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Analysis of Mono- and Oligosaccharides in Ionic Liquid Containing Matrices

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Abstract

Ionic liquids (ILs), *i. e* salts with melting points < 100 °C, have recently attracted a lot of attention in biomass processing due to their ability to dissolve lignocellulosics. In this work, we studied how two imidazolium-based, hydrophilic, cellulose dissolving ionic liquids 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP and 1-ethyl-3-methylimidazolium acetate [EMIM]AcO affect the usually employed analytical methods for mono- and oligosaccharides, typical products from hydrolytic treatments of biomass. HPLC methods were severely hampered by the presence of ILs with loss of separation power and severe baseline problems, making their use for saccharide quantification extremely challenging. Problems in DNS photometric assay and chromatography were also encountered at high ionic liquid concentrations and many capillary electrophoresis (CE) methods did not allow an efficient analysis of saccharides in these matrices. In this paper we describe an optimized CE method with pre-column derivatization for the qualitative and quantitative analysis of mono- and oligosaccharides in sample matrices containing moderate (20 – 40 % (v/v)) concentrations of ILs. The IL content and type in the sample matrix was found to affect both peak shape and quantification parameters. Generally, the presence of high IL concentrations (≥ 20 % (v/v)) had a dampening effect on the detection of the analytes. IL in lower concentrations of < 20 % (v/v) was, however, found to improve peak shape and/or separation in some cases. The optimized CE method has good sensitivity in moderate concentrations of the ionic liquids used, with limits of detection of 5 mg/L for cellooligomers up to the size of cellotetraose and 5 – 20 mg/L for cellopentaose and cellohexaose, depending on the matrix. The method was used for analysing the action of a commercial β-glucosidase in ILs and for analysing saccharides in the IL containing hydrolysates from the hydrolysis of microcrystalline cellulose with *T. reesei* endoglucanase Cel5A. According to the results, [DMIM]DMP and [EMIM]AcO] showed clear differences in enzyme inactivation.

Keywords: Ionic liquid; Saccharide; Derivatization; Beta-glucosidase; Endoglucanase; Capillary electrophoresis

1. Introduction

Ionic liquids (ILs) have recently received a great deal of interest as a new media for biomass dissolution and chemical modification. ILs are defined as salts with melting points < 100 °C[1]. In 2002, Swatloski *et al.* described the dissolution of cellulose in ionic liquids[2]. The dissolution of wood, in the form of saw dust, has also been demonstrated in ILs[3]. This dissolution ability of ILs can also be exploited in pre-treatment of lignocellulosics prior to enzymatic total hydrolysis and fermentation used in the production of ethanol from renewable feed stocks. Dadi *et al*.[4] described a pre-treatment process in which the biomass is first dissolved in IL and then precipitated by the addition of an anti-solvent such as water or alcohol. This pre-treatment process was shown to greatly enhance subsequent enzymatic hydrolysis rates. Kamiya *et al.*[5] reported an alternative process where the cellulosic substrate was first dissolved in ionic liquid and then enzymatically hydrolysed in the same vessel after addition of buffer. After the introduction of these two concepts a number of papers dealing with enzymatic hydrolysis of cellulosics in combination with IL treatments have been published.

Only a few reports have been published on the IL compatibility of analytical methods for saccharides. ILs have, however, been proposed to interact with photometric assays and High

Performance Liquid Chromatography (HPLC) methods[6], which are generally used for analysing the soluble saccharides formed in enzymatic hydrolysis of lignocellulosics. Recently, Hyvärinen *et al.*[7] discussed difficulties caused by the high salt content in chromatographic saccharide analysis in IL containing sample matrices. On the other hand, ILs have previously been used as auxiliaries in both chromatographic and electrophoretic separation techniques, predominantly as column stationary phases or mobile phase additives (chromatography) or as organic modifiers in background electrolytes solutions (BGEs) and capillary coatings in capillary electrophoresis (CE)[8]. Recently, Vaher *et al.* demonstrated that ILs in low concentrations could act as chromophores for indirect UV detection in the sensitive analysis of small saccharides[9]. According to Vaher *et al.* the presence of low amounts of ILs (10 – 50 mM) in the BGEs increased the resolution between the saccharide peaks, but increasing the content of IL further led to baseline fluctuation. Another observation was that ILs with long hydrocarbon chains may actually function as surfactants and reverse the electroosmotic flow. High sensitivity saccharide analytical methods in high content IL matrices have not been described previously.

Saccharides are non-ionic compounds in their natural state. To enable the resolution of neutral, non-derivatized saccharides by electric fields as in CE, alkaline borate buffers are used to form charged borate-saccharide complexes[10,11,12]. The detection (usually measured at 195 nm) is considerably improved by the formation of borate-saccharide complexes[12]. Also indirect detection of saccharides in CE analysis is possible. In this case, a UV absorbing compound (such as sorbic acid[13] or 2,6-pyridinedicarboxylic acid[14]) is added to the BGE and the saccharides are analysed under basic conditions.

The resolving power of CE with pre-column derivatization has previously been demonstrated for mixtures of monosaccharides, including uronic and hexenuronic acids, and small xylo- and cellooligomers, in aqueous solutions[10,11,15]. Maltooligosaccharides with degrees of polymerization (DP) of up to 13 have been separated employing CE techniques[16]. Good separation results for derivatized saccharides have also been obtained employing micellar electrokinetic capillary chromatography (MEKC)[17]. CE has been used in the separation of monosaccharide mixtures in matrices containing *N*-methylmorpholine-*N*-oxide (NMMO) used as industrial cellulose solvent[18]. In this study, monosaccharides were analysed in aqueous matrices containing roughly 10 % NMMO prior to derivatization and analysis. The presence of NMMO was reported to interfere neither with the derivatization reaction nor with analysis with CE.

Advantages of sample derivatization include a manifold increase in detectability. Usually derivatized saccharides have absorption maxima with wavelengths greater than those of underivatized analytes, which increases also the selectivity of detection. Commonly encountered carbohydrate derivatization reagents are *e. g.* 4-aminobenzoic acid ethyl ester (ABEE)[10,11,15], 4-aminobenzonitrile (ABN)[15], 6-aminoquinoline (6-AQ)[19], and 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)[16]. The derivatization proceeds *via* reductive amination and needs a free reducing end of the analyte. Reductive amination works well for aldoses, but ketoses such as fructose are not well derivatized[10,16]. Great excesses of derivatization reagent are usually used. In the derivatization method described by Dahlberg *et al.*[11], the derivatization reaction is quenched by addition of alkaline borate buffer, which is suggested to form highly water soluble saccharide-borate complexes at the same time as the excess ABEE reagent is precipitated. Alkaline borate buffers are generally employed as BGEs for the separation of ABEE, ABN and similar saccharide derivatives in CE, with

some variations in the alkalinity and borate concentration. Occasionally, additives such as surfactants and alcohols are added to the BGEs to improve resolution between adjacent peaks[15]. Both normal[10] and reverse polarity[15] modes have been employed.

Our work on developing IL compatible analytics for saccharide identification and quantification was started to allow us to study the action of hydrolytic enzymes on cellulose in imidazolium-based ILs[20]. The ionic liquids studied were 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP and 1-ethyl-3-methylimidazolium acetate [EMIM]AcO. The primary aim was to find a method that allows the sensitive quantification of cellooligomers up to the size of cellohexaose in these IL containing matrices. In this paper, we discuss how the presence of [DMIM]DMP and[EMIM]AcO affect the routine methods, such as DNS assay, different chromatography methods and CE in saccharide analysis. We present an optimized method for cellooligomer analysis in significant contents of ILs (20 – 40 % (v/v)) employing CE with pre-column derivatization. The separation power of this method is demonstrated for both mono- and oligosaccharides obtained from wood-derived biomass and results for the quantification of the water soluble cellooligomers glucose, cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose in four different matrices are presented. The usefulness of the method is illustrated by two studies, in the first of which the action of a commercial β-glucosidase preparation is studied in [DMIM]DMP and [EMIM]AcO matrices on cellooligomeric substrates, and in the second of which the partial enzymatic hydrolysis of microcrystalline cellulose by an endoglucanase is followed for different time points in IL matrices.

2. Materials and Methods

2.1 Chemicals

[DMIM]DMP was prepared as described in the literature[21]. [EMIM]AcO (purity > 98%) was purchased from Ionic Liquid Technologies (Heilbronn, Germany) and used without further purifications. The halide content of the [EMIM]AcO determined by ion chromatography was: chloride < 100 mg/kg and bromide < 50 mg/kg.

Cello- manno- and xylooligomers in the range of bioses to hexaoses were purchased from Megazyme International (Wicklow, Ireland). Boric acid, sodium hydroxide (NaOH), 1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide (hexadimethrine bromide), xylose, galactose, mannose and arabinose were obtained from Sigma-Aldrich (Steinheim, Germany). Glucose was from VWR International (Leuven, Belgium), galacturonic acid (from citrus origin) was purchased from BDH Chemicals (Poole, UK). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). For the preparation of 3,5-dinitrosalicylic acid (DNS) reagent solution according to Sumner[22], DNS and potassium sodium tartrate tetrahydrate were acquired from Merck (Darmstadt, Germany). All chemicals were used as received if not otherwise stated.

β-Glucosidase (Novozym 188) was obtained from Novozymes (Bagsvaerd, Denmark) and used as such. β-Glucosidase, xylanase and endoglucanase activities were measured for the crude β-glucosidase preparation and determined to 5900 nkat/mL, 2970 nkat/mL and 740 nkat/mL, respectively. The unit katal (kat) is defined by the International Union for Pure and Applied Chemistry (IUPAC) as the number of catalysed reactions per time unit as mol/s[23]. β-Glucosidase activity was measured according to Bailey and Linko[24] and xylanase activity according to Bailey *et*

al.[25] but at pH 5.0. Endoglucanase activity measurements were carried out according to the HEC assay[26] but using carboxymethylcellulose (CMC) in buffer at pH 5.0.

2.2 Chromatography and DNS Assay

Reversed-phase chromatography was carried out based on experimental conditions described by Yasuno *et al.*[27]. Analyses were carried out on a Dionex Ultimate 3000 HPLC system equipped with a Phenomenex C-18 Gemini-NX 3 µm 110A 150x2mm column and a diode array detector. The eluent was a 0.2M potassium borate buffer at pH 9 with 5% MeOH.

DNS photometric assay was carried out according to the IUPAC standard procedure[26] with the DNS reagent solution prepared as described by Sumner[22] using a Hitachi U-2000 spectrophotometer for absorption measurements at 540 nm. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was done according to our previously published in-house method[28].

2.3 Capillary Electrophoresis

2.3.1 Derivatization Prior to CE Analysis

The saccharides were derivatized with 4-aminobenzonitrile (ABN, samples in aqueous solution or containing [DMIM]DMP) or 4-aminobenzoic acid ethyl ester (ABEE, samples containing [EMIM]AcO), according to a modified procedure based on Dahlman *et al.*[11]. The reagent solution was prepared by dissolving ABN (0.29 g) or ABEE (0.40 g) in 10 mL methanol containing acetic acid (AcOH, 1.0 g). Sodium cyanoborohydride ($NaCNBH_3$, 0.1 g) was added to the reagent solution immediately prior to derivatization. Samples containing > 40 % [DMIM]DMP were diluted with distilled water to a final concentration of 40 % [DMIM]DMP and samples containing > 20 % [EMIM]AcO were diluted with distilled water to a final concentration of 20 % [EMIM]AcO. The sample was mixed with a 1 g/L galactose solution (internal standard) to yield a sample with a final galactose concentration of 20 mg/L. The sample with internal standard (1 part) was mixed with derivatization reagent solution (1 part) in a sealable glass vial and the vial was kept at 80 °C for 60 min. The reaction completed, 1 part of alkaline boric acid buffer (450 mM, pH = 8.5) was added, the mixture was vigorously vortexed, transferred to plastic Eppendorf tubes and centrifuged (14 000 rpm, 5 min) in order to sediment any precipitation. Aliquots of the supernatant were then as such taken for injection to the CE.

2.3.2 CE Running Conditions

Capillary electrophoresis was carried out with a P/ACE MDQ capillary electrophoresis instrument (Beckman-Coulter, Fullerton, CA, USA) equipped with a photodiode array (PDA) UV/Vis detector. Absorption of derivatized saccharides was measured at wavelengths of 284 nm for ABN derivatives and 305 nm for ABEE derivatives. The employed fused silica capillary had a total length of 60 cm, an effective length of 50 cm to the detector and an inner diameter of 50 µm. The injection was performed applying a pressure of 0.5 psi (34.5 mbar) for 30 s. The applied voltage was 20 kV in reverse polarity mode and the capillary cartridge was kept at a temperature of 30 °C. Every run was preceded by rinsing the capillary 3 min with 0.1 M HCl, 1 min with 0.1 M NaOH and 5 min with the running electrolyte. The capillary was conditioned before running any samples by flushing 10 min with 0.1 M HCl, 10 min with 0.1 M NaOH and 10 min with water. All rinsing actions were carried out at a pressure of 20 psi (1 380 mbar). As background electrolyte (BGE) solution was used a 438 mM sodium borate solution (pH = 9.7), containing 0.001 % (w/v) of 1,5-dimethyl-1,5-diaza-

undecamethylene polymethobromide (hexadimethrine bromide). Each sample was analysed with two repetitions.

2.3.3 Quantitation

Standard curves were acquired for glucose, cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose in the range of 5 – 200 mg/L (cellotetraose, cellopentaose, cellohexaose) or 5 - 300 mg/L (glucose, cellobiose, cellotriose), all the standards containing 20 mg/L galactose as internal standard. Galactose was chosen as internal standard in our work, as it migrates before the expected mono- and oligosaccharides in our samples and has a good detector response when derivatized. Every calibration measurement was repeated 6 times. The Beckman-Coulter 32 Karat Software version 8.0 was used for evaluation of migration times and peak areas. The peak areas were corrected by dividing the measured area by the migration time. For determining linearity ranges and response factors for the saccharides towards the internal standard (ISTD), x and y values were calculated according to:

x = Conc(Analyte)/Conc(ISTD)

y = Area(Analyte)/Area(ISTD)

where Conc(Analyte) is the concentration of the analyte of interest and Conc(ISTD) the concentration of the internal standard (20 mg/L), and Area(Analyte) denotes the corrected area of the peak of interest and Area(ISTD) the corrected area of the internal standard. By plotting y against x linearity zones and the response line equations could be determined. The concentration of cellooligomers could thus be evaluated using the above described relationship and solving it for Conc(Analyte), correcting with the sample dilution coefficients. Limits of detection (LOD) and Limits of Quantification (LOQ) were evaluated by comparing the signal to noise ratio (S/N) values of the calibration points peak area to the limits being LOD = 3 (S/N), LOQ = 10 (S/N).

2.4 Hydrolysis of Cellooligomers with β-Glucosidase in Aqueous Ionic Liquid Solutions

Buffer (0.050 M citrate pH 5.0 or 0.1 M phosphate pH 6.0) and the cellooligomer solution (150 mg/L) were mixed in a 2 mL plastic Eppendorf tube. The defined amount of ionic liquid was added followed by the enzyme preparation as a 1:400 dilution of the commercial enzyme solution, the enzyme dosage thus corresponding to a an activity of 1000 nkat/g of cellooligomer substrate. The hydrolysis was carried out in a FinePCR Thermo Micromixer (Mxi4t) at a shaking rate of 300 rpm in 45 °C for 20 h. The hydrolysis was stopped by heating the reaction tubes to 98 °C in a block heater for 10 min. The cellooligomer composition of the hydrolysates was analysed according to the CE method described in section 2.3. All the hydrolysis experiments were carried out with two parallel samples and one reference where no enzyme was added. The pH values of the hydrolysis media were measured with a with a Knick pH meter 766 Calimatic equipped with a Mettler-Toledo 110 Inlab Semi-Micro electrode (pH range 0-12).

2.5 Hydrolysis of Microcrystalline Cellulose with *Trichoderma reesei* Cel5A in Aqueous Ionic Liquid Matrices

The cellulase preparation of *Trichoderma reesei* Cel7B was produced, isolated and purified at VTT according to Suurnäkki *et al.*[29]. The Cel5A activity was determined by the standard HEC assay[26] but

using carboxymethylcellulose (CMC) as substrate in buffer at pH 5.0. The enzyme dosage per gram of cellulose (dry weight) was 2000 nkatal. Hydrolysis mixtures were prepared with 0, 20, 40, 60, 80 and 90 % (v/v) IL dosage in sodium citrate buffer (50 mM, pH = 5.0), making the total sample volume 3 mL, and the hydrolysis time was 2, 24, 48 or 72 h. 30 mg (dry weight) of microcrystalline cellulose was measured into a test tube, the defined amount of buffer was added and the mixture was stirred to homogeneity. The hydrolysis was carried out at 45 °C in closed test tubes in a water bath with continuous magnetic stirring. The hydrolysis was stopped by boiling the sample for 600 s to denature the enzyme. After cooling to room temperature, the reaction tube was centrifuged 3000 rpm for 10 min and the clear supernatant was separated from the solid cellulose residue and further prepared for CE analysis as described in section 2.3.

3 Results and Discussion

3.1 Effect of Ionic Liquid on DNS Assay and Chromatographic Methods for Saccharide Analysis

DNS assay[22,30] is a well-known and often employed analytical method for the estimation of the total amount of reducing saccharides in aqueous solutions. This method has also been shown to be reliable in up to 20 % (v/v) of [EMIM]AcO in the solution matrix[31]. In our experiments, it was, however, found that the commercial [EMIM]AcO used especially at high concentrations of over 40 % (v/v) caused serious colouration which gave strong absorption at 540 nm, leading to problems in background calibration. It could not be concluded whether the colour formation was due to the actual IL or impurities in it. [DMIM]DMP was also found to cause some colouration, but not to the same extent as [EMIM]AcO. DNS assay also has its limitations as although it gives an estimate of the total amount of reducing ends it is not able to distinguish different mono- and oligosaccharides from each other.

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) is known to be an excellent method for saccharide analysis in aqueous systems. Clear benefits of this method are the good sensitivity and the fact that there is no need to derivatize the samples[16]. In our experiments, saccharides like glucose, cellobiose and cellotriose could not be detected in the presence of even 20 % (v/v) of [DMIM]DMP. It is probable that the high content of ions in the sample matrix interferes with the anion exchange capability of the column and furthermore the IL ions may have an impact on the detection at the PAD detector.

We tried the use of reversed-phase HPLC systems for samples with ABEE and ABN derivatized samples. In aqueous solutions the monosaccharides and some disaccharides could be separated but for larger oligosaccharides a good separation could not be achieved. When the experiment was repeated in matrices containing 40 % (v/v) [DMIM]DMP or [EMIM]AcO, the monosaccharide peaks could not be detected anymore and severe baseline problems were encountered. Kamiya *et al.* quantified glucose and cellobiose as ABEE derivatives in IL containing hydrolysates with reverse phase HPLC[5], according to a method of Yasuno *et al.*[27]. The sample matrix contained 1,3-dimethylimidazolium diethylphosphate, an IL very similar to [DMIM]DMP in structure, but further details about the analysis and the actual IL content in the samples were not given.

3.2 Optimization of CE Method and Calibration for Cellooligomer Quantification in IL Matrices

Both CE methods with direct and indirect detection modes were tested for saccharide analysis in the presence of two imidazolium-based ILs. The presence of significant amounts (>20 – 40 % (v/v)) of [EMIM]AcO or [DMIM]DMP completely deleted the saccharide signals when CE analysis conditions known to be favourable for saccharide analysis in pure aqueous solutions were applied[14,32]. Derivatization of reducing saccharides with UV active tags proved to be a feasible method for improving saccharide detection in CE and a method employing pre-column derivatization was chosen for use in IL containing matrices. We optimized the CE running conditions for samples containing [DMIM]DMP and [EMIM]AcO based on a method previously described by Sartori *et al*.[15].

3.2.1 Optimization of Derivatization Conditions

The derivatization reaction was carried out with ABEE or ABN as described in section 2.3.1. The method follows a general procedure for ABEE derivatization by Dahlman *et al.*[11], with some modifications, *e.g* the method was found to be applicable for the ABN derivatization analogously. Experiments were carried out similarly with derivatization times of both 30 and 60 min at 80 °C for both glucose and cellobiose with ABEE. ABEE derivatization of samples containing glucose and galactose was carried out in 40 % (v/v) [EMIM]AcO matrices with reaction times of 60 and 120 min, as well as with doubled amount of acetic acid in the derivatization reagent solution. [EMIM]AcO was in our experiments found to be very basic[20] which motivated the attempt to acidify the derivatization conditions. Extending the reaction time from 30 min to 60 min and up to 120 min did not lead to an increase in the analyte peak areas and the use of double amounts of AcOH led to failure of the actual analysis with CE. The derivatization was complete already after 30 min for both glucose and cellobiose. No hydrolysis of cellobiose took place with 60 min reaction time which was confirmed by the absence of glucose peaks in the electropherograms of pure derivatized cellobiose standards. The possibility of partial hydrolysis of oligomeric polysaccharides during reductive amination has been proposed previously[16]. In our work no hydrolysis of any cellooligomers used was observed under the applied derivatization conditions.

The presence of [DMIM]DMP or [EMIM]AcO did not prevent the actual derivatization reaction from taking place. It was not, however, elucidated whether the saccharide conversion in the derivatization reaction is changed by the presence of ILs, as compared to the conversion in aqueous reaction matrices. The presence of ILs apparently increased the solubility of the reagents. The derivatization reaction was found to be rather robust and also the optimum conditions were similar in IL matrices to those earlier reported for aqueous solutions in terms of employed reagent solutions, reaction times and reaction temperatures[11]. We applied exactly the same derivatization protocol in derivatizing with ABN as with ABEE, but it was found that the alkaline borate buffer does not efficiently precipitate the excess ABN reagent in the quenching stage. This did, however, not have any detrimental effects on the CE analysis. Similar results have previously been reported for a method where the derivatization reaction is not quenched in any way, but the samples are simply cooled, filtered and injected to the CE for analysis[15]. The derivatized samples were found to be very stable. Properly stored (at -18 °C) we found the derivatives to give reproducible results even after months of storage. The derivatization procedure is easy to carry out in series, making it feasible for analysing large numbers of samples at once.

3.2.2 Optimization of Background Electrolyte

One reason for the general deterioration in saccharide derivative analysis results in IL matrices might be due to changes in the ion strength and thereby electrical properties of the BGE .Therefore, the effect of different borate concentrations on peak shape and separation was studied. The separation of saccharide derivatives in CE has earlier been shown to be better by employing concentrated borate buffers as BGEs[11]. In our work, BGEs with borate concentration in the range of 110 – 600 mM were studied. 450 mM borate buffer was found to be a good compromise between all the criteria for an optimized analysis. In both aqueous and [DMIM]DMP (40 % (v/v)) containing matrices, peak areas generally decreased and monosaccharide and small oligosaccharide peaks suffered of increased tailing when applying low borate concentration BGEs. In some cases, the peak shapes of the larger oligosaccharides were better when applying low borate concentrations than at the relatively high 450 mM concentration. Using extremely concentrated borate buffers did not yield better analysis results neither in pure aqueous matrices nor in IL containing matrices. In the method described by Sartori *et al.* for CE analysis of saccharides in aqueous matrices the BGE contained 5 % methanol and 5 % 1-propanol[15]. We found, that in the presence of the ILs and especially in the presence of [EMIM]AcO, the analysis results were better when no alcohol was added to the BGE. The use of hexadimethrine bromide when reversing the separation polarity was found to improve the analysis whereas the presence of another surfactant, cetyltrimethylammonium bromide (CTAB) was noticed to lead to complete failure of the analysis. The BGE pH was kept at 9.7, as it has been shown that higher alkalinities may cause worse peak resolutions and longer separation times under similar conditions[10].

3.2.3 Optimization of CE Method

In the CE analysis, the ILs were found to greatly influence the analysis results (Fig. 1). At IL concentrations of 20 – 40 % (v/v) in the sample matrix, the IL effect was mainly seen as peak deformation in the form of tailing or sometimes partial peak splitting. In addition, the presence of ILs in different concentrations seemed to influence the peak areas of the separate saccharides to different extents, necessitating calibration against the internal standard in every different sample matrix separately. [EMIM]AcO was found to affect CE analysis much more than [DMIM]DMP. Interestingly, there also seemed to be a difference in derivative to IL compatibility; ABN derivatization gave better analysis results in aqueous and [DMIM]DMP containing solutions whereas ABEE was to be preferred as derivatization reagent in samples containing [EMIM]AcO. The presence of high contents of ILs in the samples gradually led to peak dampening. Therefore, we studied the trade-off between diluting the samples and the response of the analytes in the IL containing samples. It was found, that samples containing more than 40 % (v/v) of [DMIM]DMP gained in overall sensitivity by diluting the sample to a final IL strength of 40 % (v/v). [EMIM]AcO had a much stronger dampening effect and any sample containing this IL was found to give the best overall sensitivity after diluting to a final IL content of 20 % (v/v). It should be noted, that the derivatization procedure dilutes the sample to a third of its nominal analyte and IL concentrations as the used sample/derivatization reagent solution/quenching borate buffer volume ratio is 1/1/1.

The reason for the deleterious effects of ILs in the sample matrix in CE is not known. One possible explanation may be related to the coating of the capillary walls with the IL imidazolium cation, which has previously been described and exploited in the separation of polyphenols[33].The presence of ILs did not always have a negative impact on the actual peak area. Actually, a 20 % (v/v) concentration of [DMIM]DMP seemed to give a better response factor for the smaller of the studied cellooligomers

(glucose, cellobiose, cellotriose) than was the case in pure aqueous solution. This result was not further developed in this study. The reviewed literature and our results show that under some circumstances, the ILs may in moderate contents work as organic modifiers with beneficial effects, such as improved peak shape.

The peaks migrated in the same order and roughly with the same migration times when both pure aqueous samples and IL containing samples were used, which makes it possible to use high quality electropherograms acquired from aqueous samples for the prediction of separation in IL containing matrices.

The running conditions were further developed based on a method published by Sartori *et al.*[15]. In normal polarity mode the largest oligosaccharides migrate first, after the excess reagent peak, followed by the smaller in ascending order. By applying reverse polarity mode the migration order of the saccharides is reversed, but the most important feature is that the saccharides migrate before the large excess reagent peak, which was found to seriously affect the baseline and also the integration of peaks migrating after it. Great gain in terms of peak shape was acquired by raising the temperature to 30 °C, which is higher than in the previous methods cited in this paper. Further increasing the temperature led to a complete failure of the analysis. It was also found that analysis sensitivity could be increased by using a quite long injection time of 30 s, but any further increase was not beneficial. The voltage optimum was determined to be 20 kV. It was observed that the combination of fairly concentrated buffer together with the use of surfactant and the IL in the sample caused severe capillary fouling. Efficient washing procedures needed to be developed to guarantee an acceptable capillary life length. Washing with propanol was tried as a means of cleansing the capillary from organic precipitations but no clear benefit could be demonstrated. However, the combination of acidic (HCl) and alkaline (NaOH) rinsing cycles prior to sample injection was beneficial.

3.2.4 Qualitative and Quantitative Analysis of Mono- and Oligosaccharides in IL Matrices

The optimized CE method was exploited in qualitative saccharide derivative analysis in IL containing aqueous solutions. It was shown that most common cellulose and hemicellulose derived mono- and oligosaccharides could be resolved as ABN or ABEE derivatives. The method was efficient for separating galacturonic acid, galactose and xylose (Fig. 2), but arabinose, mannose and glucose migrated partially together. As indicated earlier by Dahlman *et al.* these monosaccharides can be separated by CE by carefully optimized conditions in aqueous solutions[11]. Also most of the studied xylo-and cellooligomers (Fig. 3) could be separated. Mannooligosaccharides could not be separated from each other. In our study, the presence of ILs in the matrix was not the reason for the weak separation between arabinose, mannose and glucose. It was found that the peak separation, especially for arabinose, mannose and glucose, was better in samples containing 20 % (v/v) [DMIM]DMP than in purely aqueous sample matrices, which demonstrates that the presence of ILs in certain concentrations may indeed be beneficial for the CE analysis.

The CE method was calibrated for quantitative analysis of the cellooligomers in the range of glucose to cellohexaose in four different sample matrices: pure aqueous solution and aqueous solutions containing 20 and 40 % (v/v) [DMIM]DMP and 20 % (v/v) [EMIM]AcO. The regression data is given in Table 1. The regression data is somewhat different for the analysis in the different media and

therefore the calibration should be performed separately for each IL containing media for maximum accuracy. Linearity ranges were studied in the range of 5 – 300 mg/mL for glucose, cellobiose and cellotriose, and 5 – 200 mg/mL for cellotetraose, cellopentaose and cellohexaose. The limits of detection (LoD, S/N 3) and quantification (LoQ, S/N 10) determined represent well the same level of sensitivity as compared to those results previously reported in the literature for aqueous matrixes[15]. Also LoD and LoQ values calculated for the IL containing matrices show good sensitivities, considering the difficulty of the matrix. Because the detection is based on quantifying the reducing end groups as UV active derivatives, the different oligomers were detected corresponding to their molar concentration rather than their mass concentrations, which leads to higher detection and quantification limits expressed in mass per sample volume according to the molecular weight of the saccharide. The cellooligomers showed good linearity ranges in the studied concentration intervals. It needs to be stressed that the linearity ranges given in this paper do not represent the whole linearity range of the different analytes.

Reductive amination as well as other derivatization methods and also DNS assay require a free reducing end group on the saccharide. Ebner *et al*.[34] have shown that the imidazolium C2 carbon in 1-alkyl-3-methylimidazolium ILs may react to various degrees with the reducing end of saccharides in IL solutions. The reaction is proposed to proceed *via* the deprotonation of the acidic proton at C2 in the imidazolium ring, giving a highly nucleophilic carbene which reacts with the reducing end group of a saccharide[35]. In the case of glucose, glucose-imidazolium adducts have been reported to be formed in up to 15 – 20 % yield of the glucose under fairly mild conditions and the addition of base has been found to greatly accelerate the reaction rate[34]. The reaction has also been concluded to be reversible, especially under basic conditions. Acetate ILs have been suggested to be especially susceptible for this kind of reaction due to their alkalinity[35]. Consequently, quantification results may be too low if this side-reaction takes place and if it is not reversed at some point during the sample treatment. Up to date, it has not to our knowledge been elucidated whether carbene formation of imidazolium ILs with subsequent addition to saccharide reducing ends takes place in aqueous solutions of ILs in the same manner and to the same extent as it has been shown to do in pure IL solutions. To assess the general accuracy of any saccharide analytics in IL matrices, this reaction should be studied in more detail.

3.3 Hydrolysis of Celloligomers by β-Glucosidase in IL Matrices

In the total hydrolysis of cellulose, the employed cellulase cocktails generally consist of three different types of cellulases; endoglucanases, exoglucanases, and β-glucosidases, which catalyse the hydrolysis of the cellooligomers formed by the other enzymes to glucose. These enzymes work in synergy and their simultaneous function is vital for high hydrolysis yields[36]. Up to date, some studies have been published about the inactivating effects of ILs on endoglucanases,[20,37] whereas β-glucosidases have received relatively little attention in these matrices. Recently, Engel *et al.*[37] published inactivation data for a commercial β-glucosidase in [DMIM]DMP, where the enzyme was found to lose its activity completely at 15 % [DMIM]DMP. In this study, we followed the change caused by ILs on β-glucosidase action by analysing the individual cellooligomers in the hydrolysates.

Our results (Table 2) show that when the commercial β-glucosidase preparation is used all cellooligomers in the range of cellobiose to cellohexaose are hydrolysed to glucose in sodium citrate buffer at pH 5.0. The β-glucosidase preparation contained xylanase and endoglucanase side-activities, which may play a role in hydrolysing the larger cellooligomers to smaller fragments. The β-

glucosidase preparation seemed to be quite sensitive to the rise of pH, as the hydrolysis was not completed in phosphate buffer at pH 6.0.

The β-glucosidase preparation's action in matrices containing 20 and 40 % of [DMIM]DMP and [EMIM]AcO was studied with cellobiose and cellopentaose as substrates. When adding ILs to the hydrolysis matrix, the action of the β-glucosidase was greatly affected. In 20 % [EMIM]AcO, the action of the enzyme was extremely low (Table 2). In 40 % [EMIM]AcO, no cellooligoomer hydrolysis took place. In the presence of [DMIM]DMP considerable enzymatic action was observed in 20 % [DMIM]DMP, where both cellobiose and cellopentaose were completely hydrolysed to glucose. In 40 % [DMIM]DMP, the cellooligomeric substrates were hydrolysed only partly. In the cases where partial hydrolysis occurred on cellopentaose, the intermediary products were cellotetraose, cellotriose and glucose, whereas very little cellobiose was produced. The hydrolysis matrices contained either citrate buffer at pH 5.0 or phosphate buffer at pH 6.0 in addition to the IL. There was a clear difference in the hydrolysis results depending on the matrix buffer. In all cases, hydrolysis results were lower in the phosphate buffer, as could be predicted from the results from the pure buffer matrices, probably due to the buffers higher pH.

The changes in pH caused by the presence of the ILs (Table 2) is not considered as the main reason for the decreased enzymatic action in the presence of the ILs. [EMIM]AcO is in general to some extent more basic than [DMIM]DMP[20]. With the low contents of IL used in this work the pH is, however, practically the same for [DMIM]DMP and [EMIM]AcO in 0.1 M phosphate buffer (original buffer pH 6.0). This can be exemplified by comparing the hydrolysis of cellopentaose (Glc_5) in 20 % [EMIM]AcO/phosphate buffer (entry 7 in Table 2), where the pH was measured to 6.6, comparing to the hydrolysis of Glc_5 in 20 % [DMIM]DMP/phosphate buffer (entry 14, Table 2), where the pH was measured to the same 6.6. In the presence of [EMIM]AcO, there is almost no hydrolysis observed, whereas the hydrolysis conversion in the corresponding conditions with [DMIM]DMP present is fairly high. Similar but smaller difference was also observed in the enzymatic hydrolysis of Glc_5 in 40 % [DMIM]DMP/phosphate buffer *vs.* 40 % [EMIM]AcO/phosphate buffer (entries 16 and 8 in Table 2). It is thus concluded that [EMIM]AcO is more inactivating for the studied β-glucosidase than [DMIM]DMP and that the main reason for this difference between [EMIM]AcO and [DMIM]DMP is not in their different basicities.The enzyme preparation as such did not contain significant amounts of oligosaccharides, which could disturb the saccharide quantitation. The reference samples containing cellooligomers in the hydrolysis matrix without addition of enzyme showed no signs of IL or buffer induced hydrolysis of oligosaccharides during the 20 h incubation. Some of the saccharides initially added to the hydrolysis mixture were lost during the treatment in IL solutions (Table 2). The reason for this is currently not known, but the reaction between the saccharide reducing ends and possibly formed carbenes, as was discussed in 3.2.4, provides a possible explanation.

3.4 Hydrolysis of Microcrystalline Cellulose by *T. reesei* Cel5A in the Presence if [DMIM]DMP and [EMIM]AcO

Endoglucanases catalyse the hydrolysis of amorphous parts of cellulose resulting in oligosaccharides[36]. In our work, we used the CE method with pre-column derivatization described in this paper to analyse cellooligomers released from microcrystalline cellulose during hydrolysis with the *T. reesei* endoglucanase Cel5A (formerly known as endoglucanase II) in media containing the ILs [DMIM]DMP and [EMIM]AcO. Cel5A produced in all media where it showed activity glucose, cellobiose and cellotriose but no larger cellooligomeric products. The hydrolysis products could be

well separated and quantified, also in the presence of [DMIM]DMP and [EMIM]AcO, with the CE method, as can be seen in Fig 4. A more thorough analysis of the formed different cellooligomers in these systems has been published recently[20].

The hydrolysis yield of the Cel5A treatment of microcrystalline cellulose in the different IL containing media was calculated for four different hydrolysis times (2, 24, 48 and 72 h), by adding the by CE analysed amount of each formed cellooligomer (Fig. 5). It can be seen that the hydrolysis yield is by far the highest in the pure buffer at pH 5.0 as expected and the ILs are inactivating for the enzyme in all reported concentrations. For all time points, it is clear that [DMIM]DMP is much less inactivating for the enzyme than [EMIM]AcO. Interestingly, the hydrolysis degree seems to be increasing throughout the whole studied time interval, in pure buffer as well as in 20 % [DMIM]DMP suggesting that the hydrolysis has not yet at 72 h achieved its maximum level and some of the enzyme is still active also after 72 h incubation in 20 % [DMIM]DMP. In 20 % [EMIM]AcO the yields are much lower than in 20 % [DMIM]DMP. The enzymatic hydrolysis reaches its maximum rather early, between the 2 and 24 h time points (Fig. 5) indicating a complete inactivation of the cellulase in 20 % [EMIM]AcO in the said time interval. In 40 % of [DMIM]DMP only traces of saccharides were found and in 40 % [EMIM]AcO none at all. No saccharides were found in any of the other high IL content matrices.

4 Conclusions

Imidazolium-based ionic liquids form difficult matrices for the analysis of carbohydrates with HPLC, colorimetric and CE methods. In this paper we have described a method for capillary electrophoresis with pre-column derivatization of saccharides with an UV active derivatization reagent. The method developed allows sensitive qualitative and quantitative analysis of carbohydrate mixtures comprising both mono- and oligosaccharides in the presence of significant quantities of the ionic liquids [DMIM]DMP and [EMIM]AcO.

The CE analysis method described in this paper is expected to be of good use in the sensitive analysis of carbohydrates in IL containing sample matrices related to biorefinery applications. The usefulness of the described CE method is demonstrated here by two independent examples: the enzymatic hydrolysis of cellobiose and cellopentaose with β-glucosidase in different media containing the two cellulose dissolving ionic liquids [DMIM]DMP and [EMIM]AcO and the hydrolysis of microcrystalline cellulose with a *T. reesei* endoglucanase in the same media during a time span of 2 to 72 h. The results show that even if the employed cellulases are very different in their function, their hydrolysis efficiency is decreased in the same way by the presence of the studied ionic liquids. [EMIM]AcO was in both the experiments much more inactivating than [DMIM]DMP.

The reaction of carbenes generated in imidazolium-based ILs with the reducing end of saccharides still needs further elaboration, as this reaction would directly influence any quantification results of reducing saccharides. Very little has been published about high sensitivity analysis of carbohydrates in the presence of ILs. This study shows that taking carbohydrate analysis from aqueous systems to IL containing matrices is not straight forward and many crucial questions still need to be answered in future studies.

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Figures

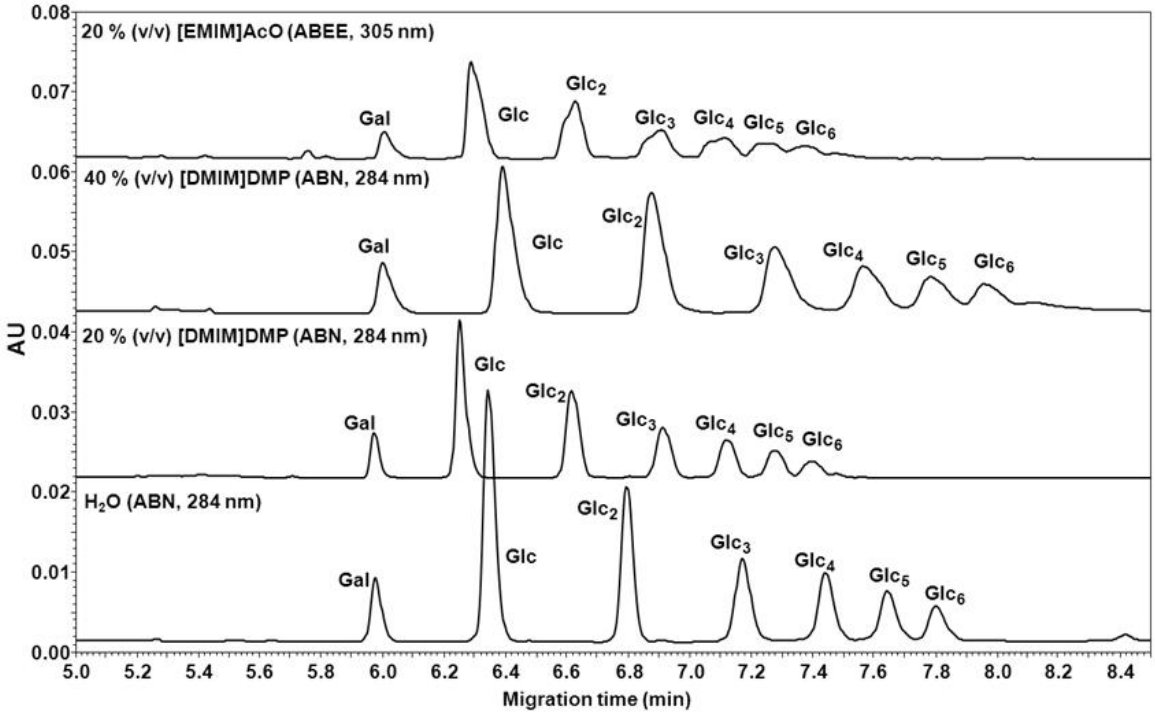

Fig. 1. Separation of cellooligomers in aqueous and IL containing matrices. Comparison of electropherograms obtained for a standard mixture of ABN or ABEE derivatives of 20 mg/L galactose (Gal) and 100 mg/L of glucose (Glu), cellobiose (Glc_2), cellotriose (Glc_3), cellotetraose(Glc_4), cellopentaose (Glc_5) and cellohexaose (Glc_6). The electropherograms have been aligned on the Gal peak (internal standard). Experimental conditions were according to section 2.3, with ABN derivatization (detection at 284 nm) for samples in aqueous and [DMIM]DMP containing matrices and ABEE derivatization (detection at 305 nm) for [EMIM]AcO containing matrices.

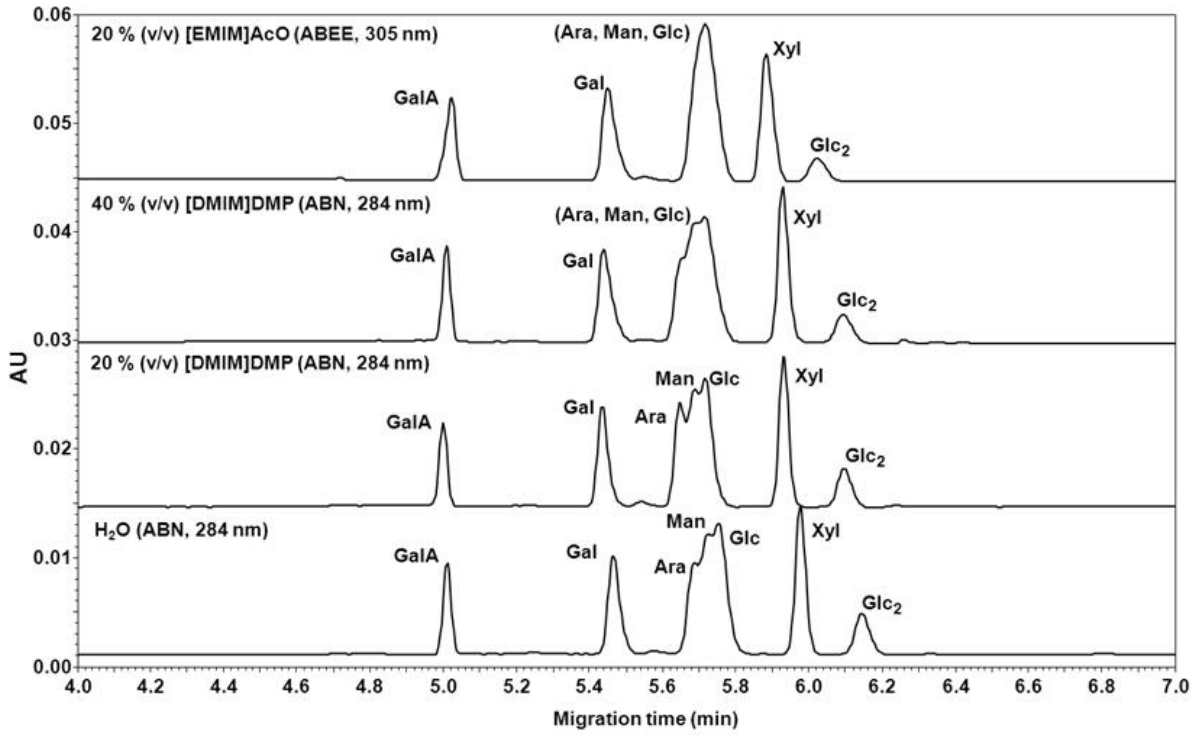

Fig. 2. Separation of galacturonic acid (GalA), galactose (Gal), arabinose (Ara), mannose (Man), glucose (Glc), xylose (Xyl) and cellobiose (Glc_2) ABN (detection at 284 nm) or ABEE (detection at 305 nm) derivatives in four different matrices. Experimental conditions as described in section 2.3. The electropherograms have been aligned on the GalA peak.

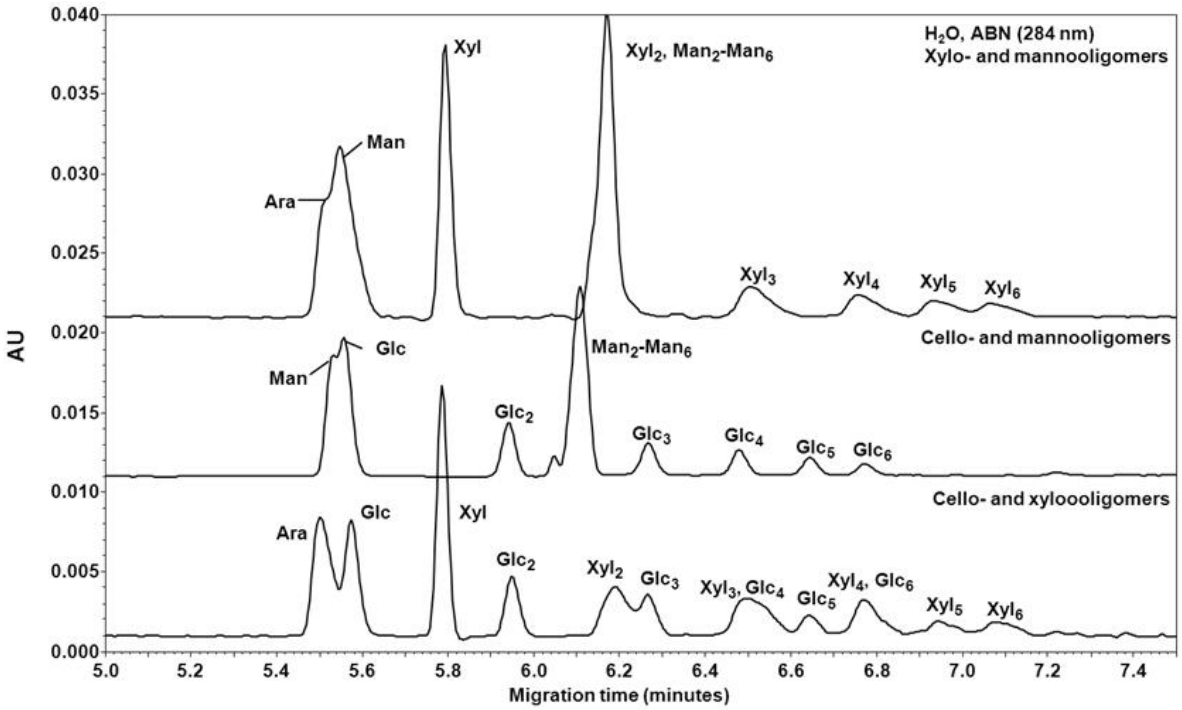

Fig. 3. Separation of cello-, xylo- and mannooligomers (ranging from monosaccharide to hexasaccharide) as ABN derivatives (detection at 284 nm) in aqueous solution. The electropherograms have been aligned on the Xyl or Glc_2 peaks. Each saccharide standard had a concentration of 100 mg/L, experimental conditions were according to section 2.3. Peak labelling is analogous to that introduced in the caption to Fig 1.

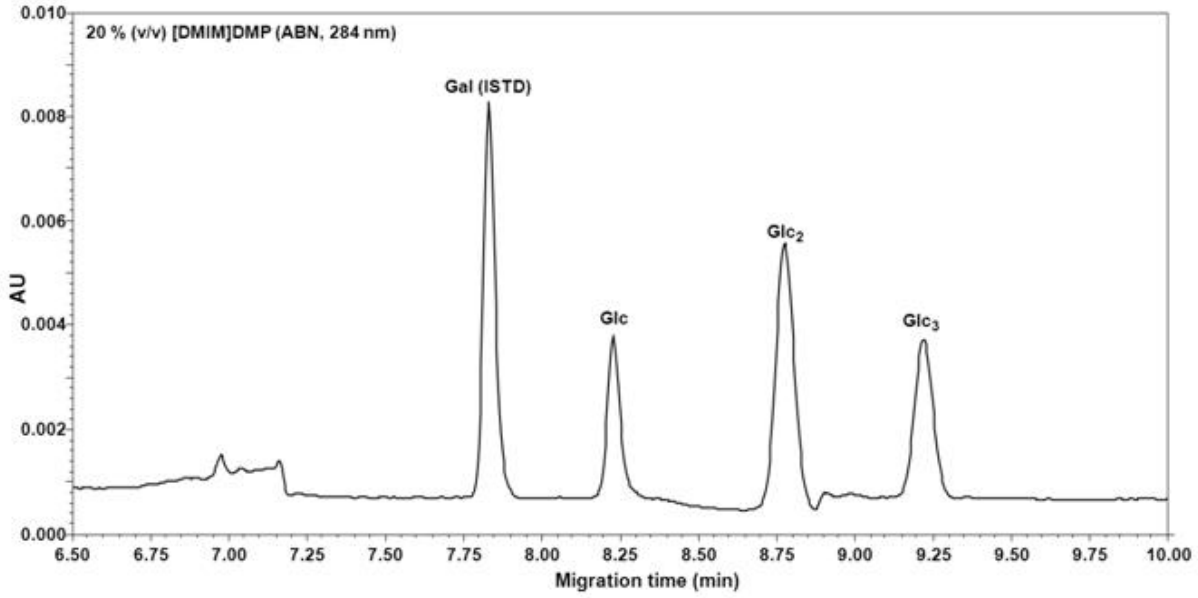

Fig. 4. Analysis of cellooligomers produced in a 24 h hydrolysis in a medium containing 20 % [DMIM]DMP. The derivatization reaction and CE conditions were as described in section 2.3. The cellooligomers in this sample were quantified to Glc: 7.0 mg/L, Glc_2: 34.4 mg/L, Glc_3: 31.4 mg/L, peak labelling is as in Fig. 1.

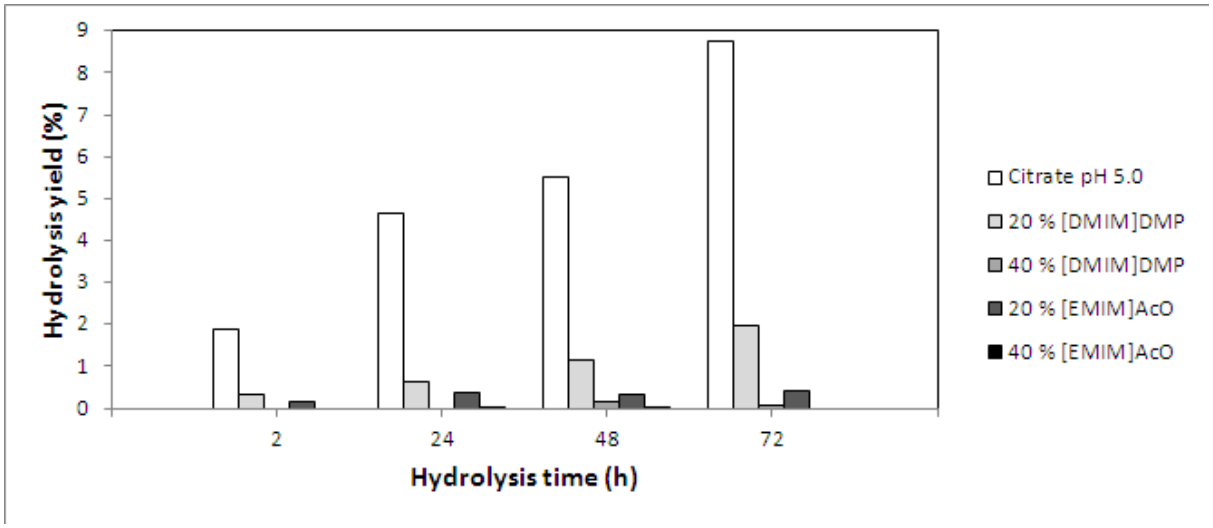

Fig. 5. Yields from hydrolysis of microcrystalline cellulose hydrolysed by *T. reesei* Cel5A after 2, 24, 48 and 72 h hydrolysis in five matrices: 0.050 M citrate buffer (pH 5.0), 20 or 40 % [DMIM]DMP or [EMIM]AcO in citrate buffer.

Saccharide/matrix	LOD (mg/L)	LR (mg/L)	RSD(low) %	RSD(high) %	Equation	R^2
Water						
Glc	< 5	5 - 150	2.38	1.45	y = 0.0429x - 0.0301	0.997
Glc_2	< 5	5 - 300	2.14	0.87	y = 0.0261x - 0.0479	0.999
Glc_3	5	10 - 300	2.16	0.54	y = 0.0165x + 0.0042	0.998
Glc_4	5	10 - 200	2.76	0.33	y = 0.0144x - 0.0557	0.999
Glc_5	10	20 - 150	2.79	3.50	y = 0.0111x - 0.06	0.998
Glc_6	10	50 - 200	5.68	1.36	y = 0.007x + 0.0075	0.989
20 % (v/v) [DMIM]DMP						
Glc	< 5	5 - 100	1.03	0.41	y = 0.039x + 0.1677	0.999
Glc_2	< 5	5 - 100	1.89	0.94	y = 0.0248x + 0.0928	0.999
Glc_3	5	10 - 300	9.04	0.98	y = 0.0159x + 0.0422	0.998
Glc_4	5	20 - 200	4.74	3.60	y = 0.0135x - 0.041	0.999
Glc_5	5	20 - 200	4.98	2.50	y = 0.0098x - 0.0047	0.999
Glc_6	5	50 - 200	9.36	3.41	y = 0.0068x + 0.0123	0.996
40 % (v/v) [DMIM]DMP						
Glc	< 5	5 - 50	2.50	0.94	y = 0.0369x + 0.1084	0.997
Glc_2	< 5	5 - 300	5.09	1.37	y = 0.0247x + 0.0728	0.996
Glc_3	5	10 - 300	10.1	1.46	y = 0.0146x + 0.0107	0.989
Glc_4	5	10 - 200	8.35	0.53	y = 0.011x + 0.0267	0.996
Glc_5	5	20 - 150	8.82	10.3	y = 0.0087x + 0.0228	0.995
Glc_6	5	50 - 150	2.50	9.02	y = 0.0065x + 0.0446	0.993
20 % (v/v) [EMIM]AcO						
Glc	< 5	5 - 100	1.25	0.47	y = 0.0385x + 0.0513	1
Glc_2	< 5	5 - 300	7.10	1.08	y = 0.0273x - 0.0659	0.998
Glc_3	5	50 - 300	2.74	2.19	y = 0.0175x - 0.0909	0.997
Glc_4	5	50 - 200	3.59	4.05	y = 0.0136x - 0.0449	0.998
Glc_5	20	50 - 200	2.42	5.01	y = 0.0095x + 0.0271	0.999
Glc_6	20	50 - 200	2.50	1.56	y = 0.0071x + 0.0182	0.999

Table 1. Quantification and regression data for cellooligomers ranging from glucose to cellohexaose (saccharide labelling as in Fig 1) in four different matrices. Data has been acquired by six independent injections of the standard solution. LOD = Limit of Detection, LR = Linearity Range, RSD(low) relative standard deviation for the response factor at lowest determined point on calibration line, RSD(high) relative standard deviation of the response factor at highest determined point on calibration line, R^2 = regression coefficient. The response factor is defined as the parameter ratio y/x.

Substrate	Matrix	pH	Products (mg/L)				
			Glc	Glc_2	Glc_3	Glc_4	Glc_5
Glc_2	C	5.0	146.9	Traces			
Glc_3	C	5.0	142.9				
Glc_4	C	5.0	165.6				
Glc_5	C	5.0	172.9				
Glc_6	C	5.0	167.6				
Glc_5	P	6.0	112.0				35.9
Glc_5	20 m-% [EMIM]AcO+P	6.6	Traces			Traces	69.6
Glc_5	40 m-% [EMIM]AcO+P	7.4					83.2
Glc_2	20 m-% [DMIM]DMP+C	5.4	120.4				
Glc_5	20 m-% [DMIM]DMP+C	5.4	135.2				
Glc_2	40 m-% [DMIM]DMP+C	6.3	45.8	46.2			
Glc_5	40 m-% [DMIM]DMP+C	6.3	43.4		Traces	17.9	45.6
Glc_2	20 m-% [DMIM]DMP+P	6.6	161.9	16.8			
Glc_5	20 m-% [DMIM]DMP+P	6.6	89.7	4.5	Traces	11.3	6.9
Glc_2	40 m-% [DMIM]DMP+P	7.2	25.4	60.2			
Glc_5	40 m-% [DMIM]DMP+P	7.2	17.0			Traces	57.4

Table 2. Oligosaccharide composition and pH value of β-glucosidase hydrolysates in buffer or in ionic liquid containing matrices (C = 0.050 M citrate buffer (pH 5.0) and P = 0.100 M phosphate buffer (pH 6.0)).