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Quantification of Solubilized Hemicellulose from Pretreated Lignocellulose by Acid Hydrolysis and High-Performance Liquid Chromatography

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Abstract

An investigation of the acid hydrolysis and HPLC analysis have been carried out in order to optimise the quantification of the solubilized hemicellulose fraction from wheat straw lignocellulose after pretreatment. Different acid hydrolyses have been performed in order to identify which conditions (concentrations of acid and hydrolysis time) gave the maximal quantification of the solubilized hemicellulose (measured as monosaccharides). Four different sugars were identified: xylose, arabinose, glucose and galactose. Some hydrolyses were carried out on aqueous samples and some using freeze-dried samples. The best overall hydrolysis was obtained by treatment of an aqueous sample with 4 %w/v sulfuric acid for 10 minutes. However, these conditions were not optimal for the determination of glucose, which was estimated by using a correction factor.

A purification step was needed following the acid hydrolysis, and included a sulfate precipitation by barium hydroxide and an elimination of remaining ions by mixed-bed ion exchange. The level of barium hydroxide addition significantly reduced the recovery of the sugars. Therefore, lower than equivalent amounts of barium hydroxide were added in the purification step.

For monosaccharide analysis two different HPLC columns, *i.e.* Aminex HPX-87P and HPX-87H with different resin ionic forms, lead (Pb^{2+}) and hydrogen (H^{+}), respectively. The lead column (HPX-87P) separated all four sugars in the acid hydrolyzates, but sample purification was laborious and required the removal of all interfering impurities, which resulted in a poor reproducibility and a sugar recovery below 50%. The hydrogen column (HPX-87H) separated only glucose, xylose and arabinose, whereas galactose was not separated from xylose; however, the column was less sensitive towards impurities and gave improved recovery and reproducibility. Therefore, the hydrogen column (HPX-87H) was chosen for routine quantification of the hydrolysed hemicellulose sugars.

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Preface

Since the oil crisis in the 1970s, there has been a vigourous search for alternative energy sources. Ethanol produced from lignocellulose materials such as wood and agricultural residues has been suggested as a clean, alternative renewable fuel (Buttler *et al.*, 1994). The lignocellulosic material consists mainly of cellulose, hemicellulose and lignin with variable distributions (Marko-Varga *et al.*, 1994). Lignocellulose has a rigid structure and in order to utilise the two major polysaccharide constituents, hemicellulose and cellulose, pretreatment of the material is necessary, *e.g.* by steam explosion (Saddler *et al.*, 1993) or by wet oxidation (McGinnis *et al.*, 1983; Bjerre *et al.*, 1996a; 1996b). In both processes hemicellulose is solubilized whereas cellulose remains in the solid fraction.

Unlike cellulose, hemicellulose has a heterogeneous structure consisting of different sugars. Furthermore, hemicellulose molecules are much shorter, highly branched and usually substituted. Several types of hemicellulose exist in nature with different major constituents (*e.g.* galactose, mannose, xylose and arabinose) as backbone and like most common polysaccharides, the monosaccharides are linked together with glucosidic bonds (Whistler, 1993). The most abundant of the hemicelluloses are the xylans (Coughlan & Hazlewood, 1993). In wheat straw hemicellulose consists mainly of arabino-4-O-methylglucurono xylan with a degree of polymerisation of approximately 70 (Coughlan & Hazlewood, 1993), where the major sugars are xylose (85%) and arabinose (15%) (Fidalgo *et al.*, 1993). These sugars are potential carbon sources for microbial production of chemicals, like ethanol.

The increased research in the use of lignocellulosic materials has focused attention on the need for a rapid and reliable analysis method for these materials (Karr *et al.*, 1991). Recently developed methods for lignocellulose analysis use acid hydrolysis of the carbohydrate components to their monomeric sugars, which are then quantified (Puls, 1993). The most common catalyst for acid hydrolysis is sulfuric acid, but trifluoroacetic acid (TFA) has also been recommended as a catalyst (Kakehi & Honda, 1993) due to its volatility and mild action towards the sugars. However, Puls (1993) observed that the use of TFA carries the risk of incomplete hydrolysis when lignified

materials are degraded to monomeric sugars. As the acid hydrolysis is a compromise between incomplete hydrolysis and sugar destruction, the method has been optimised with respect to acid concentration and hydrolysis time (Puls, 1993). In wheat straw glucose derives from cellulose, whereas xylose and other monosaccharides predominantly derive from hemicellulose. The different sugar components have varied in stability under acid conditions. For optimal recovery every polysaccharide needs to be hydrolysed under different conditions. As this is impractical correction factors become necessary (Puls, 1993). For better reproducibility of the acid hydrolysis, freeze-dried wet oxidation hydrolyzate can also be used (Puls, personal communication). A promising alternative to the chemical cleavages discussed so far is the use of enzymatic hydrolysis as an analytical tool, which provides the most gentle fragmentation method for polysaccharides (Puls, 1993). However, with those enzymes available today, the degree of hydrolysis is lower than that of acid hydrolysis resulting in decreased monosaccharide yields.

During recent years high-performance liquid chromatography (HPLC) has routinely been used for sugar analysis (Karr *et al.*, 1991) with three methods being presently used in the analysis of lignocellulose-derived carbohydrates. The most commonly used method is **partition chromatography** on cation-exchange resins (Puls 1993). Resins in the hydrogen form (Pecina *et al.*, 1984) and in the lead(II) form (Voragen *et al.*, 1986), supported on a sulfonated divinyl benzene styrene copolymeric matrix, are selective for a range of monomeric sugars. Whereas resins in the calcium(II) form have found general use in the separation of monosaccharides and oligosaccharides up to a degree of polymerisation of 4 (Voragen *et al.*, 1986). The lead form is most often chosen due to its selectivity, being able to separate a mixture of sugars within 20 minutes. However, the relatively simple system set-up where the sugars are detected by refractive index can be optimised only by varying column temperature and by coupling a second column in line (Puls, 1993).

Another method is **anion-exchange chromatography** in NaOH-medium (Pettersen & Schwandt, 1991). This method is based on ionised alcoholic groups of sugars adsorbing to anion-exchange resins. The separation of the sugars can be optimised by changing the NaOH concentration or by adding sodium acetate. Due to the NaOH eluent the sugars can be oxidised and

therefore detected by pulsed amperometric detection (Puls, 1993). An acid hydrolyzate can be separated within 20 minutes but the column has to regenerate for 35 minutes after each run (Uremovic *et al.*, 1994).

The third method is **borate-complex anion-exchange** chromatography. Borate ions react with carbohydrates to produce negatively charged carbohydrate-borate complexes (Puls, 1993). These complexes can be separated on a strong anion-exchange resin with a borate solution as mobile phase. The sugar separation takes 55 minutes and the samples can be injected without any purification. Because of the high buffer strength a special post-column reagent is needed for detection (Uremovic *et al.*, 1994).

This report describes the analytical method developed for quantifying the hemicellulose-rich fraction generated from wet oxidation pretreatment of a lignocellulosic materials. In this study wheat straw was used as an example of lignocellulosic material. The method development was carried out by optimising the acid hydrolysis and comparing two cation-exchange HPLC-systems with hydrogen and lead columns.

1 Materials and Methods

1.1 Preparation of the Hemicellulose Material

The filtrates used for optimising the analysis were produced by wet oxidation in a specially designed loop-autoclave constructed at Risø National Laboratory (Bjerre & Sørensen, 1990; Bjerre & Sørensen, 1992) with a working volume of 1 L. Wheat straw was mixed with water and Na_2CO_3 before applying the oxygen pressure and subsequently the heat (Bjerre *et al.*, 1996b). The wet oxidation process conditions used to generate the hemicellulose-rich fraction are given in **Appendix A.** After pretreatment, the suspension was filtered to separate the solid cellulose-rich fraction (the filter cake) from the liquid hemicellulose-rich fraction (the filtrate).

1.2 Acid Hydrolysis

The filtrate from wet oxidation treatment was divided into small portions. The 10 mL (or 15 mL) of filtrate was mixed with 10 mL (or 5 mL) H_2SO_4. All acid hydrolyses were carried out at 121°C but with variable final acid concentrations (4 and 7 %w/v) and hydrolysis times. After acid hydrolysis the solutions were filtered (0.45 µm) in order to remove the water-insoluble residue (Karr & Brink, 1991; Puls, 1993).

1.3 Freeze-dried Filtrate

The filtrate was frozen to -74°C on an ice bath consisting of iso-propanol and dry ice (CO_2), and dried in a Heto FD 3 freeze dryer. Approximately 0.2 g freeze-dried material was dissolved in 10 mL water and 10 mL 8 %w/v H_2SO_4. All acid hydrolyses were carried out at 121°C and 4 %w/v H_2SO_4 and a variable hydrolysis time.

1.4 Analysis of Furfurals

2-Furfural and 5-hydroxymethyl-2-furfural (5-HMF) in the acid hydrolyzates after purification were determined by HPLC (Nucleosil 5C-18 column) with a linear eluent gradient of methanol (10-90%) at pH 3 (Bjerre *et al.*, 1996b). The recovery of the furfurals in the purification step (described in section **2.5**) was determined by 3 levels of standard additions of the two compounds to the samples using the purification procedure in triplicate.

1.5 Purification of Acid Hydrolyzate

In order to avoid interference from salts and non-sugar substances in the HPLC analysis with subsequent damage to the column, acid hydrolyzates should be deionised before injection (Kaar *et al.*, 1991; Puls, 1993). The sulfate ions in the 5 mL hydrolyzates were precipitated by either 0.63 g or 1.20 g barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$). The precipitated $BaSO_4$ was removed by centrifuging (8000 rpm, 5 min.). Any remaining ions were eliminated by an ion-exchange treatment in a mixed bed resin of Amberlite CG-120 (100-200 mesh, H^+) and Dowex 1x4 (50-100 mesh, OH^-) (both Fluka) (**Appendix B**). The recovery of this purification step was determined by standard additions of glucose, xylose and arabinose using the same purification procedure at 3 different levels of addition in five replicates (**Table 7**). All measured sugar concentrations given in this report have been corrected for the recovery, unless stated otherwise.

1.6 HPLC-system 1

The first chromatographic system for analysis of sugars consisted of an HPLC pump (Knauer, Model 364.00), an injection valve with a 20-µL loop (Rheodyne, Model 7725), a differential refractometer index detector (Knauer) and an integrator (HP, Model 3395). The analytical column was a pre-packed Aminex HPX-87H strong cation-exchange resin column (hydrogen (H^+) column) (300 x 7.8 mm I.D.) supported on a sulfonated divinyl benzene styrene copolymeric matrix fitted with an ion-exclusion Micro-Guard refill cartridge (BioRad 125-0129) as pre-column. The Aminex column was held at 63°C in a

chromatographic oven (MikroLab, Århus). The mobile phase was 0.004 M H_2SO_4 at a flow-rate of 0.6 mL/min. The system is shown in **Figure 1.**

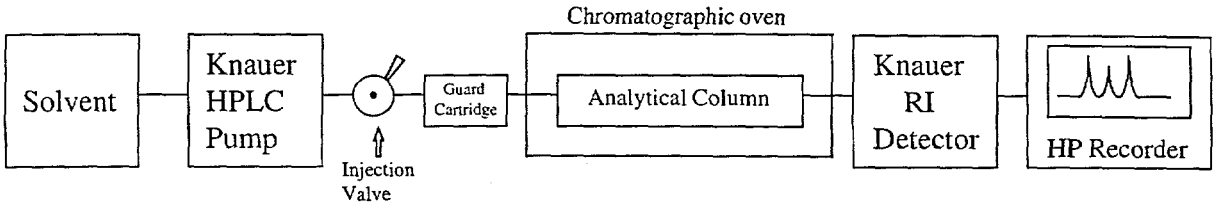

Figure 1. The arrangement of the HPLC system.

1.7 HPLC-system 2

The set-up of the second HPLC system was similar to the first system except for the columns. The analytical column was an Aminex HPX-87P cation-exchange resin column (lead (Pb^{2+}) column) (300 x 7.8 mm I.D.) supported on a sulfonated divinyl benzene styrene copolymeric matrix fitted with a Micro-Guard refill cartridge deashing column (BioRad 125-0119) as pre-column. In this system, the separation was carried out at 81°C. The mobile phase was water at a flow-rate of 0.6 mL/min.

2 Results and Discussion

2.1 Acid Hydrolysis

2.1.1 Optimisation

Different acid hydrolyses had been carried out in order to identify which conditions gave the best quantification of the solubilized hemicellulose (measured as monosaccharides) after pretreatment using both aqueous and freeze-dried samples of hemicellulose. In general, hydrolysis with 4 %w/v H_2SO_4 appeared to give a better quantification of hemicellulose compared to the 7 %w/v H_2SO_4 treatment (**Figure 2**). The level of monosaccharides at all three hydrolysis conditions tended to decrease with hydrolysis time after passing through a maximum at about 5 minutes of hydrolysis. This may be explained by the increasing sugar degradation taking place under harsh conditions (Puls, 1993).

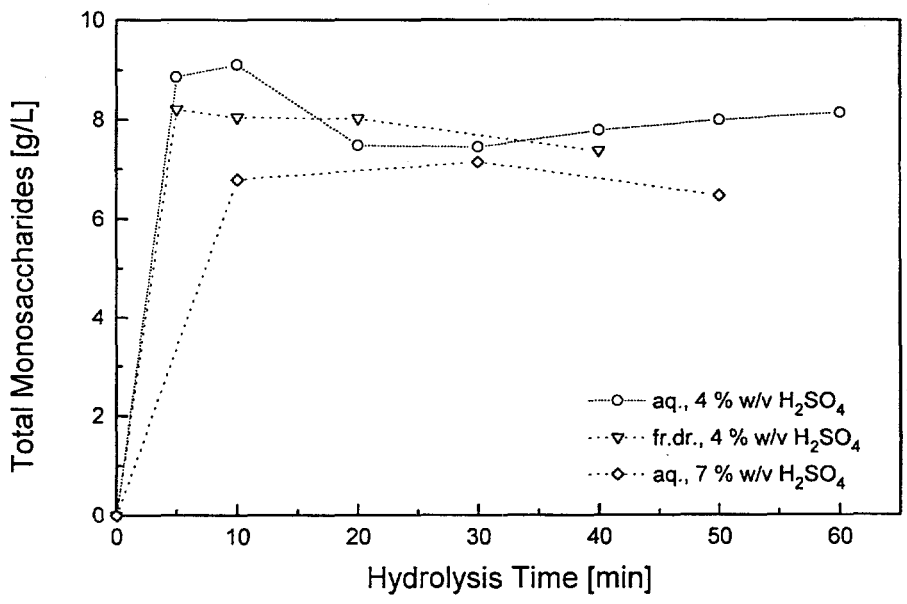

Figure 2. Acid hydrolysis of the solubilized hemicellulose fraction (Filtrate: B31) measured as total monosaccharides under 3 different hydrolysis conditions (aq. = aqueous sample; fr.dr. = freeze-dried sample) at 121 °C.

The three different hydrolysis conditions all gave a different optimal hydrolysis time. For both aqueous and freeze-dried samples in 4 %w/v H_2SO_4 the optimal hydrolysis time was about 5-10 minutes. For aqueous samples in 7 %w/v H_2SO_4 the effect of hydrolysis time was minimal. The best overall hydrolysis was one with 4 %w/v sulfuric acid for 5-10 minutes of an aqueous sample determining about 9 g monosaccharides/L.

In **Tables 1** to **3** the amount of the individual sugars is shown, where xylose was the major sugar (> 5 g/L) and arabinose and glucose the minor sugars (about 1.2 and 0.6 g/L, respectively). **Table 1** shows that at 4 %w/v H_2SO_4 (aqueous sample) xylose and arabinose, the pentoses, had the same optimal hydrolysis time of approximately 10 minutes giving 7.2 and 1.4 g/L, respectively, after which their concentration decreased to some extent. However, for glucose the concentration was low in the beginning of the hydrolysis and then increased slightly reaching a constant level after approximately 20 minutes hydrolysis. When using the freeze dried sample (**Table 2**) the optimal hydrolysis time for the pentoses was shorter, namely 5 minutes, giving 6.1 g/L xylose and 1.4 g/L arabinose. After this optimum the concentrations decreased. The aqueous sample seems to give a higher yield of the pentoses from hemicellulose.

Table 1. *Acid hydrolysis (4 %w/v H_2SO_4; aqueous sample; 121°C) of the solubilized hemicellulose fraction (Filtrate: B31) measured as monosaccharides.*

Time (min.)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)
5	0.55	6.96	1.35
10	0.57	7.18	1.36
20	0.67	5.52	1.29
30	0.65	5.62	1.19
40	0.68	5.85	1.26
50	0.65	6.15	1.21
60	0.67	6.23	1.26

Table 2. *Acid hydrolysis (4 %w/v H_2SO_4; freeze-dried sample; 121°C) of the solubilized hemicellulose fraction (Filtrate: B31) measured as monosaccharides.*

Time (min.)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)
5	0.73	6.09	1.39
10	0.75	5.95	1.35
20	0.75	5.93	1.34
40	0.73	5.44	1.21

The concentration of glucose was constant over the different hydrolysis times studied. The glucose concentration was higher for the freeze dried sample (**Tables 1** and **2**), whereas the xylose concentration was significantly lower compared to the aqueous sample. The arabinose concentration was similar in both cases. When using a higher concentration of sulfuric acid on aqueous samples, the concentrations of the pentoses were considerably lower than when hydrolysed in 4 %w/v H_2SO_4 (**Table 3**). However, the glucose behaved differently from the two pentoses and gave the highest concentration for the aqueous sample hydrolysed at 7 %w/v H_2SO_4 and 60 minutes, although a high concentration was also obtained after 30 minutes hydrolysis.

Table 3. *Acid hydrolysis (7 %w/v H_2SO_4; aqueous sample; 121°C) of the solubilized hemicellulose fraction (Filtrate: B31) measured as monosaccharides.*

Time (min.)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)
10	0.59	5.41	0.79
30	0.72	5.42	1.01
50	0.58	4.93	0.96
60	0.92	5.29	1.09

As seen in **Figure 2**, aqueous samples in 4 %w/v H_2SO_4 for 10 minutes gave the best total sugar yield and also the highest xylose concentration. Therefore, these hydrolysis conditions were chosen as the optimal hydrolysis of the hemicellulose fraction. However, as these conditions were not optimal for the determination of glucose, a correction factor for glucose was used. The correction factor (F_C) was calculated as the ratio of the glucose concentration where hydrolysis was optimal for glucose to the glucose concentration obtained by the overall optimal hydrolysis as indicated in equation (1):

$$F_c = \frac{C_{7\%,\ aq.,\ 60\,\mathrm{min}}}{C_{4\%,\ aq.,\ 10\,\mathrm{min}}} \qquad (1)$$

$C_{7\%,\ aq.,\ 60\ min.}$: Glucose concentration after hydrolysis: 7 %w/v H_2SO_4, 60 min., aqueous sample

$C_{4\%,\ aq.,\ 10\ min.}$: Glucose concentration after hydrolysis: 4 %w/v H_2SO_4, 10 min., aqueous sample

Table 4. *Correction factor (F_C) from the glucose concentrations for aqueous samples hydrolysed with 4 and 7 %w/v sulfuric acid for 10 and 60 minutes, respectively.*

Sample Code	Replications	$C_{4\%,\ aq.,\ 10\ min.}$ (g/L)	$C_{7\%,\ aq.,\ 60\ min.}$ (g/L)	F_c
B31	2	0.77	1.07	1.39
B41	2	0.78	0.85	1.09
B42	2	0.71	0.87	1.22
B43	2	0.67	0.89	1.33
B44	2	0.67	0.70	1.03
B45	2	0.68	0.76	1.12
B46	2	1.14	1.23	1.08
B49	2	0.59	0.74	1.26
B50	2	0.58	0.68	1.16
B54	2	0.77	0.88	1.14
B55	2	0.66	0.90	1.36
B91	10	0.74±0.04	0.81±0.05	1.09

The correction factor for glucose found from equation (1) varied with the sample analysed (**Table 4**), but was always greater than 1. By using the average value of the correction factors from the 12 different samples hydrolysed under the 2 different conditions a good average correction factor for future studies of acid hydrolysis of solubilized hemicellulose from wheat straw may be established. This overall correction factor was calculated to be 1.19±0.12.

2.1.2 Sugar degradation

As well as adding a correction factor for a non-optimal hydrolysis of glucose, a factor to counter balance the sugar losses during the acid hydrolysis may be included. The sugars tended to degrade to some extent (**Figure 3**); hence, their degradation products, 2-furfural and 5-hydroxymethyl-2-furfural (5-HMF), were determined in the purified acid hydrolyzates.

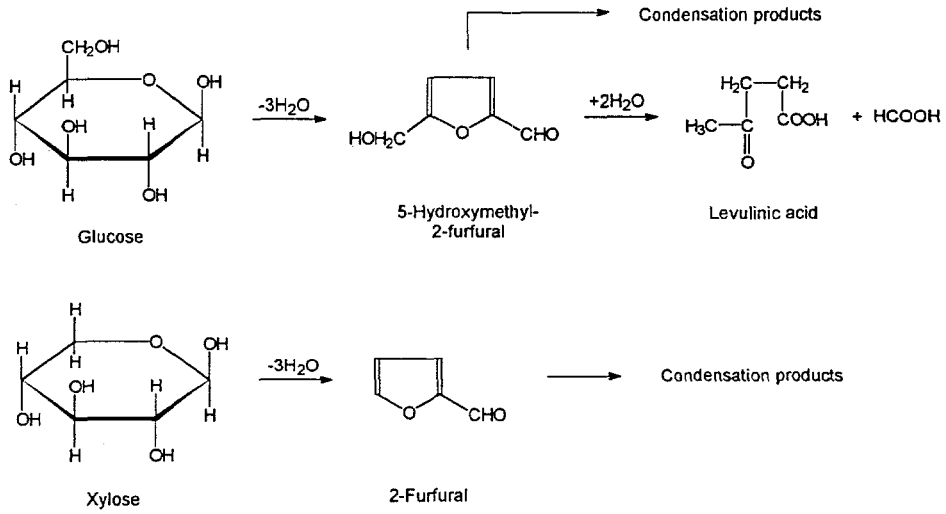

Figure 3. *Formation of 2-furfural, 5-hydroxymethyl-2-furfural, levulinic acid and formic acid from the degradation of monosaccharides in acid medium.*

Due to the possibility that the sugar degradation products may precipitate with the barium sulfate or bind to the ion exchange matrices during the purification procedure a recovery experiment was carried out for 2-furfural and 5-HMF at three levels of standard addition in triplicate purifications (**Table 5**). Each

purified sample was analysed in duplicate by HPLC. The recovery factor (R_I) was calculated by equation (2):

$$R_I = \frac{C_{h+s} \cdot 100\%}{C_s + C_h} \qquad (2)$$

C_{h+s}: Furfural concentration in acid hydrolyzate with standard addition (ppm)

C_s : Furfural concentration in the standard (ppm)

C_h : Furfural concentration in acid hydrolyzate without standard addition (ppm)

Table 5. *The recovery factor (R_I) in the purification procedure for 2-furfural and 5-HMF (Filtrate: B72).*

Furfurals Addition		2-Furfural		5-HMF	
2-Furfural (ppm)	5-HMF (ppm)	R_I (%)	R_{ave}[a] (%)	R_I (%)	R_{ave}[a] (%)
11.6	1.3	97.0 97.6 100.5	98.4±1.9	75.8 78.9 81.3	78.6±2.8
58.0	4.75	98.7 97.6 99.1	98.4±0.8	65.5 64.8 64.0	64.7±0.8
580	50	75.7 78.6 77.5	77.3±1.5	42.1 45.5 43.9	43.8±1.7

a: R_{ave} = average recovery factor (±SD).

The recoveries of 2-furfural and 5-HMF varied with the concentration of the furfurals and no overall recovery factor could be found. However, in practice the concentration of furfurals in samples analysed following acid hydrolysis was low, hence, as a recovery of 98.4% was found for 2-furfural and 78.6% for 5-HMF. In order to correct the measured monosaccharide concentration for the degradation occurring during acid hydrolysis (**Figure 3**) the two major degradation products were measured (**Table 6**). The correction factors for the pentoses (FCP) and the glucose (FCG) were calculated by equations (3) and (4), respectively, as seen in **Table 6**.

$$FCP = \frac{C_{pentoses} + C_{furfural} \cdot \left(\frac{M_{pentoses}}{M_{furfural}} \right)}{C_{pentoses}} \quad (3)$$

$$FCG = \frac{C_{glucose} + C_{HMF} \cdot \left(\frac{M_{glucose}}{M_{HMF}} \right)}{C_{glucose}} \quad (4)$$

Usually only a very small part of the hemicellulose underwent degradation during acid hydrolysis; about 1-2% of the pentoses (xylose and arabinose) and about 0.6% of the glucose (**Table 6**). Puls (1993) also found that the correction factor for glucose was smaller than for the pentoses. Even when monosaccharides instead of solubilized hemicellulose were subjected to the same acid hydrolysis the degradation was low though slightly higher than for the hemicellulose. Consequently, for most purposes the degradation to furfurals during the acid hydrolysis can be ignored; bearing this in mind the small correction factors found in **Table 6** could be used.

Table 6. *Monosaccharides and furfurals in purified acid hydrolyzates for calculation of the correction factors for the pentoses (FCP) and the glucose (FCG) by using equations (3) and (4).*

Sample Code	Furfural[a] (ppm)	5-HMF[a] (ppm)	Pentoses (g/L)	Glucose (g/L)	FCP	FCG
B58	56.6	2.6	4.4	0.59	1.0203	1.0063
DTU1	45.4	3.5	7.3	0.75	1.0097	1.0066

a: Corrected for recovery (**Table 5**)

2.2 Purification Procedure

2.2.1 Optimisation

In order to increase the sensitivity of the acid hydrolysis, a series of experiments was carried out in which the dilution factor was reduced during both the hydrolysis and subsequent purification steps. The initial dilution by acid addition was reduced 30% by adding 16 %w/v sulfuric acid instead of 8 %w/v H_2SO_4 to a final concentration of 4 %w/v H_2SO_4. These experiments showed that the initial reduction in the dilution factor that occured by increasing the sample-acid volume ratio had no significant effect on the recovery. However, as a higher quantity of sugars was injected on the column the peaks rose and the quality of the chromatogram was improved. As illustrated in **Table 7** experiments were carried out using different precipitation and purification conditions. The amount of barium hydroxide used in the precipitation step affected the recovery of the monosaccharides. When using the equivalent amount of barium hydroxide (1.15 g) the recovery and reproducibility were considerably lower than by using a non-equivalent amount (0.63 g). The reason for this observation could be that the monosaccharides were absorbed to the precipitate at the high barium hydroxide concentration. Furthermore, the ion-exchange step was also an essential step in the purification, and changes in wash volume and sample size resulted in different recoveries. Experiments carried out using a sample size of 3 mL instead of 2 mL with wash volumes of 1½ mL for the former, instead of 2 mL for the latter, showed a reduction in recovery in the purification step.

As mentioned earlier, purification of the samples was necessary since the used HPLC columns were sensitive towards sulfate and other salts. Therefore, prior to analysis all sulfate and other salts should be removed to insure stability and durability of the columns. The effect of the amount of barium hydroxide on the column performance was evaluated (**Figure 4**). As illustrated in **Table 7** and **Figure 4** experiments performed on the HPX-87H column showed that when an equivalent amount of barium hydroxide was applied to precipitate the sulfate ions, high standard derivations and very low recoveries (< 50%) were achieved.

Table 7. Sugar yields and standard derivation using different dilution factors (DF) in the acid hydrolysis and different purification steps, analysed on the HPLC system 1 (HPX-87H column, 63°C, 0.004 M H_2SO_4, 0.6 mL/min.) (Filtrate: B91).

Precip.[a] (g)	IE[b] (mL)	Filtrate[c] (mL)	DF	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Total sugar (g/L)	Total[d] (%)
0.63	2	10	5	0.67 ± 0.02	5.81 ± 0.03	1.47 ± 0.02	7.94 ± 0.08	100
0.63	2	15	3.33	0.61 ± 0.01	5.60 ± 0.55	1.54 ± 0.06	7.75 ± 0.63	98
0.63	3	15	2.22	0.61 ± 0.04	4.74 ± 0.16	1.54 ± 0.04	6.89 ± 0.24	87
1.15	2	10	5	0.43 ± 0.15	3.91 ± 1.49	0.85 ± 0.15	5.18 ± 1.78	65
1.15	2	15	3.33	0.52 ± 0.05	5.14 ± 0.05	1.19 ± 0.08	6.85 ± 0.18	86
1.15	3	10	3.33	0.43 ± 0.08	4.60 ± 0.85	0.97 ± 0.08	5.99 ± 1.01	76
1.15	3	15	3.33	0.54 ± 0.01	4.70 ± 0.09	1.34 ± 0.04	6.59 ± 0.13	83
1.20	3	15	2.22	0.54 ± 0.01	4.61 ± 0.03	1.40 ± 0.07	6.54 ± 0.10	82

a: Amount of barium hydroxide used in the precipitation
b: Sample volume applied on the ion exchange column
c: Filtrate volume in the acid hydrolysis
d: The total sugar quantified by the different procedures as a percentage of the sugar quantified by the standard procedure (**Appendix B**)

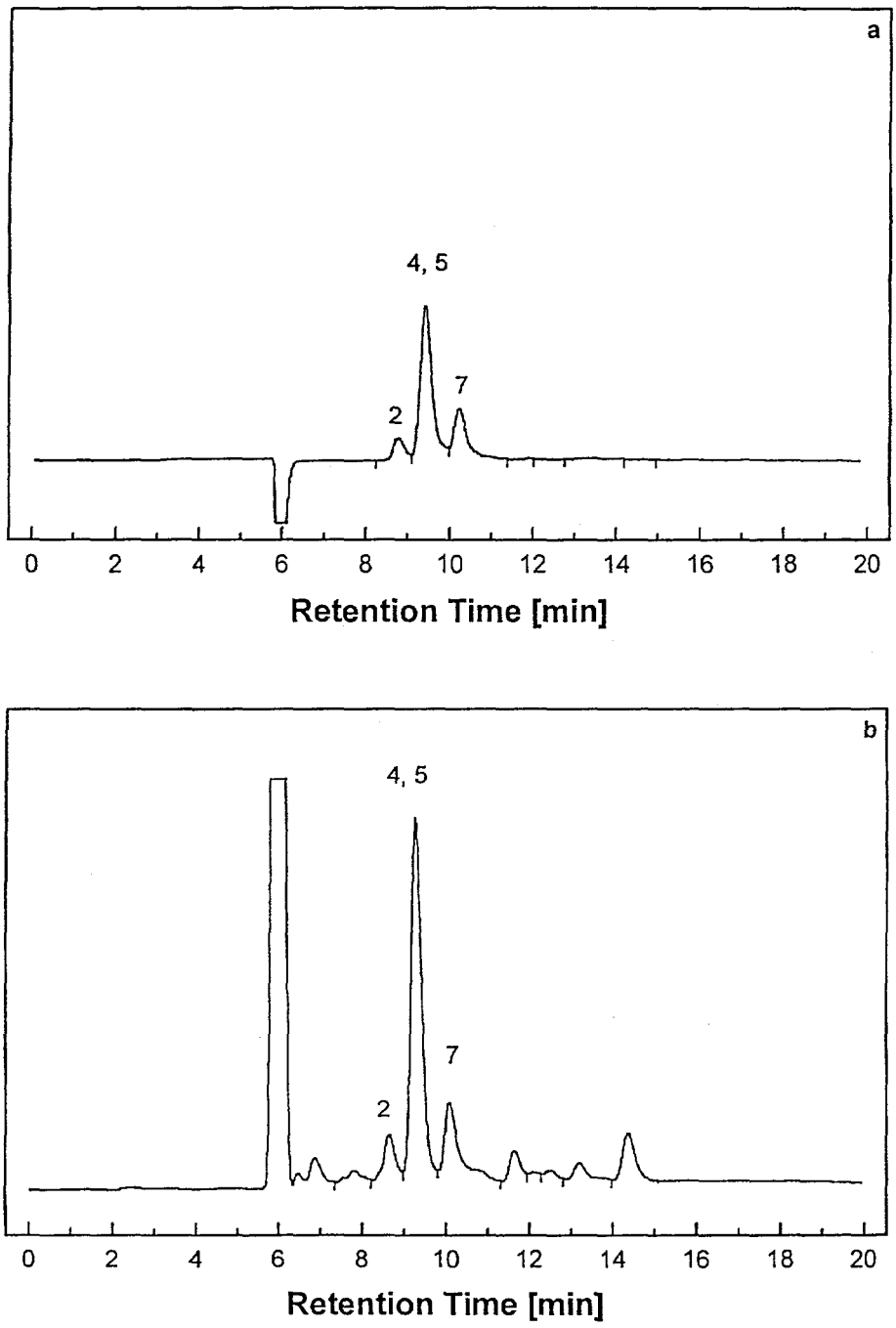

Figure 4. *Sugar profiles of wet oxidised wheat straw (Filtrate: B96) after acid hydrolysis and purification, using equivalent and non-equivalent amounts of barium hydroxide to precipitate the sulfate ions, analysed on HPLC system 1 (HPX-87H column, 63°C, 0.004 M H_2SO_4, 0.6 mL/min.). a) equivalent (1.15 g); b) non-equivalent (0.63 g). 2: Glucose; 4: Galactose; 5: Xylose; 7: Arabinose.*

The chromatograms in **Figure 4** clearly demonstrated the efficiency of the precipitation procedure using the equivalent amount of barium hydroxide (**Figure 4a**) by eliminating the majority of the peaks. The salt peak at a retention time of 5.8 minutes disappeared together with several of the minor peaks including acetic acid. Not all the peaks in **Figure 4a** were identified, but may be products of the acid hydrolysis, possibly xylo-oligosaccharides and/or other sugars.

2.2.2 Recovery

The applied purification procedure for the HPX-87H column was laborious and time-consuming but resulted in an efficient recovery of the sugars. However, when the samples were eluted from the mixed bed ion-exchange resin, some of the sugars were probably retained on the columns by absorption to the resin. Therefore, the results were adjusted with a factor for the recovery. In order to calculate this recovery factor in the purification step, known amounts of monosaccharides were added to an acid hydrolyzate. These samples were then purified by the same procedure as the sample, and the sugar concentrations were determined. The recovery factor (R_{II}) was calculated by equation (5):

$$R_{II} = \frac{C_{h+s} \cdot 100\%}{C_s + C_h} \qquad (5)$$

C_{h+s}: Sugar concentration in acid hydrolyzate with the standard addition (g/L)

C_s : Sugar concentration in the standard (g/L)

C_h : Sugar concentration in acid hydrolyzate without standard addition (g/L)

Three different levels of standard additions were analysed in five replicates (**Table 8**). The level of the standard addition did not significantly affected the average recovery factors (R_{ave}), hence, overall recovery factors ($R_{overall}$) for glucose, xylose and arabinose were calculated as 86.0%, 92.0% and 93.3%, respectively. This agreed with Marko-Varga *et al.* (1994), who obtained a recovery of 88-94% for similar sugars and the HPLC method. All results presented in this work have already been corrected for these recoveries.

Table 8. Recovery factor (R_{II}) in the purification procedure for the different monosaccharides (Filtrate: B31).

Sugar Addition (g/L)	Glucose		Xylose		Arabinose	
	R_{ave} (%)	$R_{overall}$ (%)	R_{ave} (%)	$R_{overall}$ (%)	R_{ave} (%)	$R_{overall}$ (%)
0.5	91.4±1.7		96.9±1.4		96.1±2.3	
2.5	82.1±2.1	86.0±4.4	89.2±2.6	92.0±3.9	91.7±4.0	93.3±3.3
5	84.2±1.4		90.2±1.4		92.0 ±1.7	

2.3 HPLC Analysis

2.3.1 Column Efficiency

In order to compare the ability of the 2 different analytical HPLC columns to separate and quantify, a mixture of sugars, cellubiose, glucose, mannose, galactose, xylose, rhamnose, and arabinose, was examined on the column individually and in a mixture. In **Figure 5** the chromatograms of the sugars from the two HPLC-systems are compared, which show that the HPX-87P column separated the sugars better than the HPX-87H column, the former giving additional peaks. However, the sugars eluted later and the peak widths were generally broader than using the HPX-87H column. The run time on the HPX-87P column was 18 minutes, whereas that of the HPX-87H column was 6 minutes shorter. Wet oxidised wheat straw will also contain some acetic acid, which eluted after 15 and 20 minutes on the HPX-87H and HPX-87P, respectively.

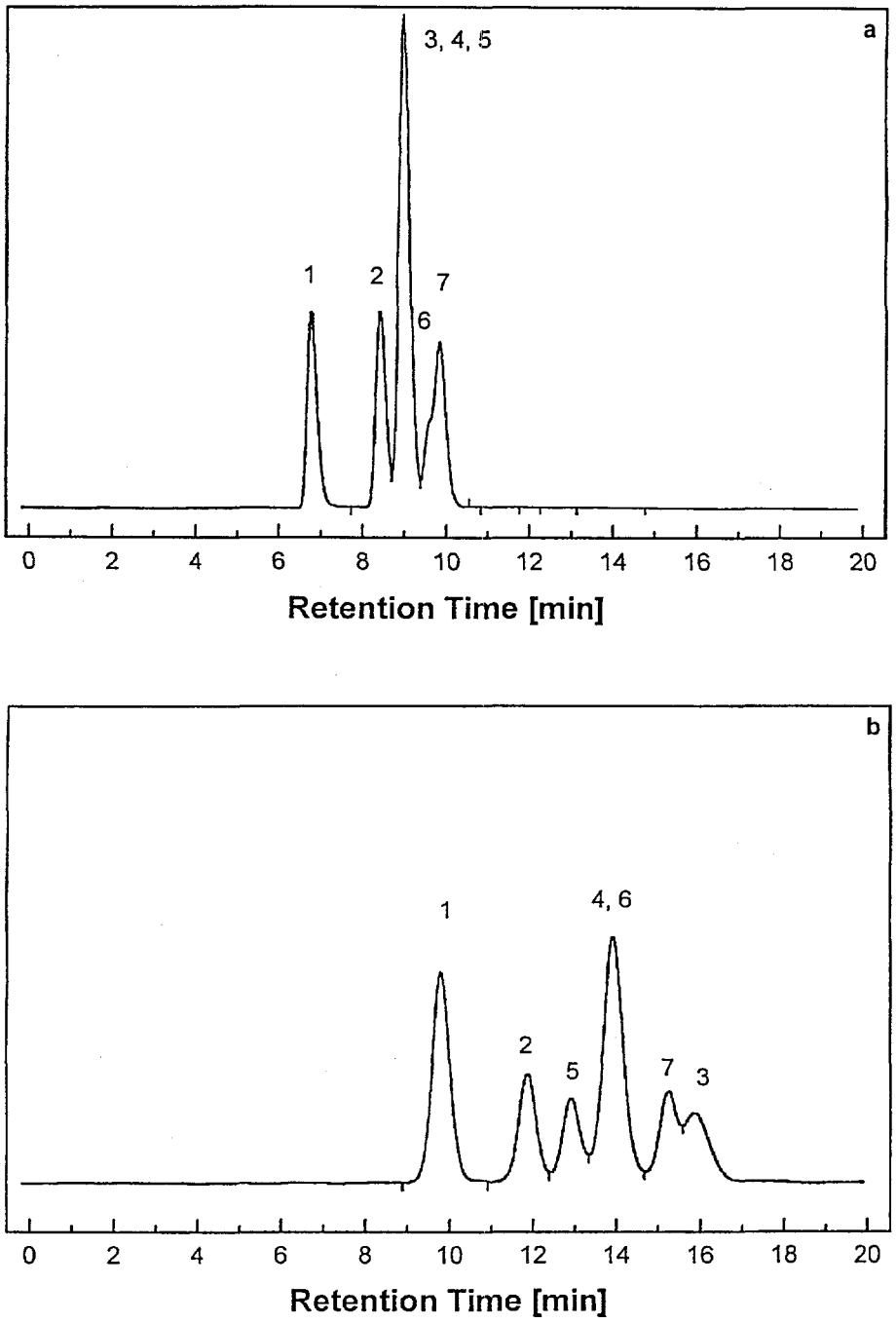

Figure 5. *Authentic mixture of sugars as separated by:* ***a)*** *HPLC system 1 (HPX-87H column, 63°C, 0.004 M* H_2SO_4*, 0.6 mL/min.);* ***b)*** *HPLC system 2 (HPX-87P column, 81°C, water, 0.6 mL/min.). 1: Cellubiose (3 mM); 2: Glucose (3 mM); 3: Mannose (3 mM); 4: Galactose (3 mM); 5: Xylose (3 mM); 6: Rhamnose (6 mM); 7: Arabinose (3 mM).*

From the chromatograms (**Figure 5**) of each of the individual sugars the peak widths and retention times were determined in order to calculate the theoretical plates (N) and resolutions (R_S) of the two columns: HPX-87H (**Table 9**) and HPX-87P (**Table 10**) for those common sugars in pretreated lignocellulose by using equations (6) and (7), respectively.

$$N = 16\left(\frac{V_R}{w}\right)^2 \qquad (6)$$

$$R_s = 2 \cdot \frac{V_{R_B} - V_{R_A}}{w_A + w_B} \qquad (7)$$

V_R : Retention volume (mL)

w : Peak width at half peak height (mL)

As shown in the chromatograms (**Figure 5**), the highest retention times and resolutions (**Tables 9** and **10**) were obtained by using the HPX-87P column. However, the HPX-87H column had more theoretical plates than the HPX-87P column. The former column only separated cellubiose and glucose well, whereas mannose, galactose and xylose almost had the same retention time and was seen as one peak (**Figure 5a**). Rhamnose and arabinose were not fully separated in the chromatogram. The HPX-87P column (**Figure 5b**) separated cellubiose, glucose and xylose well, whereas galactose and rhamnose eluted at the same time. Also arabinose and mannose were not completely separated. Furthermore, the sugars eluted in different orders on the two different columns.

In general, the HPX-87P column gave an improved separation of a range of monosaccharides compared with the HPX-87H column, but the lower number of theoretical plates resulted in broader peaks and the analysis time was also longer. For precise quantification of galactose and xylose the HPX-87P column may be favourable. According to standard curves xylose and galactose gave approximately the same peak height when applied in the same concentration. When applying a completely different HPLC system based on a borate-complex anion-exchange, about 0.5 g/L galactose was found for acid hydrolyzates from wet oxidised wheat straw (Puls, 1993) corresponding to galactose contributing about 6% of the peak.

Table 9. *Characteristic of the HPLC analysis of the authentic mixture of sugars using the HPLC system 1 (HPX-87H column; 63°C; 0.004 M H_2SO_4; 0.6 mL/min.).*

Sugars	Retention Time (min.)	Retention Volume (mL)	Peak Width (mL)	Theoretical Plates	Resolution
Cellubiose	6.9	4.14	0.30	3047	
					3.4
Glucose	8.6	5.16	0.30	4733	
					1.1
Mannose	9.1	5.46	0.27	6543	
					0.0
Galactose	9.1	5.46	0.33	4380	
					0.19
Xylose	9.2	5.52	0.30	5417	
					1.0
Rhamnose	9.7	5.82	0.30	6022	
					0.57
Arabinose	10.0	6.00	0.33	5289	

Table 10. *Characteristic of the HPLC analysis of the authentic mixture of sugars using the HPLC system 2 (HPX-87P column; 81°C; water; 0.6 mL/min.).*

Sugars	Retention Time (min.)	Retention Volume (mL)	Peak Width (mL)	Theoretical Plates	Resolution
Cellubiose	9.8	5.88	0.42	3136	
					3.0
Glucose	11.9	7.14	0.42	4624	
					1.4
Xylose	12.9	7.74	0.48	5434	
					1.3
Galactose	13.8	8.28	0.44	6218	
					0.35
Rhamnose	14.1	8.46	0.59	3181	
					0.94
Arabinose	14.9	8.94	0.48	7249	
					0.95
Mannose	15.8	9.48	0.69	2774	

2.3.2 Quantification of Purified Samples

Shown in **Figure 6**, the HPLC analysis of acid hydrolyzates from pretreated wheat straw using the HPX-87H and the HPX-87P column and the same purification procedure (**Table 7**: 1.15 g barium hydroxide, DF: 5). The two HPLC-systems displayed different sensitivities towards impurities. A more comprehensive purification procedure was necessary for the HPX-87P column to insure the removal of all interfering impurities; hence, excess barium hydroxide was used. The HPX-87H column resulted in a stable baseline and narrow peaks (**Figure 6**), so that the purification procedure proved to be efficient. By using the HPX-87P column a stable baseline could not be obtained probably due to the high sensitivity of the column toward salts and other impurities. The comprehensive purification procedure resulted in a reduced recovery of the sugars and decreased reproducibility (**Table 7**). Due to this lowered recovery a higher correction factor would be needed contributing to an increased uncertainty in the quantification of the sugars. In spite of efforts to remove the impurities the HPX-87P column remained unstable, with drifting retention times and reduced resolution. Hence, the production of new standard curves prior to analysis became necessary, making this analytical method time consuming. The HPX-87H column was less sensitive and kept its chromatographic properties after many analyses, whereas the HPX-87P column changed its chromatographic properties after only a few analyses.

The chromatogram from the HPX-87H column (**Figure 6a**) showed the presence of glucose, arabinose and then a peak consisting of xylose, galactose and/or mannose according to the retention times obtained from the standard chromatogram (**Table 9** and **Figure 5a**). No rhamnose was identified, either due to the absence of rhamnose in the sample or a concentration of rhamnose lower than the detection limit (0.001 g/L). In **Figure 6b** four separate peaks were identified as glucose, xylose, galactose and arabinose. No mannose peak was found. Therefore, solubilized wheat straw hemicellulose does not seem to contain any mannose or rhamnose in detectable amounts. The quantification of the different sugars by the two columns could not be compared due to difficulties in the quantification on the HPX-87P column, which gave an unstable baseline, so that the more comprehensive purification procedure was not very reproducible.

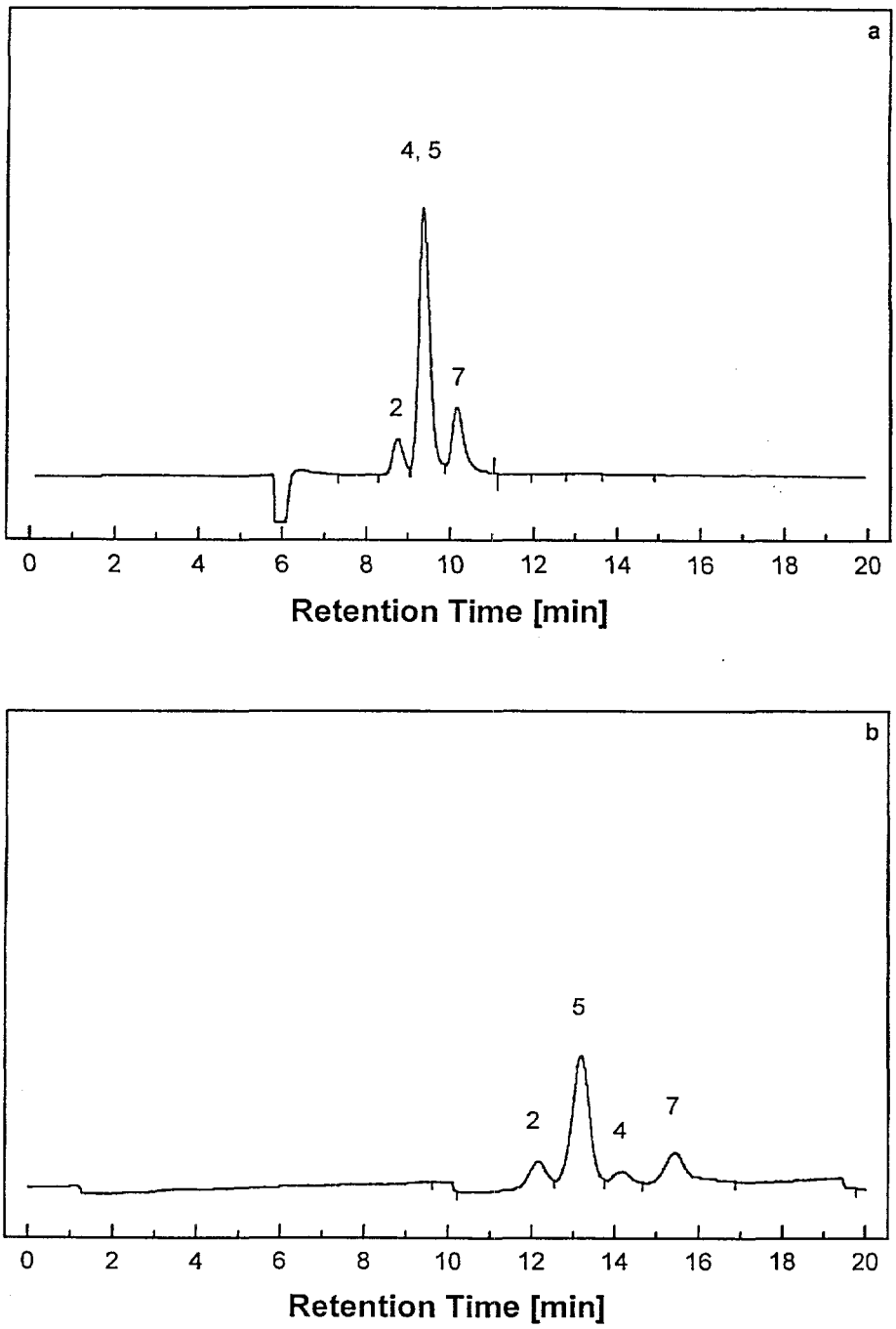

Figure 6. *Sugar profiles of wet oxidised wheat straw (B91) after acid hydrolysis and purification as separated by:* ***a)*** *HPLC system 1 (HPX-87H column, 63°C, 0.004 M* H_2SO_4*, 0.6 mL/min.),* ***b)*** *HPLC system 2 (HPX-87P column, 81°C, water, 0.6 mL/min.). 2: Glucose; 4: Galactose; 5: Xylose; 7: Arabinose.*

3 Conclusions

A method was developed for quantifying the sugars from the solubilized hemicellulose from wheat straw lignocellulose. The best overall hydrolysis was found to be one with 4 %w/v sulfuric acid for 10 minutes of an aqueous sample. By reducing the dilution rate in the acid hydrolysis step it was possible to improve the sensitivity of the method, but it resulted in low recovery. After acid hydrolysis a purification procedure was developed using sulfate precipitation by barium hydroxide and eliminating the remaining ions by mixed-bed ion exchange. The amount of barium hydroxide used in the precipitation step had a critical effect on recovery and reproducibility of the method. When an equivalent amount of barium hydroxide was applied to precipitate the sulfate ions, the recovery was lower than 50%. In the precipitation process the monosaccharides may form complexes with the precipitated barium sulfate residue. Furthermore, other compounds like acetic acid were completely removed. By using lower than the equivalent amount of barium hydroxide the recovery of sugars was in the range of 89-94%.

Two different HPLC systems (columns: HPX-87H and HPX-87P) were used to analyse a purified acid hydrolyzates from pretreated wheat straw for monosaccharide composition. HPLC system 2 (HPX-87P) showed superiority in separating monosaccharides, but it was very sensitive towards impurities. In order to remove interfering impurities, it was necessary to precipitate the present sulfate ions with excess barium hydroxide, but no satisfactory chromatograms could be obtained. Furthermore, the addition of more barium hydroxide resulted in an extensive variation in the recovery of the monosaccharides. Its main application may be occasional quantification of galactose. Separation of the hydrolysis products from wheat straw on the HPX-87H column gave a lower selectivity for monosaccharides; however, this column was more stable and less sensitive to impurities than the HPX-87P column. Overall, the HPX-87H column was chosen as the standard HPLC method for analysing monosaccharides in wet oxidised wheat straw because the method displays reproducibility and stability and was less time consuming.

Acknowledgement

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APPENDIX A: Wet Oxidation Conditions

Table A1. Wet oxidation pretreatment conditions used in preparing the hemicellulose material.

Sample Code	Straw (g/L)	Na_2CO_3 (g/L)	O_2-pressure (bar)	Temperature (°C)	Reaction Time (min.)
B31	60	6.5	12	185	15
B41	60	6.5	12	170	15
B42	60	6.5	12	185	10
B43	60	6.5	12	185	20
B44	60	6.5	12	185	0[a]
B45	60	6.5	12	185	25
B46	60	0	12	185	15
B49	60	9	12	185	15
B50	60	13	12	185	15
B54	60	6.5	12	200	0[a]
B55	60	6.5	12	200	5
B58	60	6.5	12	185	15
B72	60	6.5	6	185	15
B91	60	6.5	6	185	15
B96	60	4	3	185	15
DTU1	60	6.5	12	185	15

a: The reaction mixture was heated to the reaction temperature and then cooled.

APPENDIX B:
RISØ STANDARD PROCEDURE
Quantification Of Solubilized Hemicellulose

B1 Acid Hydrolysis

B1.1 Procedure

- 10 mL of wet oxidised filtrate was added to 10 mL of 8 %w/v sulfuric acid.
- Minimum 2 cm of water was added to the autoclave
- The autoclave was closed and locked by turning the red knob.
- The samples were autoclaved for 10 minutes at 120°C.
- After cooling the samples was filtered though a 0.45 µm filter.

B1.2 Reagents

- 8 %w/v sulfuric acid: 80 mL 95-97% H_2SO_4 was added to a 1 L volumetric flask containing some water. The total volume was made up to 1 L with water.

B2 Precipitation

B2.1 Procedure

- 10 mL filtrate was added to 0.63 g barium hydroxide octahydrate in a 25 mL beaker.
- The suspension was stirred vigorously until white precipitate was formed.
- The suspension was transferred to a 10 mL centrifuge tube and covered with aluminium foil.
- The suspension was centrifuged for 5 minutes at approximately 4000 rpm.
- The supernatant was purified by ion exchange.

B3 Ion Exchange

B3.1 Preparation of the Ion-Exchange Columns

- The column was made of a 1 mL plastic pipette tip.
- A little piece of glass fibre was placed in the pipette tip using a forceps and paper clip.
- The pipette tip was filled to the bending point of the pipette tip with Dowex 1x4 (50-100 mesh, OH^-) (**Figure B1**).
- On top of the Dowex approximately 1 cm of Amberlite CG-120 (100-200 mesh, H^+) was applied (**Figure B1**).

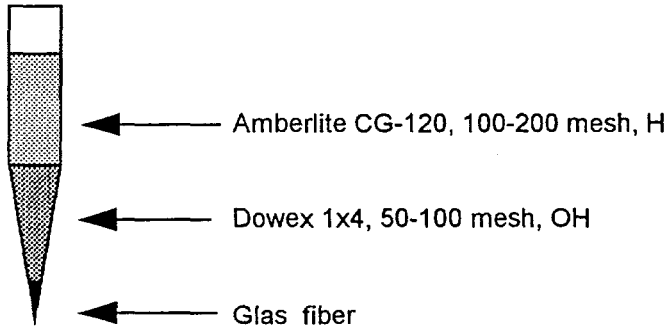

Figure B1. An illustration of the small ion exchange columns.

B3.2 Procedure

- The column was drained and placed in a 5 mL volumetric flask.
- 2x1 mL supernatant was applied to the column and then washed with 2x1 mL water.
- The column was allowed to drain.
- The 5 mL volumetric flask was filled with water to a total volume of 5 mL.
- Prior to HPLC analysis the pH of the samples were checked (pH 1-3).

B3.3 Reagents

- 0.5 M NaOH: 20.0 g NaOH was dissolved in water in a 1 L volumetric flask.
- 1 M HCl: 82.5 mL 37% HCl was transferred to a 1 L volumetric flask and diluted with water to a total volume of 1 L.

B3.4 Dowex 1x4 (50-100 mesh)

- The Dowex ion exchange material was suspended for 1 hour in a beaker containing 0.5 M NaOH (Dowex-NaOH volume ratio: 1:5).
- The fluid was removed by decanting and water was added.
- The ion exchange material was allowed to settle.
- The fluid was removed by suction.
- This was repeated until all non-settling particles were removed.
- The ion exchange material was placed in a büchner funnel fitted with a 2 L suction flask.
- The material was filtered by vacuum using a fast filter paper.
- The ion exchange material on the funnel was regenerated with 0.5 M NaOH (Dowex-NaOH volume ratio: 1:10).
- The material washed with water until the pH of the filtrate was neutral.
- The ion exchange material was stored at 4°C and could keep for 1 month.

B3.5 Amberlite CG-120 (100-200 mesh)

- The Amberlite ion exchange material was suspended for 1 hour in a beaker containing 0.5 M NaOH (Amberlite-NaOH volume ratio: 1:5).
- The ion exchange material was placed in a büchner funnel fitted with 2 L suction flask.
- The material was filtered by vacuum using a fast filter paper.
- The material washed with water until the pH of the filtrate was neutral.
- The material was washed with 96% ethanol (Amberlite-ethanol volume ratio: 1:5).
- The ion exchange material was regenerated on the funnel with 1 M HCl (Amberlite-HCl volume ratio: 1:10).
- The material washed with water until the pH of the filtrate was neutral.
- The ion exchange material was stored at 4°C and could keep for 1½ month.

B4 HPLC Analysis

B4.1 Equipment

Pump:	Knauer HPLC-pump, model 364.00	
Columns:	Biorad Guard Column no. 125-0129	
	Biorad Aminex HPX-87H Column	
Run parameters:	Maximum pressure 10 mPa	
	Maximum flow rate 0.2 mL/min at room temperature	
	Maximum flow rate 0.6 mL/min at 63°C	
	Maximum temperature 65°C	
	pH 1-3	
Detector:	Knauer Differential Refractometer	
Integrator:	HP 3395	
Integrator set-up:	Zero = 10	Reset of base line
	ATT2 = 0	Attenuation. Set of range of peak heights
	CHT SP = 1.0	Chart speed
	AR REJ = 0	Threshold for rejection of area
	THRSH = 3	Threshold for rejection of peaks
	PK WD = 0.50	Expected peak width at half height
Integrator time:	5.000 INTG = 2	Set base line at all valleys
	7.000 INTG = 8	Turn on start/stop marks
	15.000 STOP	Stop integration
Eluent:	0.004 M H_2SO_4	

B4.2 Start-Up Procedure

- The flow [P4] (**Figure B2**) was turned off by lowering the flow rate gradually by 0.1 mL/min allowing the pressure to stabilise.
- The solvent tube was removed from the solvent and wrapped in aluminium foil.
- The solvent was treated in an ultrasonic bath for 5 minutes.
- Meanwhile the *pump* [P16] was washed with 5 mL water.
- The *detector* reference cell [D9] was filled with treated solvent with a 10 mL syringe by removing one of the ends of the plastic tube and injecting (5-10 mL) solvent until all the air was removed.

- The solvent tube was placed in the treated solvent and the *detector* outlet tube [D10] was connected to the waste container.
- The valve [P18] on the *pump* was turned left and a beaker was held under the outlet [P21].
- The purge button [P13] was pressed for approximately 2 minutes to remove trapped air from the system.
- The valve [P18] was closed by turning to the right.
- In the case the *pump* did not start ([P6] will then show a red light) the pump was reset by switching the reset button [P2].
- The flow rate was gradually raised to 0.2 mL/min and the column oven was turned on.
- The column was heated to 63°C before the flow rate gradually was raised to 0.6 mL/min.
- To allow the HPLC system to stabilise the HPLC was left running with eluent for 15 minutes.
- The *detector* was reset to 000 mV by pressing the FINE button [D5] and turning the COARSE knob [D4].

B4.3 Sample Run

- The integrator set-up parameters was checked by pressing [LIST] [LIST] on the *integrator*.
- The integrator time parameters was checked by pressing [LIST] [TIME] [ENTER] on the *integrator*.
- A 1 mL syringe was washed twice with water and once with the sample.
- A 0.7 mL sample was taken.
- The trapped air in the top of the syringe (piston end) was removed by tapping the on the syringe.
- The *injector valve* was turned to the [LOAD] position and the sample was loaded into the 20 µL loop by injection through the membrane (**avoiding injection of air**).
- The syringe was left in the membrane.
- The *injector valve* was then turned to the [INJECT] position without touching the syringe. The sample in the loop was injected on to the column.
- Simultaneously the [START] button was pressed on the *Integrator*.
- The analysis time was 15 minutes.

B4.4 Shut-Down Procedure

- The flow rate was gradually lowered [P5] to 0.2 mL/min
- The column oven was switched off.
- The *detector* outlet tube [D10] was placed in the solvent flask for recycling of eluent.
- The eluent was running continuously through the system in order to avoid the column to dry out.
- The *pump* [P16] was washed with 5 mL of water.

B4.5 Reagents

Eluent:

- 0.004 M sulfuric acid: 213 µL 95-97% H_2SO_4 was added to a 1 L volumetric flask containing some water and the volume was made up to 1 L with water.
- The eluent was filtered through a 0.45 µm filter.

Sugar Standards:

- 6 mM glucose and arabinose standard: 0.0540 g glucose and 0.0450 g arabinose was transferred to a 50 mL volumetric flask. Water was added to a total volume of 50 mL.
- 6 mM xylose standard: 0.0450 g xylose was transferred to a 50 mL volumetric flask. Water was added to a total volume of 50 mL.

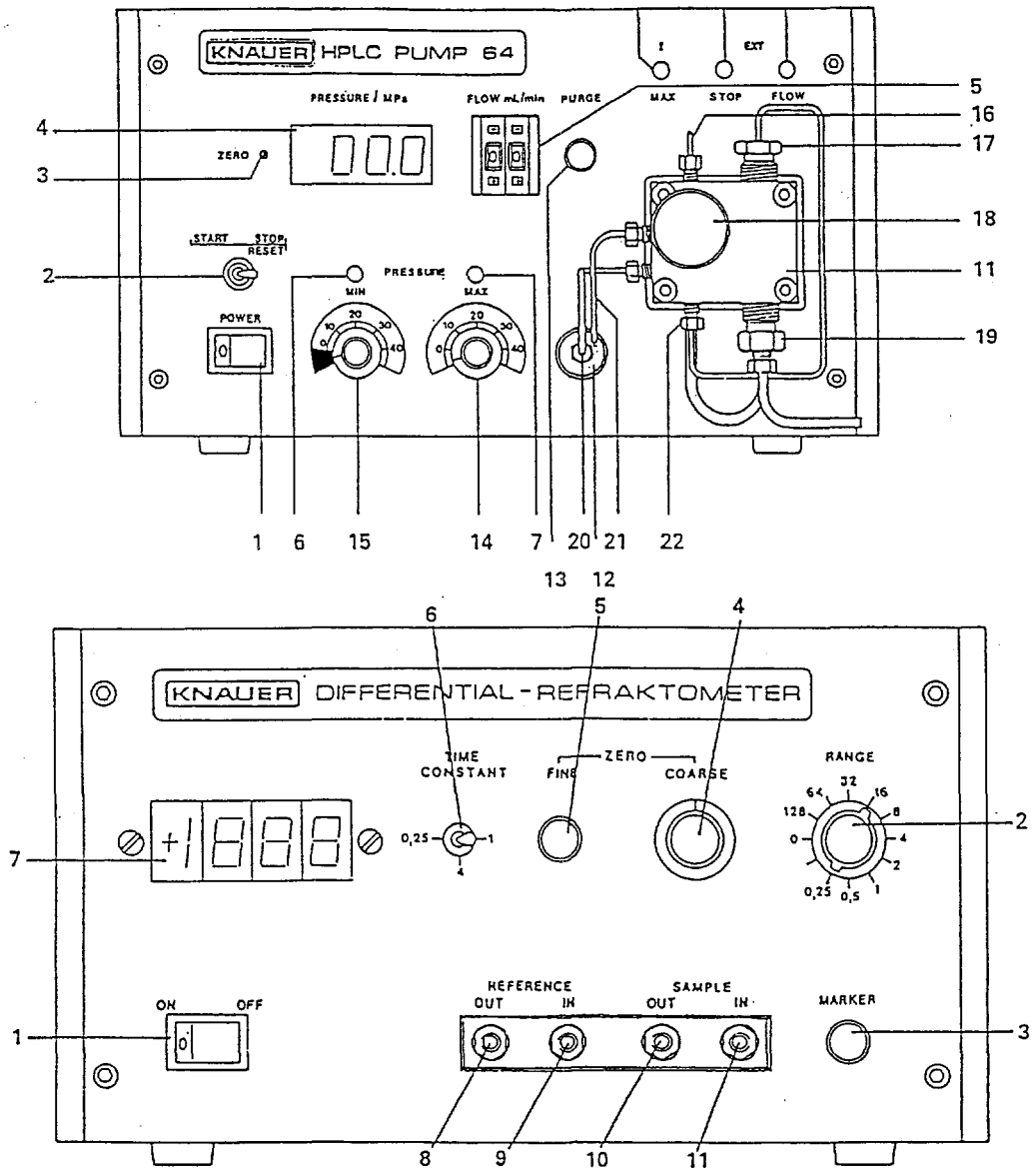

Figure B2. *The control panel of the pump [P] (Knauer, model 364.00) and the detector [D] (Knauer Differential Refractometer) used in this study.*

Bibliographic Data Sheet **Risø-R-855(EN)**

Title and author

Quantification of Solubilized Hemicellulose from Pretreated Lignocellulose by Acid Hydrolysis and High-Performance Liquid Chromatography

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Abstract (max. 2000 characters)

An investigation of the acid hydrolysis and HPLC analysis have been carried out in order to optimise the quantification of the solubilized hemicellulose fraction from wheat straw lignocellulose after pretreatment. Different acid hydrolyses have been performed in order to identify which conditions (concentrations of acid and hydrolysis time) gave the maximal quantification of the solubilized hemicellulose (measured as monosaccharides). Four different sugars were identified: xylose, arabinose, glucose and galactose. Some hydrolyses were carried out on aqueous samples and some using freeze-dried samples. The best overall hydrolysis was obtained by treatment of an aqueous sample with 4 %w/v sulfuric acid for 10 minutes. However, these conditions were not optimal for the determination of glucose, which was estimated by using a correction factor.

A purification step was needed following the acid hydrolysis, and included a sulfate precipitation by barium hydroxide and an elimination of remaining ions by mixed-bed ion exchange. The level of barium hydroxide addition significantly reduced the recovery of the sugars. Therefore, lower than equivalent amounts of barium hydroxide were added in the purification step.

For monosaccharide analysis two different HPLC columns, *i.e.* Aminex HPX-87P and HPX-87H with different resin ionic forms, lead (Pb^{2+}) and hydrogen (H^+), respectively. The lead column (HPX-87P) separated all four sugars in the acid hydrolyzates, but sample purification was laborious and required the removal of all interfering impurities, which resulted in a poor reproducibility and a sugar recovery below 50%. The hydrogen column (HPX-87H) separated only glucose, xylose and arabinose, whereas galactose was not separated from xylose; however, the column was less sensitive towards impurities and gave improved recovery and reproducibility. Therefore, the hydrogen column (HPX-87H) was chosen for routine quantification of the hydrolysed hemicellulose sugars.

Descriptors

WET OXIDATION; WHEAT STRAW; STANDARD PROCEDURE; PURIFICATION; SAMPLE PREPARATION; RECOVERY; SUGAR DEGRADATION

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