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Analysis of carbohydrates in wood and pulps employing enzymatic hydrolysis and subsequent capillary zone electrophoresis

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Abstract

An efficient method for determining the carbohydrate composition of extractive-free delignified wood and pulp is described here. The polysaccharides in the sample are first hydrolyzed using a mixture of commercially available preparations of cellulase and hemicellulase. The reducing saccharides in the hydrolysate thus obtained are subsequently derivatized with 4-aminobenzoic acid ethyl ester and thereafter quantitated by capillary zone electrophoresis (CZE) in an alkaline borate buffer with monitoring of the absorption at 306 nm. All reducing sugars (i.e., neutral monosaccharides and uronic acids) which occur as structural elements in the polysaccharides of wood and pulp can be quantitated in a single such analytical run, which can also determine the contents of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid (HexA) residues present in pulps obtained from alkaline processes. CZE analyses were performed using linear regression of standard curves over a concentration range spanning approximately three orders of magnitude. Carbohydrate constituents constituting approximately 0.1% of the dry mass of the sample could be quantitated. The overall precision of this analytical procedure – involving enzymatic hydrolysis, derivatization and CZE – was good (RSD=2.2–7.5%), especially considering the heterogeneity of the wood and pulp samples. The total yield of carbohydrates (93–97%) obtained employing the procedure developed here was consistently higher than that obtained upon applying the traditional procedure for carbohydrate analysis (85–93%) (involving acid hydrolysis and gas chromatographic analysis) to the same pulps. The trisaccharide HexA-xylobiose was the only HexA-containing saccharide detected using the conditions for enzymatic hydrolysis developed here (i.e., 30 h incubation at pH 4 and 40°C); whereas mixtures of HexA-xylobiose and HexA-xylotriose were obtained when the incubation was performed at pH 5 or 6. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Chemical and mechanical pulping of wood constitutes the major supply of fiber for the manufacture of paper worldwide. Polysaccharides, i.e., cellulose and different hemicelluloses, are the primary constituents of wood and wood pulps [1]. Cellulose is a homopolymer of D-glucose units, joined by β-(1-4) glycosidic linkages, whereas the hemicelluloses are heteroglycans containing several different types of neutral (pentose and hexose) and acidic (uronic acid) monosaccharides as structural elements [1].

During chemical pulping, the uronic acid residues originally present in hemicelluloses become chemi-

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cally modified and, to various extents, destroyed. Unsaturated uronic acid residues, i.e., 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid (HexA), are formed from 4-*O*-methyl-D-glucuronic acid units (4-*O*-MeGlcA) under the conditions employed in connection with alkaline pulping processes [1,2]. Consequently, determination of the complete carbohydrate composition of such materials requires an analytical procedure capable of quantitating all neutral monosaccharide and uronic acid (both ''native'' and chemically modified) residues, preferentially in a single analytical run.

In a preliminary report, we have described a successful approach towards the development of such an analytical procedure applicable to chemical pulp [3]. This present paper describes the full experimental details concerning this method for analysis of the carbohydrates in extractive-free delignified wood and pulps.

Traditionally, determination of the carbohydrate composition of extractive-free wood and pulp is accomplished by initially swelling and dissolving the ground-up material in strong sulfuric acid. After diluting this mixture with water, a second hydrolysis is performed at elevated temperature [4]. After subsequent derivatization to their corresponding alditol acetates, the monosaccharides released by this two-stage acid hydrolysis are usually separated and quantitated by gas chromatography (GC) [5,6]. This approach has been adopted as the procedure for carbohydrate analysis by the Technical Association of the Pulp and Paper Industry [7]. Such two-stage acid hydrolysis has also been combined with subsequent determination by ion-exchange chromatography [8–14].

Although widely utilized, this acid hydrolysis destroys the monosaccharides released to various extents [4,5,15], while achieving the desired hydrolytic cleavage of glycosidic bonds. Under the conditions applied, uronic acid residues are released to only a minor extent, because of the relatively high stability of glycosidic bonds involving this sugar. Consequently, the disaccharide 4-*O*-methyl-glucuronosyl-xylose can be detected after acid hydrolysis [16]. On the other hand, unsaturated uronic acid residues (HexA) are destroyed completely by the acid hydrolysis [17,18] and can thus not be analyzed by this procedure. Therefore, an improved procedure for hydrolyzing wood and pulp is desirable.

Glycosidic bonds involving uronic acids are more efficiently cleaved by methanolysis, employing a strong acid in anhydrous methanol, than they are by acid hydrolysis. Decomposition of the uronic acid released is also less extensive during such methanolysis [15]. Therefore, it is tempting to apply methanolysis for carbohydrate analysis of cellulose-containing materials [19]. However, a recent study demonstrated that the carbohydrate yields obtained from wood and pulp samples following methanolysis are low, i.e., 13–32% [20]. Only hemicelluloses and pectins in the samples analyzed were depolymerized to monosaccharide methylglycosides; whereas the cellulose component remained largely unaffected by the methanolysis procedure [20]. Furthermore, HexA residues present in the kraft pulp could not be analyzed by this technique [20].

Enzymatic hydrolysis is an established approach to determining the structure of polysaccharides [21] and the modes of action of various enzymes on cellulose-containing fibers and on hemicelluloses have been investigated in detail [22–24]. Enzymatic hydrolysis in combination with subsequent quantitation by liquid or ion-exchange chromatography has been used to characterize different hemicelluloses originating from wood [25–27], as well as to analyze carbohydrates in kraft pulp [2,18,28]. This approach has also been employed to determine uronic acid and hexenuronic acid residues in kraft pulps [29,30].

We have previously described the application of capillary zone electrophoresis (CZE) for analysis of carbohydrates obtained by enzymatic hydrolysis of hemicelluloses prepared from mechanical [31,32] and chemical pulps [32–34]. CZE has also been used in conjunction with acid hydrolysis to analyze the carbohydrate contents of plant materials [35–37], wood hemicelluloses [32,38] and materials in effluents from kraft pulp bleaching [39]. Recently, El Rassi [40,41] and Novotny [42] have reviewed the progress made in applying CZE to carbohydrate analysis.

The present paper describes the development of a procedure for carbohydrate analysis based on enzymatic hydrolysis and subsequent CZE, a procedure which has proven to be very useful and which is frequently requested by customers of our service

laboratory. This method is applicable to extractive-free delignified wood and pulps. It quantitates all of the monosaccharides present as structural elements in the samples analyzed, i.e., both the neutral (pentose and hexose) and the acidic (uronic acid and HexA) residues. Optimal conditions for the enzymatic hydrolysis were developed using a fractional factorial experimental design. A modified CZE procedure was employed in order to achieve rapid separation and quantitation, in a single run, of the reducing saccharides present in the enzymatic hydrolysates.

2. Experimental

2.1. Reagents

All reagents used were of analytical grade. 4-Aminobenzoic acid ethyl ester (ABEE) and sodium cyanoborohydride (employed in the reductive amination of saccharides prior to analysis by CZE) were obtained from Aldrich (Milwaukee, WI, USA). Water for the preparation of reagents and buffer solutions was purified utilizing a Millipore Milli-Q Plus apparatus (Millipore, Milford, MA, USA). The PD-10 gel filtration columns were purchased from Pharmacia Biotech (Uppsala, Sweden).

2.2. Reference compounds

Commercially available monosaccharides and uronic acids were procured from Sigma (St. Louis, MO, USA), Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany). 4-*O*-Methylglucuronic acid (4-*O*-MeGlcA) was synthesized by Christian Krog-Jensen at the Stockholm University. The trisaccharide containing 4-deoxy-L-*threo*-hex-4-enopyranosyl-uronic acid β-linked to xylobiose, i.e., β-L-HexA*p*(1→2)-β-D-Xyl*p*(1→4)-D-Xyl (denoted HexA-xylobiose), was obtained in a manner similar to that described previously by Teleman et al. [28]. In short, a hexenuronoxylan extracted with dimethylsulfoxide from unbleached softwood kraft pulp [43] was hydrolyzed with a mixture of cellulases and hemicellulases as described below in Section 2.6. Subsequently, the trisaccharide HexA-xylobiose was isolated from the hydrolysate obtained by selective adsorption and desorption using ion-exchange columns. First, this hydrolysate was run through a column containing a cation-exchange resin (Amberlite IR-120, H^+ form) to yield the desired acid saccharide in its protonated form. This acid saccharide was then adsorbed onto an anion-exchange resin (IRA-93, carbonate form) and neutral saccharides removed from the column by washing with water. Thereafter, the acid saccharide was released from the column by elution with aqueous acetic acid. Lyophilization of this eluate yielded the product HexA-xylobiose as a white powder. The data obtained by ^{1}H-nuclear magnetic resonance (NMR) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the isolated HexA-xylobiose were as reported earlier [28,34].

2.3. Buffer solutions

The buffer solutions employed for enzymatic hydrolysis were prepared by dissolving sodium acetate in water to give a concentration of 50 mmol l^{-1}, followed by the addition of enough acetic acid to obtain the desired pH. The running electrolyte buffer for CZE was an aqueous solution of sodium hydroxide (300 mmol l^{-1}) and boric acid (438 mmol l^{-1}). The dilution buffer was similar, except that the final concentrations of sodium hydroxide and boric acid were 125 mmol l^{-1} and 438 mmol l^{-1}, respectively.

2.4. Enzymes

Commercially available solutions of cellulases and hemicellulases (Celluclast 1.5 L, Novozym 188 and Novozym 431 obtained from Novo Nordisk, Bagsvaerd, Denmark) were employed throughout this investigation. According to the supplier, the Celluclast 1.5 L preparation (approximately density of 1.2 g ml^{-1}) had an activity of 1500 Novo Cellulase units per gram (NCU g^{-1}); while Novozym 188 had an activity of 250 cellobiose units per gram liquid (CbU g^{-1}). The Novozym 431 preparation was stated to have 500 units of xylanase (XYU) and 200 units of endoglucanase (EGU) activity per gram liquid. Prior to enzymatic hydrolysis of the wood and pulp samples (described in Section 2.6 below), the solutions of Celluclast 1.5 L, Novozym 188 and

Novozym 431 were further purified by gel filtration and thereafter mixed in the volume ratio of 1:2:2. This purification step was performed on a PD-10 gel filtration column with water as the eluent. This gel filtration procedure diluted the enzyme preparations to approximately twice their original volumes. The enzyme mixture thus purified was stored at 4°C for not longer than 1 week prior to use.

2.5. Samples

Wood chips and pulp samples were first air-dried and thereafter ground down to fine powders (40 mesh) in a Wiley laboratory mill. The chemical pulps were analyzed without prior extraction and delignification; whereas such pretreatment was necessary in the case of wood and mechanical pulp samples. The ground-up wood and mechanical pulp were extracted with acetone at room temperature and then delignified using an aqueous solution of sodium chlorite and acetic acid. This chlorite delignification was carried out using a general laboratory procedure, described previously [44]. All extractive-free and delignified samples were dried at a temperature of 40°C overnight and their dry masses determined prior to analysis. These dry mass values were later used to calculate the carbohydrate contents.

2.6. Enzymatic hydrolysis

To 10 mg dry extractive-free and delignified wood or pulp were added 0.5 ml of the purified enzyme mixture and 0.5 ml sodium acetate buffer, pH 4.0. The resulting suspension was stirred for 30 h at 40°C to allow complete hydrolysis of the polysaccharides present. This hydrolysate was supplemented with 0.040 ml of a ribose solution (10 mg ml^{-1}) as internal standard for quantitation by CZE. Aliquots (200 μl) of the resulting solution were removed for subsequent derivatization and analysis. This saccharide solution could be stored for long periods of time at −15°C without affecting the analytical results subsequently obtained.

2.7. Derivatization

The procedure for reductive amination of saccharides suggested by Vorndran et al. [35] was employed, with slight modification designed to remove excess ABEE reagent prior to analysis by CZE. The stock solution was prepared by dissolving ABEE (100 mg ml^{-1}) and acetic acid (100 mg ml^{-1}) in methanol. Immediately prior to use, 10 mg sodium cyanoborohydride was added to 1 ml of this stock solution to obtain the ABEE reagent solution.

A 200-μl aliquot of the enzymatic hydrolysate (containing approximately 10 mg saccharide ml^{-1}) and 240 μl of the reagent solution were mixed in a glass tube with a plastic screw cap. The cap was then screwed on and the tube placed in a heating block and maintained at 80°C for 60 min. Thereafter, the mixture was removed from the heating block, immediately diluted with 200 μl alkaline borate buffer (pH 8.5) and vortex-mixed vigorously for several seconds. During this step, excess ABEE reagent precipitated in the form of a fine white powder. After cooling the sample to room temperature, this precipitate was removed using a membrane filter (Millipore). The resulting clear, colorless solution was then analyzed by CZE within 24 h, in order to minimize degradation of the derivatized saccharides.

2.8. Capillary zone electrophoresis

In this study analysis by CZE was performed with two different instruments. Most of the analyses were carried out employing a Dionex capillary electrophoresis system (designated as CZE system A) equipped with a variable-wavelength UV–Vis detector (Dionex, Sunnyvale, CA, USA). Absorption of the derivatized saccharides at 306 nm was determined with this detector placed at the cathode 5 cm from the end of the capillary column. The uncoated fused-silica capillary had a total length of 44 cm and an inner diameter of 30 μm. Injection was performed in the hydrodynamic mode with the sample vial being held at an elevation of 75 mm for 10 s. The power applied to obtain separation was 1200 mW. Data sampling and peak integration were achieved using EZ-CHROM chromatography software (Nordlab, Stockholm, Sweden).

The second CZE system employed was a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) (designated as system B) equipped with a diode-array UV detector. In this case as well, absorption at 306 nm

was used for quantitation of the derivatized saccharides. The capillary column was the same as the one employed in system A, except that its total length was 40 cm and on-column detection was performed 10 cm from the cathode end. Injections were performed by applying 0.3 p.s.i. pressure for 5 s (1 p.s.i.=6894.76 Pa). The power used for separation was 2500 mW and the capillary column was thermostated at 20°C. Data sampling and peak integration were achieved using the Beckman chromatography software.

2.9. The fractional factorial experimental design

Experimental design based on the fractional factorial approach was performed with the MODDE 4.0 software from Umetri (Umeå, Sweden).

2.10. MALDI-TOF-MS

MALDI-TOF-MS was carried out in the positive mode on a Hewlett-Packard G2025 A MALDI-TOF system, with 1–5 μJ energy pulses of UV light (337 nm) from the laser beam and using 2,5-dihydroxybenzoic acid (DHB) as the matrix. To a 1% (w/v) solution of DHB in methanol, an equal volume of the enzymatic hydrolysate was added. After mixing, approximately 0.5 μl of the resulting solution was pipetted onto the MALDI probe and the solvent then evaporated. In this manner, the probe surface was covered with a thin layer of matrix crystals, with the sample distributed within the matrix. Routinely, 30–100 single pulse raw spectra were averaged and transformed into a MALDI spectrum.

2.11. Comparison to carbohydrate analysis employing acid hydrolysis and gas chromatography

For purposes of comparison, carbohydrate analysis on selected samples was also carried out according to a reference procedure utilized in our laboratory. This method involves hydrolysis with sulfuric acid, followed by derivatization to alditol acetates and gas chromatographic quantitation. It is essentially the same as the procedure described by Sjöström et al. [5], except that it involves the modified acetylation step (using 1-methylimidazole as catalyst) suggested by Theander and Westerlund [45]. Gas chromatographic separation of alditol acetates is achieved on a fused-silica capillary column (SGE Analytical Products, BP225, 12 m) having an inner diameter of 0. 32 mm and a film thickness of 0.25 μm.

3. Results and discussion

3.1. Enzymatic hydrolysis

The physical and biochemical factors to be considered in connection with the enzymatic degradation of wood fibers have been reviewed in several articles (see Ref. [46] and references therein). One prerequisite for achieving successful enzymatic hydrolysis is that the polysaccharides in the wood or pulp are sufficiently accessible to enzymatic attack, i.e., not embedded in lignin or lipophilic extractives. The microstructure and porosity of the wood material are major determinants of this accessibility [46]. Grinding up or milling the sample increases the surface area, thereby facilitating enzymatic hydrolysis [46,47]. Pretreatment designed to loosen the fiber matrix and increase its porosity also facilitates the enzymatic degradability of wood fibers [46,48]. Finally, enzymatic hydrolysis is improved by a high degree of swelling and a low degree of crystallinity in the cellulose [24,47,49].

Chemical pulping results in removal of most of the lignin and extractives from the fibers and enhances swelling and porosity of the fibers. Consequently, the cellulose and hemicelluloses in chemical pulps can be effectively hydrolyzed to their monosaccharide components by enzymes. In contrast, HexA residues bound to the xylan present in pulps obtained using alkaline processes cannot be released by cellulases and hemicellulases, since these enzymes cannot cleave the β-L-(1-2)-glycosidic bond which links these HexA residues to the xylan backbone. Consequently, two HexA-containing oligosaccharides, i.e., HexA-xylobiose and HexA-xylotriose [β-L-HexA-*p*(1→2)-β-D-Xyl*p*(1→4)-D-Xyl and β-L-HexA-*p*(1→2)-β-D-Xyl*p*(1→4)-β-D-Xyl*p*(1→4)-D-Xyl, respectively], have been detected upon ion-exchange chromatographic analysis of enzymatic hydrolysates of kraft pulps [29,30]. In this case, HexA residues were quantitated as the sum of HexA-xylobiose and HexA-xylotriose [30].

In an earlier study on birch kraft xylans, we detected β-L-HexA*p*(1→2)-β-D-Xyl*p*(1→4)-D-Xyl (HexA-xylobiose) as the only HexA-containing product after hydrolysis with hemicellulases [34]. For purposes of analytical detection and quantitation, it is, of course, advantageous to obtain a single product containing HexA residues. Therefore, we looked for conditions under which enzymatic hydrolysis resulted in HexA-xylobiose as the only HexA-containing product, as well as in optimal yields of the other neutral monosaccharides and ''native'' uronic acids.

Five-factor two-level fractional factorial experimental design (2^5-FED) [50,51] was employed to optimize the enzymatic yields of the monosaccharides and HexA-xylobiose from an unbleached softwood kraft pulp. The experimental factors involved were length of hydrolysis, temperature, pH and the concentrations of enzymes and pulp (see Table 1).

In a fractional factorial experiment involving two values for each parameter examined, the values selected must be sufficiently different to clearly reveal the effect of the change [50]. At the same time, the values selected should not be so different as to fall outside of the normal acceptable range for the parameter in question. Hydrolysis of glycosidic bonds catalyzed by cellulolytic enzymes is usually performed using suspensions containing 1–10% (w/v) of the substrate and within the pH range of 4–9 (preferably at pH 5) [24]. We selected values for the pH and temperature that covered the ranges recommended by the supplier for the three different enzyme preparations (Celluclast 1.5 L, Novozym 188 and Novozym 431) used here. The values selected for the different parameters in our 2^5-FED experiment are presented in Table 1. Quantitation of the saccharides in the hydrolysates obtained was subsequently performed employing the CZE procedure described below in Sections 3.2 and 3.3.

This fractional factorial experiment demonstrated that the incubation temperature had a marked influence on the yields of all of the saccharides investigated. Fig. 1A is a diagram of the scaled and centered coefficients for the total yield of carbohydrates, calculated from the 2^5-FED using the MODDE software. As can be seen from this diagram, although the incubation temperature had a pronounced effect on this yield, changes in the enzyme concentration and length of hydrolysis exerted only a minor influence and the other parameters investigated were without significant effect.

Thus, incubation of the softwood pulp at the lower

Table 1
Parameters varied in our fractional factorial experimental design

Parameter	Value	
	Low	High
Length of hydrolysis (h)	6	30
Temperature (°C)	40	65
pH	4	6
Enzyme concentration (%, v/v)	5	50
Pulp concentration (mg ml^{-1})	2	10

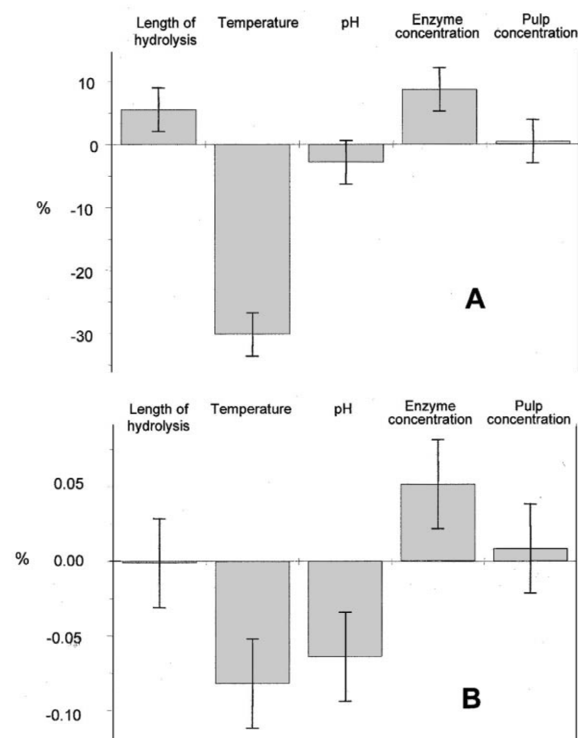

Fig. 1. The scaled and centered coefficients for (A) the total yield of carbohydrates and (B) the yield of HexA-xylobiose, as calculated from the five-factor two-level fractional factorial experimental design. The bars indicate the magnitude of the influence of each parameter on the yield. A negative bar shows that the yield was higher when the lower value of the parameter was used. The 95% confidence interval is indicated for each parameter.

temperature (40°C) resulted in much higher yields of monosaccharides and HexA-xylobiose than did treatment at the higher temperature (65°C). The total carbohydrate yield varied between 85 and 95% when incubation was performed at 40°C using a high concentration of enzyme, compared to only ≤35% when performed at 65°C. The pH was found to be of minor significance for the yields of the neutral monosaccharides and of 4-*O*-MeGlcA. In contrast, in the case of HexA-xylobiose, evaluation of the 2^5-FED experiment (Fig. 1B) demonstrated clearly that, in addition to the incubation temperature, variations in the pH and enzyme concentration affected the yield significantly. According to the parameter evaluation illustrated in Fig. 1B, a low pH and high enzyme concentration increased the yield of this product.

In Fig. 2A, the total yields of carbohydrates and HexA-xylobiose obtained from the softwood pulp are plotted as a function of the pH employed during enzymatic hydrolysis. In this case the other experimental parameters were maintained constant at optimal levels (i.e., a temperature of 40°C, high concentrations of enzymes and pulp, and a hydrolysis time of 30 h). Fig. 2A is based on linear regression analysis of the data obtained from the 2^5-FED experiment. As shown, the highest yield of HexA-xylobiose was obtained after incubation at pH 4. This yield decreased by about 70% when the pH was increased to 6. The total carbohydrate yield was, however, high (approximately 95%) in both cases, i.e., this yield was largely independent of the pH within this range.

In order to confirm the 2^5-FED calculations illustrated in Fig. 2A, a series of enzymatic hydrolyses were carried out on the softwood kraft pulp (see Fig. 2B). In these experiments only a single parameter, the pH, was varied, whereas the other parameters were maintained constant at their optimal levels (i.e., the same levels as those used in constructing Fig. 2A). Fig. 2B reveals a dramatic increase in the yields of HexA-xylobiose when the pH was lowered from 6 to 4. Only minor variations in the total carbohydrate yield were observed within this same pH range. Thus, these empirical findings confirmed the conclusions drawn earlier from the 2^5-FED experiment.

The pronounced effect of pH on the yield of HexA-xylobiose after enzymatic hydrolysis was

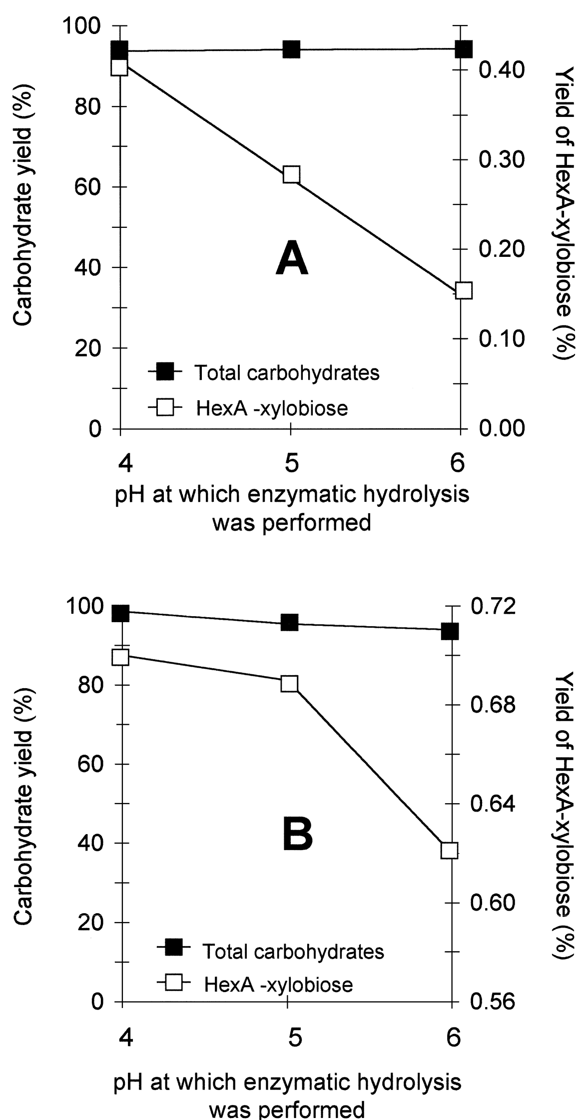

Fig. 2. Total carbohydrate yield and the yield of HexA-xylobiose after enzymatic hydrolysis of a softwood kraft pulp as a function of the incubation pH. (A) Results calculated from the data obtained from the five-factor, two-level fractional factorial experiment. (B) Empirical results obtained after enzymatic hydrolysis at different pH values.

quite surprising. To our knowledge, no such effect has been reported in previous investigations on degradation of kraft pulps by similar enzymes. In order to obtain additional information concerning the influence of pH and enzyme concentration on the formation of HexA xylooligosaccharides, further

experiments using a pure hexenuronoxylan (isolated after extraction from an unbleached hardwood kraft pulp) were carried out. This xylan was incubated with our enzyme mixture at two different enzyme concentrations and three different pH values (4, 5 and 6). After a 30-h incubation at 40°C, the levels of HexA-xylobiose and HexA-xylotriose in the hydrolysates were determined using MALDI-TOF-MS. Employing positive ion MALDI-TOF-MS, the sodium adduct ions $(M+Na)^+$ of these two HexA-oligosaccharides can be detected readily [34]. These MALDI-MS analyses provided further evidence for the presence of HexA-xylobiose and HexA-xylotriose in the hydrolysates, which were analyzed by CZE as well.

The MALDI spectra obtained with three different hydrolysates are shown in Fig. 3. Results from hydrolysis using a low enzyme concentration and pH 6 or 5 are illustrated in the spectra denoted A and B, respectively; while the corresponding spectrum with pH 4 and high enzyme concentration is presented in C. In the mass spectra in Fig. 3, the peaks appearing at 463 and 595 mass units originate from HexA-xylobiose and HexA-xylotriose, respectively. As is evident, HexA-xylotriose is the predominant HexA-containing saccharide present after hydrolysis using a low enzyme concentration and pH 6 (spectrum A). In contrast, HexA-xylobiose is the only HexA-containing saccharide detectable in the corresponding hydrolysate with high enzyme concentration and pH 4 (spectrum C). Incubation of the xylan with a low enzyme concentration at pH 5 yielded both of these saccharides in almost equal amounts (spectrum B).

In Fig. 4 the yields of HexA-xylobiose and HexA-xylotriose (determined by CZE as their ABEE derivatives) after hydrolysis with high or low concentrations of enzymes are plotted as a function of pH. Diagram A presents the yields of HexA-xylobiose with low (unfilled squares) and high (filled squares) enzyme levels, while B depicts the corresponding data for HexA-xylotriose. The results in Figs. 3 and 4 together demonstrate that HexA-xylobiose was the only HexA-containing product when hydrolysis was carried out at pH 4 in the presence of a high concentration of enzymes. Under all other conditions investigated, mixtures of both HexA-xylooligosaccharides were produced.

In combination, the experiments performed here

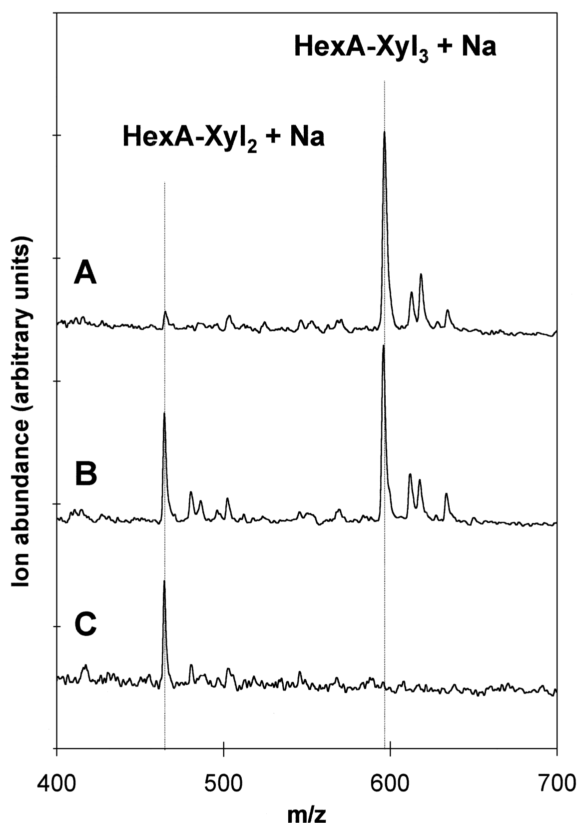

Fig. 3. Positive-ion MALDI-TOF-MS spectra obtained after enzymatic hydrolysis of hexenuronoxylan isolated from a hardwood kraft pulp. The peaks appearing at 463 and 595 mass units originate from HexA-xylobiose (HexA-Xyl_2) and HexA-xylotriose (HexA-Xyl_3), respectively. The first two spectra correspond to hydrolysis employing a low enzyme concentration and pH 6 (A) or pH 5 (B). Spectrum C represents hydrolysis performed at pH 4 with a high enzyme concentration.

on kraft pulps and an isolated hexenuronoxylan indicated the conditions for optimal enzymatic hydrolysis in connection with the procedure for carbohydrate analysis being developed. As shown below in Sections 3.3 and 3.4, these conditions provided good yields of monosaccharides and HexA-xylobiose from the soft- and hardwood chemical pulps investigated here.

As discussed above, one prerequisite for effective enzymatic hydrolysis of carbohydrate components of wood is that these polysaccharides are relatively accessible to the enzymes employed. Wood samples require pretreatment – e.g., milling, delignification or

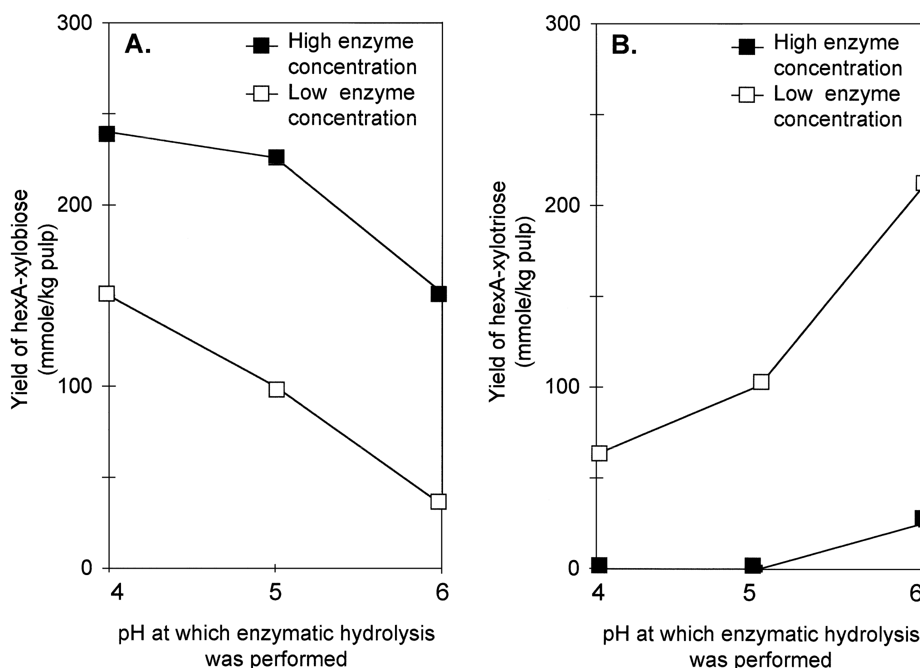

Fig. 4. Yields of HexA-xylobiose and HexA-xylotriose determined by CZE after enzymatic hydrolysis of a hexenuronoxylan from a hardwood kraft pulp at different pH values. (A) Yields of HexA-xylobiose in the presence of low (unfilled squares) and high (filled squares) concentrations of enzymes; (B) corresponding findings for HexA-xylotriose.

acidic prehydrolysis [46,52] – in order to provide such accessibility. Consequently, when wood and mechanical pulp samples which had been milled, but not delignified, were incubated with our enzyme mixture under the conditions defined above, only low yields of monosaccharides were obtained (i.e., total carbohydrate yields of 5–15%). In contrast, milled, extractive-free and chlorite delignified samples of wood and mechanical pulp could be hydrolyzed enzymatically to produce their neutral and acidic monosaccharide constituents in good yields (i.e., total carbohydrate yields of 75–100%, see Section 3.5).

3.2. Derivatization and capillary zone electrophoresis

The use of reductive amination to ‘‘tag’’ reducing saccharides with a UV chromophoric group prior to separation and detection has become one of the most popular derivatization procedures in connection with analysis of mono- and oligosaccharides by CZE [40,41,53]. In an earlier study, we derivatized neutral and acidic mono- and oligosaccharides from wood with 6-aminoquinoline and then employed CZE to separate the derivatives obtained (as their borate complexes) [32]. Although labeling with 6-aminoquinoline allowed the saccharides to be detected with high sensitivity, this reagent is somewhat unstable and tends to decompose during storage. Furthermore, we had difficulties in obtaining commercial preparations of this reagent which are sufficiently pure for CZE analysis. Therefore, in the present study we utilized ABEE instead as the chromophoric reagent to derivatize the saccharides obtained from wood and pulp samples. This reagent is readily available in pure form from several different suppliers.

The application of ABEE in connection with carbohydrate analysis by CZE was originally suggested by Vorndran et al. [35] and this reagent has since been employed in several such studies [37,54–57]. In order to obtain reproducible derivatization, addition of a large excess of the ABEE reagent to the reducing saccharides is necessary [35]. In the origi-

nal procedure [35] methanol was added at the end of the derivatization step in order to prevent precipitation of remaining ABEE reagent during cooling of the reaction mixture. However, the presence of large quantities of methanol and ABEE may interfere with subsequent CZE analysis [56]. Therefore, two groups of investigators [56,57] have removed excess ABEE from the reaction mixture by solvent extraction prior to CZE. In the present study, selective precipitation and filtration was shown to effectively remove excess ABEE. Thus, after derivatization of the reducing saccharides, the reaction mixture was diluted with an alkaline borate buffer (pH 8.5) and then shaken vigorously for a few seconds. As a result, the excess ABEE precipitated in the form of a fine white powder. After cooling the mixture to room temperature, this precipitate could be removed using a membrane filter.

Use of an alkaline borate buffer in this connection is essential. If the reaction mixture is diluted with water alone, the ABEE derivative of glucose (i.e., the most abundant component in wood and pulp hydrolysates) precipitates together with remaining reagent, thereby interfering with quantitation of glucose. The alkaline borate buffer prevents such precipitation of the glucose derivative, probably by forming a soluble borate–saccharide complex. ABEE itself does not form such a complex and, thus, precipitates even in the alkaline borate solution.

A system involving a concentrated alkaline borate buffer is required to achieve efficient separation of the monosaccharides glucose, mannose and arabinose by CZE after derivatization using reductive amination [32]. These three monosaccharides are among the most abundant components of hydrolysates derived from polysaccharides in wood and pulp. The electropherogram in Fig. 5A depicts the separation obtained by CZE employing the borate buffer (175 mmol l^{-1} borate, pH 10.5) suggested earlier for use with ABEE derivatives [35,54,55]. This procedure resulted in little or no resolution of the ABEE derivatives of glucose, mannose and arabinose.

Reliable quantitation of these three monosaccharides in hydrolysates from wood and pulp by CZE requires their complete separation. By employ-

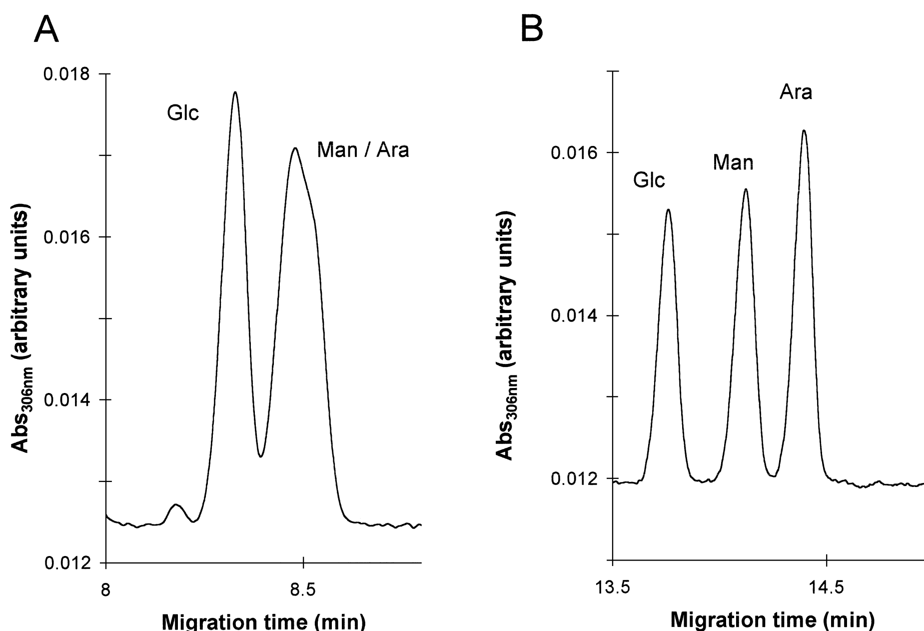

Fig. 5. Electropherogram illustrating separation of the ABEE derivatives of glucose (Glc), mannose (Man) and arabinose (Ara) by CZE. This separation was obtained using CZE system A with (A) 175 mmol l^{-1} borate, pH 10.5, or (B) 438 mmol l^{-1} borate, pH 11.5.

ing a more concentrated borate buffer (i.e., 438 mmol l^{-1}) and a pH of 11.5, such complete separation of the ABEE derivatives of glucose, mannose and arabinose by CZE could be accomplished (Fig. 5B).

Use of the more concentrated buffer increased the migration times for these saccharides. Fig. 6 illustrates an electropherogram (from migration times of 8–26 min) in which separation of these saccharides from wood and pulp (except for galacturonic acid, whose migration time in this system is 36 min) was successfully achieved. This electropherogram was obtained using CZE system A, the characteristics of which (including application of a constant power of 1200 mW) are documented in Table 2. The electrophoretic mobility, μ, and the resolution between two adjacent monosaccharide peaks, R_s, was calculated as described previously [32].

CZE system B was equipped with an efficient liquid cooling device to maintain the capillary column at a temperature of 20°C. This more efficient cooling allowed the application of a higher voltage (corresponding to a constant power of 2500 mW) across the capillary column without overheating. Fig. 7 depicts separation of the same saccharides shown in Fig. 6, except that in Fig. 7 CZE system B was employed.

The migration times for the saccharides derived from wood and pulp obtained with CZE system B were about half as long as those obtained with CZE system A (compare Figs. 6 and 7). In Table 2, migration times, electrophoretic mobilities and resolution between adjacent peaks are documented. The resolution was only slightly lower using the high-power CZE system B, with which migration times were considerably shorter than with the low-power CZE system A.

In conclusion, with the modified procedure employed for ABEE derivatization, together with the use of a concentrated alkaline borate buffer during separation by CZE, efficient and rapid analysis of all saccharides commonly produced by enzymatic hydrolysis of wood and pulp can be achieved. Separation and detection of the saccharides investigated here could be accomplished within a running time of <20 min using CZE system B.

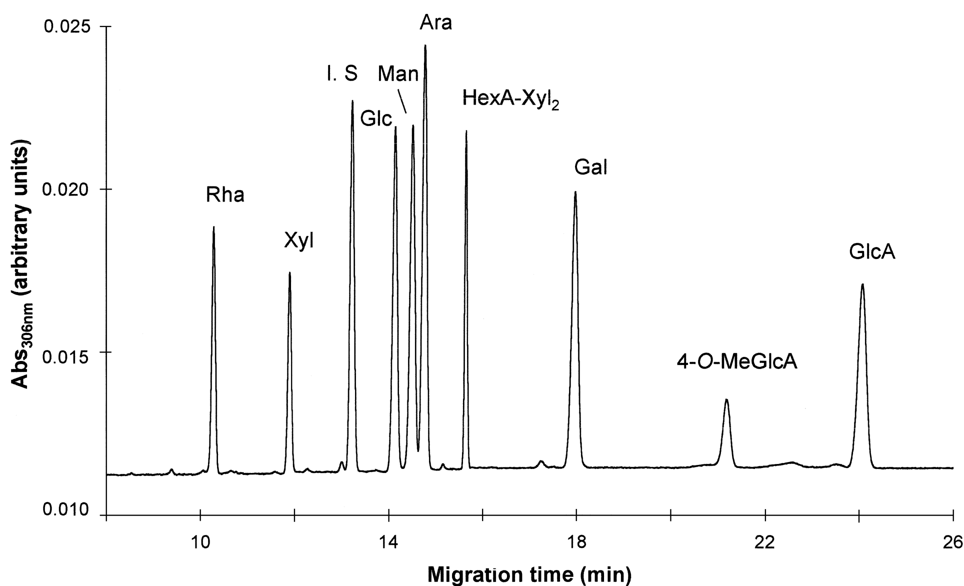

Fig. 6. Electropherogram (from migration times of 8–26 min) illustrating the separation of ABEE derivatives of saccharides commonly found in wood and pulp by CZE. This separation was obtained using CZE system A with 438 mmol l^{-1} borate, pH 11.5. Rha=Rhamnose, Xyl=xylose, I.S.=internal standard (ribose), Glc=glucose, Man=mannose, Ara=arabinose, HexA-Xyl$_2$=HexA-xylobiose, Gal=galactose, 4-*O*-MeGlcA=4-*O*-methyl-glucuronic acid, GlcA=glucuronic acid.

Table 2
Migration times, electrophoretic mobilities (μ) and resolution between adjacent peaks (R_s) obtained by capillary electrophoresis of the ABEE derivatives of the monosaccharides and HexA-xylobiose investigated[a]

Monosaccharide	Capillary electrophoresis, system A			Capillary electrophoresis, system B		
	Migration time (min)	μ (cm^2 V^{-1} s^{-1})$\cdot 10^{-4}$	R_s	Migration time (min)	μ (cm^2 V^{-1} s^{-1})$\cdot 10^{-4}$	R_s
Rhamnose	10.6	−1.21		5.3	−1.05	
			11.9			8.9
Xylose	12.2	−1.40		6.1	−1.23	
			9.0			6.5
Ribose	13.6	−1.53		6.8	−1.34	
			4.8			3.6
Glucose	14.4	−1.60		7.3	−1.40	
			2.2			1.2
Mannose	14.7	−1.62		7.4	−1.42	
			1.6			1.3
Arabinose	15.0	−1.64		7.6	−1.44	
			5.6			5.2
HexA-xylobiose	15.8	−1.69		8.0	−1.49	
			13.7			11.6
Galactose	18.0	−1.82		9.1	−1.59	
			17.4			12.0
4-*O*-Me-glucuronic acid	21.1	−1.95		10.8	−1.70	
			11.3			9.7
Glucuronic acid	23.8	−2.04		12.2	−1.77	
			72.6			28.7
Galacturonic acid	35.9	−2.26		18.8	−1.97	

[a] The conditions employed were as follows: 438 mmol l^{-1} borate buffer, pH 11.5; a fused-silica capillary column with I.D.=30 μm. CZE system A was operated applying a constant power of 1200 mW, while system B was operated with a constant power of 2500 mW and column cooling to 20°C.

3.3. Quantitation and repeatability

The precision of this procedure, including the derivatization step, with respect to eight monosaccharides investigated here is documented in Table 3. A calibration curve was constructed for the ABEE derivative of each monosaccharide by plotting the relative amount (y) as a function of the relative peak area (x) compared to the internal standard (i.e., the ABEE derivative of ribose). Linear calibration curves were obtained in the concentration range of $2\cdot10^{-6}$–$5\cdot10^{-3}$ mol l^{-1} for all saccharides, except for galactose, which gave a linear curve in the range of $2\cdot10^{-6}$–$0.2\cdot10^{-3}$ mol l^{-1}.

In wood and pulp samples glucose is the major saccharide constituent, accounting for as much as 70–80% of the total carbohydrate content of softwood. The analytical precision with respect to this component was therefore investigated for a range of concentrations between $9\cdot10^{-6}$ and $25\cdot10^{-3}$ mol l^{-1}. The pentose xylose, which is the second most abundant saccharide constituent of hardwoods, was also calibrated up to a concentration of $25\cdot10^{-3}$ mol l^{-1}.

In Table 3, regression equations (having the form $y=kx+m$) and regression coefficients (r^2) (obtained by least-squares linear regression analysis) for each saccharide are presented. In addition, the relative standard deviations (RSDs) in the determination of each saccharide at both low and high concentrations are provided. These values were obtained from six independent determinations, each including the ABEE derivatization. As seen from Table 3, quantitation is linear over a concentration range of three orders of magnitude. These results demonstrate that this CZE analysis provides good repeatability, in most cases also both at the high and low ends of the concentration range investigated.

In order to examine the precision with respect to the entire procedure for carbohydrate analysis, in-

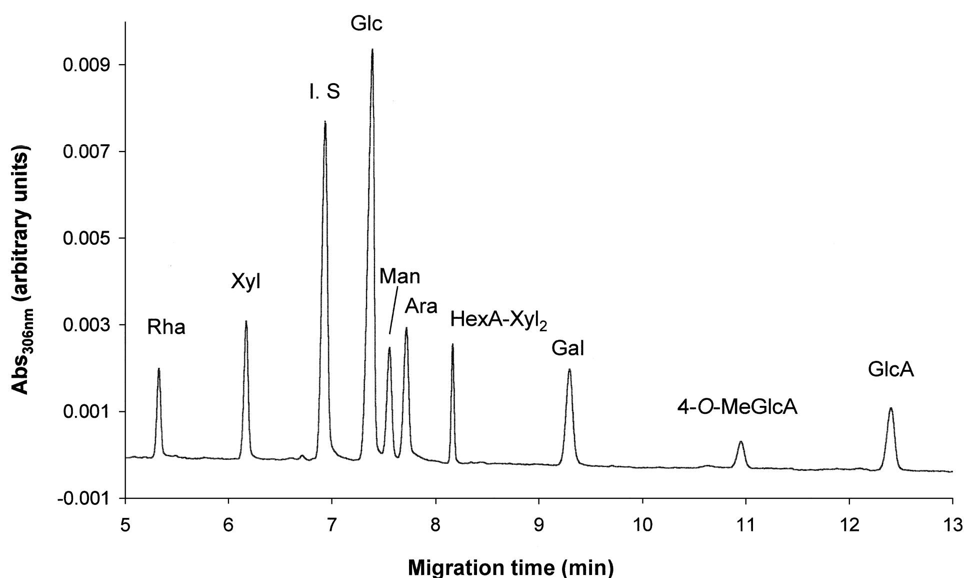

Fig. 7. Electropherogram (from migration times of 5–13 min) illustrating the separation of ABEE derivatives of saccharides commonly found in wood and pulp by CZE. This separation was obtained using CZE system B with 438 mmol l^{-1} borate, pH 11.5. The peaks are labeled as in Fig. 6.

cluding the enzymatic hydrolysis step, five chemical pulp samples were analyzed (Table 4). Four of these samples were unbleached or fully bleached softwood and hardwood pulps obtained from different Scandinavian kraft pulp mills. The fifth sample was an unbleached pulp from a Swedish sulfite pulp mill. For each sample, the entire analysis commencing with the enzymatic hydrolysis step was repeated six times.

In all cases total yield of carbohydrates was high, i.e., 93–98% of the dry mass of the samples, with the exception of the unbleached softwood kraft pulp. This sample was an unbleached pulp intended for liner board manufacturing which had a high content

Table 3
Repeatability of the CZE analytical procedure: relative standard deviations (RSDs) and regression equations and coefficients for six independent quantitations of eight monosaccharides (as their ABEE derivatives) in standard mixtures

Monosaccharide	Quantitation: RSD (%) at a concentration of		Regression	
	$2\cdot10^{-6}$ mol l^{-1}	$5\cdot10^{-3}$ mol l^{-1}	Equation ($y=kx+m$)	Coefficient (r^2)
Rhamnose	8.8	3.5	$y=1.497x-0.001$	0.997
Xylose	2.7	2.4[b]	$y=1.157x-0.096$	0.999
Glucose	4.4[a]	1.4[b]	$y=0.931x-0.109$	0.999
Mannose	4.9	5.4	$y=0.820x+0.026$	0.999
Arabinose	6.0	4.3	$y=0.825x+0.046$	0.999
Galactose	9.6	4.3	$y=0.652x+0.057$	0.998
Glucuronic acid	11.8	2.6	$y=0.935x+0.038$	0.995
Galacturonic acid	2.6	6.7[c]	$y=0.949x+0.011$	0.998

[a] $9\cdot10^{-6}$ mol l^{-1}.
[b] $25\cdot10^{-3}$ mol l^{-1}.
[c] $0.2\cdot10^{-3}$ mol l^{-1}.

Table 4
Carbohydrate analyses of five unbleached or bleached chemical pulps performed using enzymatic hydrolysis and subsequent capillary electrophoresis[a]

Sample	Saccharide % dry mass (RSD, %)							
	Xylose	Glucose	Mannose	Arabinose	Galactose	Hexanuronic acid (HexA)	4-*O*-Me-glucuronic acid	Total
Hardwood kraft pulp	25.4	70.1	1.0	n.d.	n.d.	1.3	0.4	98.2
Unbleached	(1.2)	(2.1)	(21.3)	–	–	(2.8)	(9.2)	(2.0)
Hardwood kraft pulp	24.0	71.7	0.9	n.d.	n.d.	0.6	0.3	97.6
Bleached	(2.0)	(2.5)	(30.8)	–	–	(2.9)	(3.9)	(2.6)
Softwood kraft pulp	8.5	77.8	6.0	0.7	0.3	0.4	0.1	93.8
Bleached	(2.5)	(2.8)	(5.1)	(5.1)	(5.6)	(3.4)	(14.2)	(2.9)
Softwood kraft pulp	8.9	64.3	6.2	1.0	0.5	0.9	0.4	82.3
Unbleached	(2.1)	(2.0)	(2.0)	(5.3)	(6.2)	(5.1)	(3.8)	(1.7)
Softwood sulfite pulp	4.7	76.4	11.5	n.d.	n.d.	n.d.	0.7	93.2
Unbleached	(3.1)	(3.9)	(4.6)	–	–	–	(6.4)	(3.9)
Average standard deviation (%)	2.2	2.7	2.9[c]	5.2	5.9	3.5	7.5	2.6

[a] The results and relative standard deviations are mean values of six independent analyses on each pulp sample.
n.d.=Not detectable, i.e., $<0.05\%$.
[c] Hardwood samples excluded.

of lignin, approximately 10% of the dry mass (i.e., one third of the lignin content originally present in softwood) (Table 4). The repeatability of the procedure is very good considering the heterogeneity of fibers present in commercial chemical pulps. This repeatability compares favorably to that reported previously for analysis involving acid hydrolysis in combination with gas chromatographic [6] or ion-exchange chromatographic separation and quantitation [13].

The average standard deviation of the values obtained for the seven neutral and acidic carbohydrates varied from 2.2 to 7.5%, if the determinations of mannose residues in the hardwood pulps are excluded. The low degree of precision of these latter measurements is due to the low mannose content in these hardwood pulps, as well as to the fact that the enzyme preparation employed contained a small amount of mannose, even after purification. For mannose determination in the case of the softwood samples, the contribution from the enzyme preparation was relatively small and could simply be subtracted. Accurate quantitation of each saccharide component was possible if it constituted at least 0.1% of the dry mass of the sample.

Fig. 8 depicts the electropherogram obtained in connection with CZE analysis of the saccharides present in the enzymatic hydrolysate from the bleached hardwood pulp sample. This electropherogram is shown at both high and low magnification. As can be seen, well-separated peaks were obtained, with sufficient sensitivity to allow the minor components to be quantitated with a good signal-to-noise ratio.

3.4. Comparison to the traditional procedure

As pointed out earlier, wood pulps are somewhat heterogeneous in composition. The carbohydrate composition of different types of fibers within the same species of wood may vary quite considerably [58]. Thus, carbohydrate analyses provide average values for the different components in all of the fiber types present in the sample. Reference materials of uniform and well-defined fiber composition are not available.

Therefore, the procedure for carbohydrate analysis developed here was compared to the traditional procedure involving acid hydrolysis and GC. The same four different chemical pulp samples – unbleached and bleached hardwood kraft, bleached softwood kraft and unbleached softwood sulfite pulps

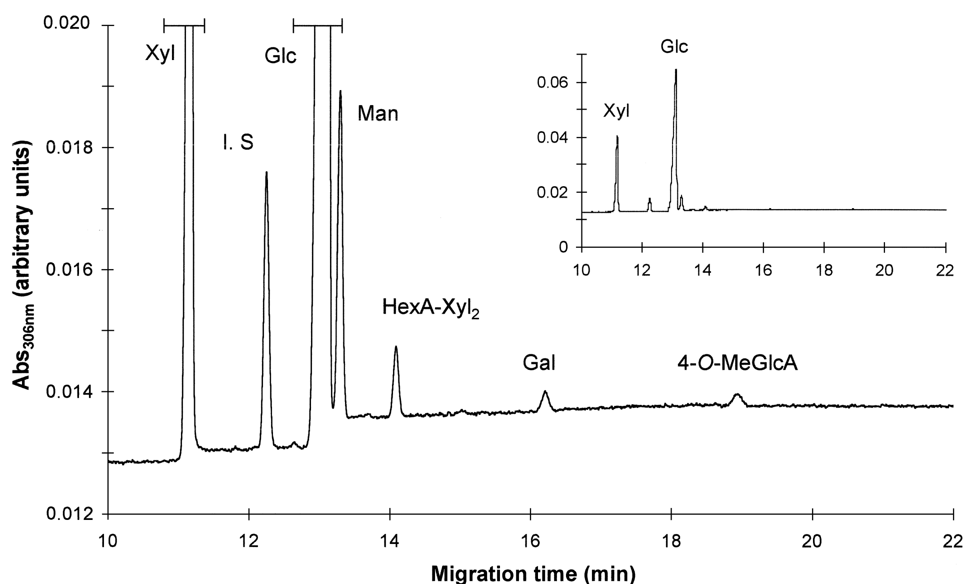

Fig. 8. Electropherogram illustrating separation of the ABEE derivatives of saccharides present in the enzymatic hydrolysate from the bleached hardwood pulp by CZE. This separation was obtained using CZE system A with 438 mmol l^{-1} borate, pH 11.5. The peaks are labeled as in Fig. 6.

– were analyzed using both of these procedures. Since the traditional procedure only measures the neutral carbohydrate components, acidic uronic acid components were excluded from this comparison.

A good correlation between the procedure developed here and the traditional procedure was obtained with respect to the levels of individual saccharides detected in each pulp sample. Least-squares linear regression analysis of these data gave the regression equation $y=1.0595x$ and coefficient $r^2=0.9979$. The slope of this regression equation indicates that the yields obtained employing the procedure developed here are consistently higher than those obtained applying the traditional procedure for carbohydrate analysis.

As evident from Table 5, the yields of xylose

Table 5

Comparison between carbohydrate analyses (neutral monosaccharides only) of pulps performed employing sulfuric acid hydrolysis–gas chromatography (traditional procedure) and enzymatic hydrolysis–capillary electrophoresis (the procedure developed here)

Anhydrosaccharide	Content (% of pulp dry mass)							
	Hardwood kraft pulp, unbleached		Hardwood kraft pulp, bleached		Softwood kraft pulp, bleached		Softwood sulfite pulp, unbleached	
	Acid hydrolysis and GC[a]	Enzymatic hydrolysis and CZE[b]	Acid hydrolysis and GC[a]	Enzymatic hydrolysis and CZE[b]	Acid hydrolysis and GC[a]	Enzymatic hydrolysis and CZE[b]	Acid hydrolysis and GC[a]	Enzymatic hydrolysis and CZE[b]
Xylose	20.2	25.4	21.4	24.0	7.0	8.5	3.9	4.7
Glucose	64.1	70.1	70.4	71.7	74.1	77.8	73.3	76.4
Mannose	0.4	1.0	0.4	0.9	5.8	6.0	10.9	11.5
Arabinose	n.d.	n.d.	n.d.	n.d.	0.8	0.7	n.d.	n.d.
Galactose	n.d.	n.d.	n.d.	n.d.	0.5	0.3	n.d.	n.d.
Total	84.7	96.5	92.2	96.7	88.2	93.3	88.1	92.5

[a] Mean of two separate determinations.
[b] Mean of six separate determinations.
n.d.=Not detected.

obtained with the procedure developed here were, however, significantly higher than the corresponding values obtained with the traditional procedure. This was expected, since parts of the xylose residues remain linked to uronic acids after the acid hydrolysis [16,59] and are thus not analyzed by the subsequent GC. Furthermore, in most cases slightly higher contents of the other neutral saccharides quantitated in these pulps were also found using the enzymatic hydrolysis–CZE procedure.

It is known that a portion of the saccharides released during acid hydrolysis is decomposed [4,5]. This is corrected for by subjecting the monosaccharide standards in the calibration mixtures to identical acid hydrolysis [5,7]. Evidently, this correction does not compensate fully for the loss of saccharides that occurs. Consequently, the higher contents of saccharides detected by the enzymatic hydrolysis–CZE procedure are apparently more accurate.

3.5. Analysis of wood and mechanical pulps

As expected, when wood and mechanical pulp samples (extracted and grounded) were analyzed directly using enzymatic hydrolysis and capillary electrophoresis, only low yields of carbohydrates were obtained. The polysaccharides present in these samples were obviously not sufficiently accessible to the enzymes employed. Therefore, in order to render wood and mechanical pulp more susceptible to enzymatic hydrolysis, these samples were first delігninfied using chlorite.

Chlorite treatment is a widely used technique that removes the lignin more or less selectively from the wood sample. This technique is however quite species-dependent and the extent of delignification obtained by the treatment may vary between different hard- and softwoods. In order to avoid extensive degradation of the saccharides, the chlorite treatment must be terminated when a few percent of the lignin still remains in the sample [44,60,61]. In addition, water-soluble polysaccharides (e.g., pectins and other polyoses), which are usually present in wood at low levels (except for the arabinogalactan in larch wood), are washed away to various extents during this treatment [60–62].

After pretreatment with chlorite, delignified mechanical pulp and wood samples could be hydrolyzed enzymatically to produce their constituent saccharides in good yields (total carbohydrate yield of 75–100%), especially considering the fact that some lignin remained in these samples. Table 6 documents the carbohydrate analyses performed on a thermomechanical pulp and four different wood samples (spruce, pine, birch and larch wood). These results clearly demonstrate that the analytical procedure developed here is applicable to wood samples subjected to suitable pretreatment, e.g., chlorite delignification.

Table 6
Carbohydrate analyses of milled, extracted and delignified thermomechanical pulp and wood samples employing enzymatic hydrolysis and capillary electrophoresis[a]

Anhydrosaccharide	Content (% of dry mass)				
	Thermomechanical pulp* (TMP)	Spruce wood	Pine wood	Birch wood	Larch wood
Arabinose	0.7	1.3	1.7	0.2	1.0
Galactose	0.6	1.5	1.1	0.4	0.6
Glucose	80.0	68.3	65.5	43.0	75.0
Mannose	9.1	8.2	8.1	2.3	9.7
Xylose	8.1	8.2	8.1	25.5	5.9
Glucuronic acid	0.2	0.1	0.1	n.d.	0.2
4-*O*-Me-glucuronic acid	1.8	2.4	2.1	3.3	1.3
Galacturonic acid	n.d.	0.3	n.d.	n.d.	n.d.
Total yield	100.5	90.3	86.7	74.7	93.7

[a] These values reported are the means of two separate determinations (*=mean of three determinations).
n.d.=Not detected.

4. Conclusion

An efficient procedure for analyzing the carbohydrate composition of extractive-free delignified wood and pulp has been developed. By optimizing the incubation conditions, chemical pulps can be effectively hydrolyzed into their constituent saccharides using a mixture of cellulase and hemicellulase preparations. Under these conditions, HexA-xylobiose is the only HexA-containing saccharide detected in the hydrolysates. These conditions are also suitable for hydrolysis of wood and mechanical pulp samples presubjected to chlorite delignification. The reducing saccharides in the enzymatic hydrolysates obtained can be separated rapidly (i.e., within 20 min) as their 4-aminobenzoic acid ethyl ester derivatives by CZE employing an alkaline borate buffer. Quantification of the saccharides is achieved with high precision (RSD=2.2–7.5%) down to levels as low as 0.1% of the dry mass of the sample. In contrast to previous procedures involving acid hydrolysis and GC or ion-exchange chromatography, the procedure developed here can be used to quantitate both neutral monosaccharides and uronic acids, including the 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid residues, in a single analytical run. The procedure for carbohydrate analysis described here utilizes commercially available reagents, enzymes and CZE apparatus.

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