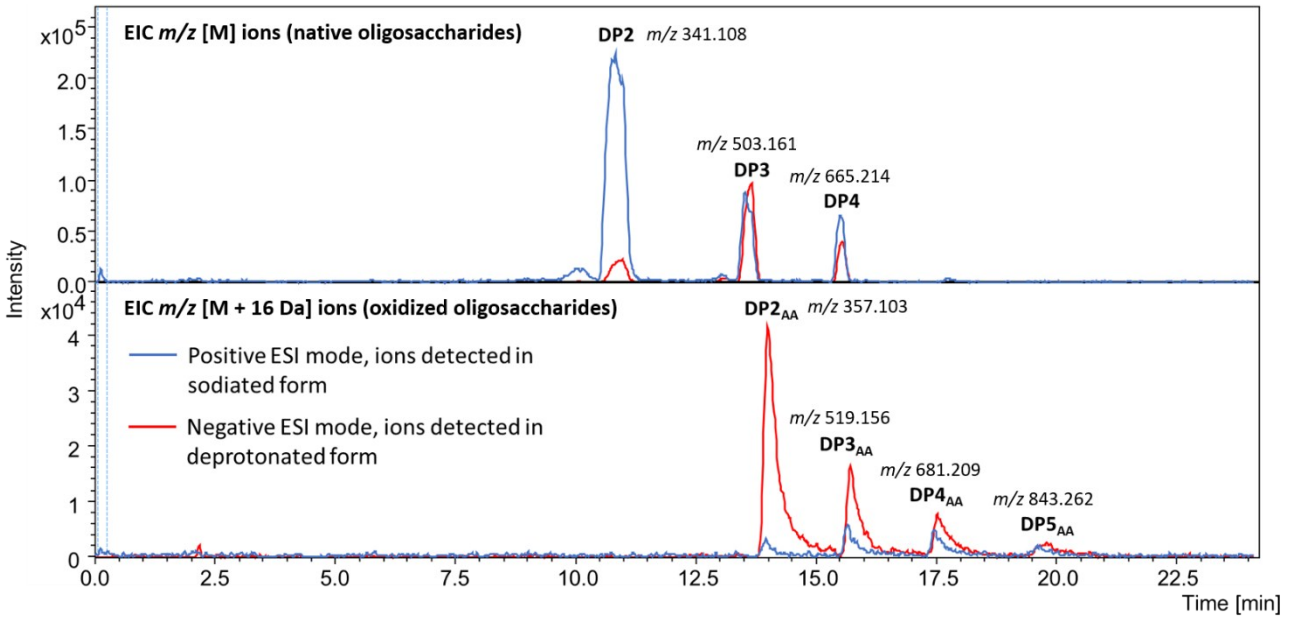


**Figure S8.** Comparison of positive and negative electrospray ionization (ESI) modes on the detection of neutral (*m/z* [M] ions) and oxidized cello-oligosaccharides (*m/z* [M + 16 Da] ions) produced by *Tr*AA9A activity on cellulose in hydrophilic interaction UHPLC-ESI-MS analyses. Peaks are labeled with their respective *m/z* value and their putative annotation. All compounds were detected in deprotonated form ($[M - H]^-$) in the negative ESI mode and in sodiated form ($[M + Na]^+$) in the positive ESI mode. The *m/z* [M – 2 Da] and [M + 14 Da] ions (putatively annotated as C4-oxidized products and doubly-oxidized products) were not detected in the positive ESI mode and therefore were not shown in this figure. EIC = extracted ion chromatogram

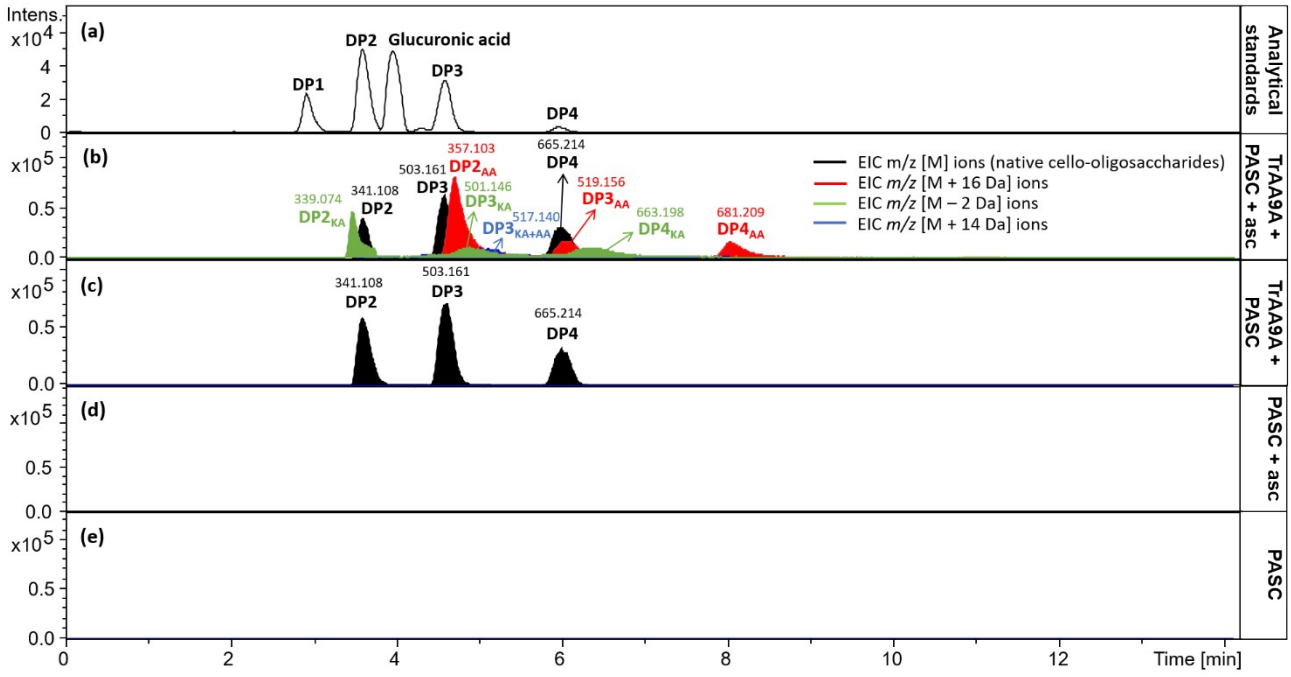


**Figure S9.** Hydrophilic interaction UHPLC-ESI-MS analysis of *Tr*AA9A assay (b) in comparison to negative controls performed in the absence of ascorbic acid (asc) (c), enzyme (d), or both (e). A mixture of standard mono- and oligosaccharides is shown in (a). EIC = extracted ion chromatogram. All compounds were detected in deprotonated form ($[M - H]^-$). Peaks are labeled with their respective *m/z* value and their putative annotation (see text). Oligosaccharides are abbreviated according to Table 1.

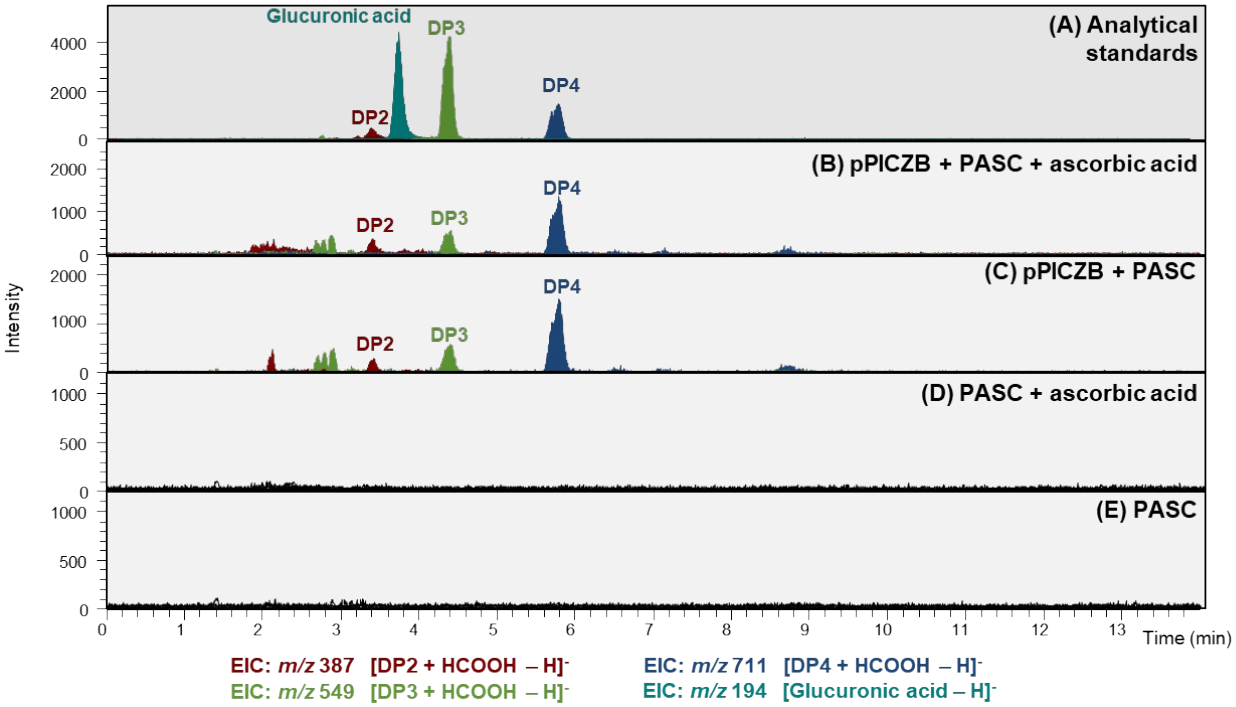


**Figure S10. Endoglucanase activity in the crude extract of *K. phaffii* expressing the empty pPICZB vector.** Hydrophilic interaction UHPLC-ESI-MS analysis of the enzymatic assay performed with the crude extract of *K. phaffii* expressing the empty pPICZB vector as the enzyme source. A mixture of standard mono- and oligosaccharides (10 µg/mL) is shown in (A). The complete assay (containing *K. phaffii* crude extract, PASC and ascorbic acid) is shown in (B), whereas negative controls performed in the absence of ascorbic acid (C), enzyme (D), or both (E) are also shown. All compounds were detected in the form of deprotonated formic acid adduct ([M + HCOOH – H]$^-$), except for glucuronic acid, which was detected in deprotonated form ([M – H]$^-$). Oligosaccharides are abbreviated according to Table 1. EIC = extracted ion chromatogram. Methodology: the UHPLC-ESI-MS analysis was performed in a UPLC Acquity® chromatography system (Waters) coupled to a LTQ XL Linear Ion-trap mass spectrometer via a electrospray ionization source (Thermo Scientific) using the chromatographic protocol "gradient 3" described in the text. Thermo Tune Plus and XCalibur softwares (Thermo Scientific) were used for data acquisition. The mass spectrometer was operated in negative ion mode and tuned for the detection of m/z 549 ion (corresponding to cellotriose) with Thermo Tune Plus software. Instrument settings were: sheath gas flow rate = 40; aux gas flow rate = 10; sweep gas flow rate = 0; spray voltage (kv) = 5; capillary temp (°C) = 300; capillary vontage (v) = 10; tube lens (V) = -100.