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A robust and universal NMR method for the compositional analysis of polysaccharides

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ABSTRACT

A method is presented for the detailed and accurate quantitative determination of the monomeric composition of polysaccharides. The method is a modification of the well-known Saeman hydrolysis in combination with 600 MHz ^{1}H NMR quantification. Experimental conditions for this two-step hydrolysis have been optimized for cellulose and hemicelluloses, and the method has been applied to several other polysaccharides as well. It is shown that even very resistant polysaccharides are hydrolyzed completely, while at the same time degradation of monosaccharides is kept at a minimum. The degradation of monosacharides is corrected for by subjecting a standard mixture represented in the polymer to the same conditions. This correction results in a very accurate and reproducible method with relative deviations down to 1%. It is shown that the duration of hydrolysis and the concentration of sulfuric acid in the second hydrolysis step are the most important factors to determine the reliability of the results.

1. Introduction

Polysaccharides are a very abundant and economically important class of compounds in natural products, and much research is dedicated toward their isolation, structure, function and chemical modification. Very often a precise knowledge of the monomeric composition of the polysaccharide is required, or at least desirable, and this requires a robust method for the hydrolysis of the polysaccharide and subsequent analysis of its monomers. Other important natural polymeric substances are protein and triglycerides, and in these cases very robust methods for hydrolysis, which can be applied on a routine basis, are available. There is a general consensus about the conditions of hydrolysis to decompose the polymer into its monomeric building blocks, amino acids and fatty acids, respectively. These methods are generally applicable irrespective of the type of protein or triglyceride.

Unfortunately, such a generally applicable method for the compositional analysis of polysaccharides does not exist until today. The main reason why such a method does not exist is that the building blocks of polysaccharides are rather unstable toward the harsh conditions of hydrolysis and that some glycosidic linkages require extremely harsh conditions in order to be hydrolyzed, while other glycosidic linkages can be hydrolyzed relatively easily. The methods for the quantitative analysis of polysaccharide composition make use of an acidic hydrolysis in one form or another. The most commonly employed acids are trifluoroacetic acid (TFA), hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) (Barreto-Bergter, Travassos, & Gorin, 1980; Bertaud, Sundberg, & Holmbom, 2002; Biermann, 1988; de Ruiter, Schols, Voragen, & Rombouts, 1992; Johansson et al., 2006; Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005; Saeman, Moore, Mitchell, & Millet, 1954; Sun, Lawther, & Banks, 1996). Of these acids TFA is relatively mild, and does not give rise to a great degree of monomer decomposition. Moreover, the TFA hydrolysis is very well compatible with a separation of the monomers by means of HPLC. However, TFA is not efficient enough to hydrolyze a great number of very stable polysaccharides, such as cellulose, β-glucan and chitin. HCl at high temperature, on the other hand, is more aggressive toward some of the glycosidic linkages that are difficult to hydrolyze, but gives at the same time a high degree of decomposition of the monomers, which makes a quantitative analysis quite unreliable.

Hydrolysis by means of H_2SO_4 is a more favorable method because it can hydrolyze the very strong β-(1,4) linkages of cellulose, while at the same time it is mild toward the monomers. However, since sulfuric acid is difficult to remove from the hydrolysis mixture, these solutions are less compatible with the HPLC and GLC methods that are commonly employed for the detection of mono saccharides.

It has been shown by Mittal et al. that the acidic hydrolysis with sulfuric acid can be combined with the detection by means of ^{1}H NMR (Bose, Barber, Alves, Kiemle, & Stipanovic, 2009; Mittal, Scott, Amidon, Kiemle, & Stipanovic, 2009). It is well known that ^{1}H NMR is an excellent method for quantitative purposes in all kind of samples ranging from relatively pure compounds to complex mixtures. Therefore, we investigated the combination of hydrolysis of

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polysaccharides with sulfuric acid and the quantitative detection by means of ^{1}H NMR more in depth.

The potential of sulfuric acid as an acid for the hydrolysis of cellulose was realized first by Saeman et al. (1954). He proposed a method, which starts with a presolubilization for 2 h at 20 °C in 72% sulfuric acid, followed by a dilution to 3% and a secondary hydrolysis in an autoclave for 4 h at a pressure of 15 p.s.i. Monomeric sugars were analyzed by means of paper chromatography. Since then, many authors have modified the experimental conditions of the method to make it better suitable for their own purposes. For instance Martinez, Martinez, and Almendros, 1986 analyze fungal β-glucan by a presolubilization in 72% sulfuric acid for 30 min at 5 °C followed by a secondary hydrolysis for 7 h in 1 M sulfuric acid at 105 °C, and Renard, Lahaye, Mutter, Voragen, and Thibault, 1998 prefer a presolubilization at 20 °C for 1 or 3 h followed by a secondary hydrolysis in 1 M sulfuric acid at 100 °C for 3 h for the analysis of pectin. Recently, Mittal et al. (2009) proposed for the analysis of wood mass a presolubilization in 72% sulfuric acid at 40 °C for 1 h and a second hydrolysis for 1 h at 121 °C in 4% sulfuric acid. These authors use ^{1}H NMR for the quantitative detection of monosaccharides in the final hydrolyzate.

It is the purpose of the present investigation to explore systematically the optimal conditions for the sulfuric acid hydrolysis (Saeman hydrolysis) to give maximal hydrolysis of glycosidic linkages of polysaccharides, while at the same time minimizing the degradation of monosaccharides. In addition, a correction for the degradation of the monosaccharides was added. This correction makes the method robust and applicable to polysaccharides containing labile monosacharides and uronic acids. Arabinoxylan (hemicellulose) and cellulose were chosen as model compounds to optimize the following parameters: time and temperature of the pre-hydrolysis and time and sulfuric acid concentration of the secondary hydrolysis. These parameters were varied by means of an experimental design, and the results were analyzed statistically to choose the optimal conditions.

Cellulose is a good model compound for resistant polysaccharides that require harsh conditions to obtain 100% degree of hydrolysis, while arabinoxylan is a good example of a polysaccharide with labile monomeric constituents (xylose) that requires more mild conditions. Finally, it is shown that the method is very suitable for products containing these polysaccharides such as wheat straw, acid pretreated wheat straw, and corn fiber. The method is applied to yeast β-(1,3),(1,6) glucan, linear xylan, yeast mannoproteins and starch. This group of polysaccharides comprises both very resistant compounds (glucan) and compounds with labile monomers (xylan), which adds more confidence that the hydrolytic conditions chosen here are applicable to a wide range of other polysaccharides as well.

White biotechnology, also called industrial biotechnology, has large potential to substantially impact industrial production and thereby contribute to a more sustainable future. Reductions in greenhouse gas emissions, energy, and water usage are examples of the benefits brought about by cleaner, greener, and simpler bioprocesses. White biotechnology can also reduce the dependency on fossil fuels through the utilization of renewable resources. One of the most interesting products produced by white biotechnology is bio-ethanol. In this case sugars are converted by fermentation to ethanol (Sarkar, Ghosh, Bannerjee, & Aikat, 2012). However, some carbohydrates sources might compete with the food chain. In order to circumvent it, agricultural waste can be used as carbohydrate source. Most of agricultural wastes are rich in cellulose and hemicellulose which forms the plant cell walls. These polysaccharides are enclosed in a network with lignin and difficult to access for both chemical and enzymatic hydrolysis. The method optimized in this report was applied to a series of agricultural waste samples. These samples have in some cases an acid or steam pre-treatment to loosen up the lignin network.

Furthermore, it is shown that quantitaive ^{1}H NMR is an easy and accurate method for the analysis of the complicated mixtures in a strongly acidic environment that result from the Saeman hydrolysis of polysaccharides.

2. Materials and methods

Deuterated solvents were purchased from Cambridge Isotope Laboratories. Crystalline cellulose was purchased from Sigma. Arabinoxylan was purchased from Megazymes. The 72% D_2SO_4 in D_2O (w/w) were freshly prepared. DSS was added to D_2O for calibration of the spectra. The biomass samples were lyophilized and milled for 30 s with a Janke & Kunkel analysis knife mill type A10 prior hydrolysis. A solution of approximately 10 mg/ml maleic acid of known purity in D_2O was used as internal standard for the NMR quantification experiments. Note, that the amount of maleic acid was accurately weighed to within 0.01 mg.

2.1. Experimental design

The experimental design was perfomed using Statgraphics version 5.1. A Box–Behnken design in 3 blocks with 3 center points was chosen. For practical reasons only the following variables were investigated:

- Duration presolubilization step: 20–60 min.
- Temperature presolubilization step: 30–70 °C
- Duration hydrolysis step: 60–120 min
- Sulfuric acid concentration hydrolysis step: 5–10%

Approximately 40 mg of each sample were accurately weighed in a head space vial. For the presolubilization 0.5 ml of 72% D_2SO_4 in D_2O (w/w) was added to the samples. The samples were sealed and stirred in a water bath for different times and temperatures, according to the experimental design conditions. After this step, D_2O was added to the samples until the desired final D_2SO_4 acid concentration was reached. The samples were sealed and incubated at 100 °C for different times, according to the corresponding condition of the experimental design.

After the hydrolysis, the samples were allowed to cool down to room temperature. Then 1 ml of maleic acid internal standard solution was added. If the sample contained insoluble matter after hydrolysis, it was centrifuged and the clear supernatants were measured with NMR. If the sample was clear, it was directly measured by NMR.

Sugar analysis was performed by quantitative ^{1}H NMR spectroscopy. The spectra were recorded at 280 K with a Bruker DRX 360 MHz equipped with a 5 mm triple resonance *z*-gradient probe. HOD suppression was obtained by pulse program zgcppr with 90 degrees pulse, and a mild water suppression power level corresponding to 10 Hz. For quantification a relaxation delay of 40 s was used.

Cellulose concentration was calculated applying Eq. (1). The sum of the integrals of the α- and β-anomeric proton peaks at 5.25 and 4.67 ppm and the integral of the maleic acid protons at 6.1 ppm were used for this purpose. Arabinoxylan was determined by the sum of the xylan and arabinan. Xylan and arabinan amounts were calculated using the peaks at 5.21 and 4.59 ppm and 5.26 and 4.55 ppm, respectively. The molecular weight used for cellulose was 162 and for arabinoxylan was 132. These values represent the MW of the monomeric unit in the polymer.

$$P_x = \frac{A_x}{A_{st}} \times \frac{n_{st}}{n_x} \times \frac{MW_x}{MW_{st}} \times \frac{W_{st}}{W_x} \times P_{st} \quad (1)$$

Table 1
Example of anomeric ratios described in literature.

monosaccharide	α-Furanose	β-Furanose	α-Pyranose	β-Pyranose
Glucose	–	–	0.38	0.62
Arabinose	0.025	0.002	0.60	0.355
Galactose	0.03	0.04	0.29	0.64
Xylose	<0.01	<0.01	0.365	0.63
Mannose	0.01	<0.01	0.66	0.33

A_x = integral peak of product, A_{st} = integral of internal standard peak, n_{st} = number of protons corresponding to the internal standard peak, n_x = number of protons corresponding to the product peak, MW_x = molecular weight of product, MW_{st} = molecular weight internal standard, W_{st} = weight internal standard, W_x = weight sample, P_{st} = purity internal standard.

2.2. Optimum hydrolysis procedure

Approximately 40 mg of each sample was accurately weighed in a head space vial. For the presolubilization 0.5 ml of 72% D_2SO_4 in D_2O (w/w) was added to the samples. The samples were sealed and stirred in a water bath at 30 ± 3 °C for 60 min. After this step, 3.1 ml of D_2O was added to the samples until the final concentration of 10% D_2SO_4 was reached. The samples were sealed and incubated at 100 ± 3 °C for 90 min.

After the hydrolysis, the sample was allowed to cool down to room temperature. Then 1 ml of maleic acid internal standard solution was added. If the sample contained insoluble matter after hydrolysis, it was centrifuged and the clear supernatants were measured with NMR. If the sample was clear, it was directly measured with the NMR. The final solution was pipetted into a 3 mm NMR tube.

The ^{1}H NMR spectra were recorded at 290 K with an Avance III 600 MHz equipped with a 5 mm triple resonance cryoprobe. HOD suppression was obtained by pulse program zgcppr with 90 degrees pulse, and mild water suppression of 10 Hz. For quantification a relaxation delay of 40 s was used.

The polysaccharide concentrations were calculated using at least one of the anomeric signals and Eq. (2), (the sugar recovery standard, SRS, is described in the following section. If only one of the anomeric peaks was well resolved for calculation (Fig. 1), the number of protons corresponding to the product was used taken into account the anomeric ratio of each monosaccharide, for examples see Table 1 (Köpper & Freimund, 2003; Pigman & Horton, 1980; Zhu, Zajicek, & Serianni, 2001).

$$P_x = \frac{(A_x/A_{st}) \times (n_{st}/n_x) \times (MW_x/MW_{st}) \times (W_{st}/W_x) \times P_{st}}{1 - D_x/100} \quad (2)$$

A_x = integral peak of product, A_{st} = integral of internal standard peak, n_{st} = number of protons corresponding to the internal standard peak, n_x = number of protons corresponding to the product peak, MW_x = molecular weight of product, MW_{st} = molecular weight internal standard, W_{st} = weight internal standard, W_x = weight sample, P_{st} = purity internal standard, D_x = Degradation factor (obtained from SRS Eq. (3))

Table 2
Parameters varied in optimization.

Parameter	Range
Duration presolubilization step (A)	20–60 min
Temperature presolubilization step (B)	30–70 °C
Duration hydrolysis step (C)	60–120 min
Acid concentration hydrolysis step (D)	5–10% (w/w)

2.3. Sugar recovery sample (SRS)

To 3.1 ml of a solution containing a known amount of glucose, galactose, mannose, glucosamine, xylose, arabinose (app. 7 mg/ml) was added 0.5 ml of a 72% solution of D_2SO_4 in D_2O (final acid concentration 10% sulphuric acid). This sample and the polysaccharide samples were incubated during the second hydrolysis step (at 100 ± 3 °C for 90 min).

After the hydrolysis, the sample was allowed to cool down to room temperature. Then 1 ml of maleic acid internal standard solution was added. If the sample contained insoluble matter after hydrolysis, it was centrifuged and the clear supernatants were measured with NMR. If the sample was clear, it was directly measured with the NMR. The final solution was pipetted into a 3 mm NMR tube.

A SRS solution was measured before hydrolysis and after hydrolysis conditions in order to determine the percentage of degradation. The percentage degradation of each monosaccharide is calculated according Eq. (3).

$$D_x = \frac{\left(\left((A_x/A_{st}) \times (n_{st}/n_x) \times (MW_x/MW_{st}) \times (W_{st}/W_x) \times P_{st} \right)_{\text{before hydrolysis}} - \left((A_x/A_{st}) \times (n_{st}/n_x) \times (MW_x/MW_{st}) \times (W_{st}/W_x) \times P_{st} \right)_{\text{after hydrolysis}} \right)}{\left((A_x/A_{st}) \times (n_{st}/n_x) \times (MW_x/MW_{st}) \times (W_{st}/W_x) \times P_{st} \right)_{\text{before hydrolysis}}} \times 100 \quad (3)$$

3. Results

Two model polysaccharides were used for the optimization of the Saeman Hydrolysis procedure. Crystalline cellulose was chosen a model system because the β-1,4 glycosidic linkage needs harsh conditions for hydrolysis. In addition, this polysaccharide is highly insoluble. Arabinoxylan was used as model polysaccharide for the optimization because, contrary to cellulose, the glycosidic linkage in arabinoxylan can be hydrolyzed by much milder conditions; while at the same time the formed monosaccharides, such as xylose, are very acid labile. Furthermore these two polysaccharides are the main constituents of most plant cell walls. Plant cell wall polysaccharides from agricultural waste are a very attractive source of carbohydrate for the fermentative production of bioethanol (Sarkar et al., 2012). Therefore, the two model molecules are even more interesting if one wants to determine the polysaccharide content in different agricultural waste biomass.

3.1. Hydrolysis optimization

The complete hydrolysis of polysaccharides by the Saeman hydrolysis is controlled by 6 factors, i.e. concentration, time and temperature of the presolubilization, and concentration, time and temperature of the secondary hydrolysis. The systematic variation of all factors would require an enormous number of experiments. Therefore, the following simplifications were introduced. First of all only four factors were varied (see Table 2). A range was chosen for each factor. For example, even a variation of only three points within these ranges would still give rise to 3^4 experiments. This number was reduced to 33 experiments by means of the Box–Behnken experimental design (supplementary material).

The two factors that were kept constant were: the acid concentration of the presolubilization step (72% D_2SO_4 w/w) and the

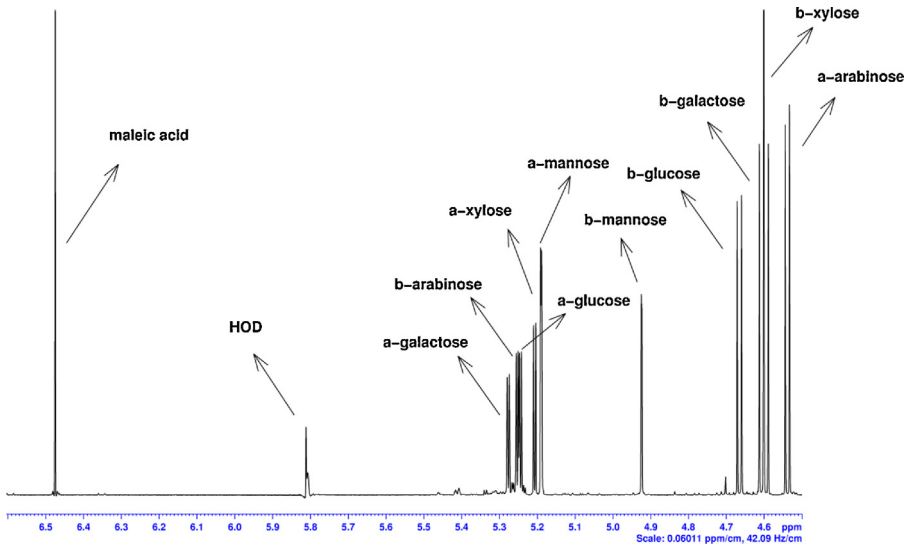

Fig. 1. 600 MHz ^{1}H NMR spectrum of the anomeric region of a SRS sample with galactose, glucose, arabinose, xylose and mannose.

temperature of the secondary hydrolysis. 72% of D_2SO_4 was chosen because at lower acid concentration only minor amounts of cellulose was solubilized even after 3 h incubation (data not shown). The choice of the temperature for the hydrolysis step was based on practical reasons and comparison to literature. The cellulose content in each experiment was calculated as described in the Eq. (1).

The total concentration of cellulose recovered varies from 41% to 90%. Pareto charts analysis of the data clearly shows that the temperature of the presolubilization step is the factor that has the strongest influence on the final hydrolysis results (Fig. 2). Interaction plots show that the temperature and duration of the presolubilization step have great influence on each other (Fig. 3). Accordingly, if the presolubilization is performed at the shorter time (curve with −) increasing the temperature will increase the total performance and if the presolubilization is done at the long incubation time (curve with +) an increase in the temperature will decrease the total hydrolysis performance.

The data was fitted in a model. ANOVA analysis of the data results in the R^2 of 95.6668 and a standard error of the estimation of 3.6442. Furthermore, this model predicted an optimal set of hydrolysis conditions (Table 3). Next, the optimal parameter set (rounded for integer) was used for hydrolysis of cellulose. Comparison between the interpolated predicted cellulose (97.5%) content and the obtained cellulose (92.2%) under these conditions showed a difference of about 5% (see Table 3).

This difference is slightly larger than the standard error of the model, however once moisture and ashes of the cellulose sample are taken into account (ashes 3% and moisture 2%) a very close mass balance was obtained. In addition, even under the optimized conditions some degradation products of the released monosaccharides, namely furfural and HMF (5-hydroxymethylfurfural), were observed.

Table 3
Calculated optimum parameters and real measurement with the optimum parameters set for cellulose.

Parameter	Calculated	Measured
Duration presolubilization step (min)	60.0	60 ± 5
Temperature presolubilization step (°C)	30.0	30 ± 3
Duration hydrolysis step (min)	94.1061	90 ± 5
Acid concentration hydrolysis step (°C)	9.9905	10 ± 1
Cellulose (w/w %)	97.5213	92.2
Cellulose (with SRS correction)	n.d.	97.5
Arabinoxylan (w/w%)	n.d.	72.1
Arabinoxylan (with SRS correction)	n.d.	78.5

n.d: not determined.

The optimized parameters set for cellulose was applied to arabinoxylan in order to evaluate the validity of the method for this polysaccharide. Arabinoxylan hydrolysis liberates xylose and arabinose. These monosaccharides are known to be acid labile. The yield of arabinoxylan after hydrolysis according to the optimized method was 72.1%, which is the sum of liberated arabinan and xylan. This rather low yield could partially be attributed to a high content of inorganics and water 19.5% ashes and 2.5% water. After correction for these contaminants the yield was 94%, which indicates that some 6% of the monosaccharides were degraded during the hydrolysis. This observation was confirmed by the large amounts of furfural and HMF observed in the NMR spectra of the hydrolyzates.

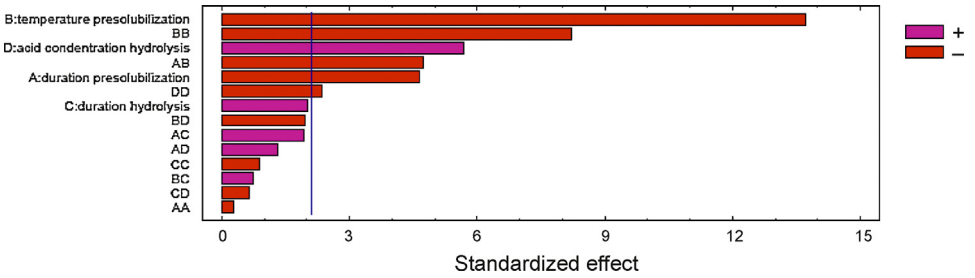

Fig. 2. Standardized Pareto chart of the cellulose optimization experiments.
(A) Duration presolubilization step; (B) temperature presolubilization step; (C) duration secondary hydrolysis step; (D) acid concentrations econdary hydrolysis step. The double letter are the interactions between the two factors.

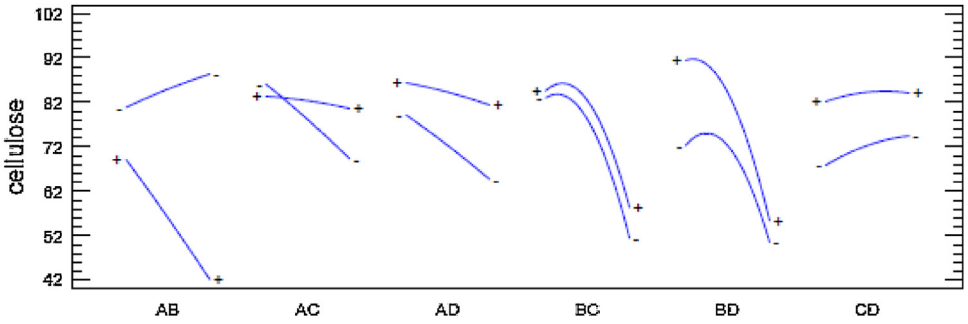

Fig. 3. Interactions plot of the cellulose optimization experiments.
(A) Duration presolubilization step; (B) temperature presolubilization step; (C) duration secondary hydrolysis step; (D) acid concentrations econdary hydrolysis step. The double letter are the interactions between the two factors.

As a consequence, a correction for the monosaccharide degradation would further improve the reliability of the method.

In order to get more insight on which moment monosaccharide degradation occurs during hydrolysis, samples taken after the presolubilization of cellulose and arabinoxylan were analyzed by NMR spectroscopy. In this case, minor amounts of furfural and HMF were observed. These results indicate that the major degradation of monosaccharides occurs during the hydrolysis step. Consequently, it is logical to introduce a correction only to the second step of degradation. A possible correction procedure could be based on the quantification of the formed degradation product such as furfural and HMF. However, in the case of heteropolysaccharides the degree of degradation is not the same for all constituent monosaccharides. In addition, in the case of uronic acid other products are formed, such as lactones. Therefore, we have chosen to add a Sugar Recovery Standard (SRS) sample to the secondary hydrolysis step. The SRS corresponds to a solution of known concentration of the monosaccharides which is incubated simultaneously according to the hydrolysis conditions in the second step of the procedure (10% deuterated sulfuric acid in D_2O, 90 min at 100 °C). The concentration of the monosaccharides in the SRS after the hydrolysis procedure was determined; and the percentage of degradation of each monosccharide was determined. The degradation of the monosaccharide forming cellulose and hemicelluloses varies between 5 and 20%, and there was in some cases a large difference between two consecutive analysis. Typical values of degradation percentages are shown in Table 4. These values may be used as a standard correction factor, however, it is recommended to determine the factors each time, a hydrolysis is performed, because they depend on the final acid concentration, which is subject to experimental error. After SRS correction, the total cellulose and arabinoxylan in the model samples were calculated as 96.3% and 78.5%, respectively.

3.2. Application of the method to diverse polysaccharides

Next, the procedure optimized in the previous section was applied to a series of compounds of different nature (Table 5). The carbohydrate amount was determined following the procedure presented above. In addition, the ashes and water content were determined according to standard procedures, and in one case (mannoprotein) also the protein content was determined by means of the Kjeldahl method and the conversion factor %protein = 6.25 × %N (total N base on method ISO (20483:2006), 2006). In all cases an SRS was used for monosaccharide degradation correction. In all cases >90% of the compound composition

Table 4
Typical degradation percentage of different monosaccharides.

Monosaccharides	Before hydrolysis (w/w %) (according Eq. (1))	After hydrolysis (w/w %) (according Eq. (1))	Degradation percentage (according Eq. (3))
Glucose	100.7	96.4	4.3
Xylose	99.8	88.8	11.0
Arabinose	97.9	91.0	6.8
Galactose	97.8	93.1	4.7
Mannose	99.3	92.6	6.7

Table 5
Carbohydrate content in w/w % of starting material.

Compound	Glucose	Xylose	Mannose	Glucosamine	Arabinose	Ash[b]	Moisture[b]	Protein[c]	Sum
β-Glucan[a]	77.7	<0.1	<0.1	<0.1	<0.1	4.1	8.6	n.d.	90.4
Oat xylan[a]	20.1	57.6	<0.1	<0.1	5.9	3.7	3.7	n.d.	91
Mannoprotein[a]	<0.1	<0.1	49.2	<0.1	<0.1	10.1	6.3	30.4	96
Maize starch[a]	87.0	<0.1	<0.1	<0.1	<0.1	3.6	8.3	n.d.	98.9
Rnase B	<0.1	<0.1	4.8	1.4	<0.1	n.d.	n.d.	n.d.	6.2

[a] It should be noted that the carbohydrates were expresses in w/w% of the polysaccharide or proteoglycan. Each monomeric unit of the polymer has a molecular weight, which is 18 Da lower than the molecular weight of the measured free monosaccharide. Thus, MWx in Eq. (1) must be 162 for hexoses (glucose and mannose), 161 for hexoamines (glucosamine), and 132 for pentoses (arabinose and xylose).
[b] Determined by thermogravimetric analysis.
[c] Determined by total N with Kjeldahl method. The residual material are mainly composed of lignin (20–30%), protein (about 10%), ashes (10% to 15%) and other small unknown compounds (data not shown).

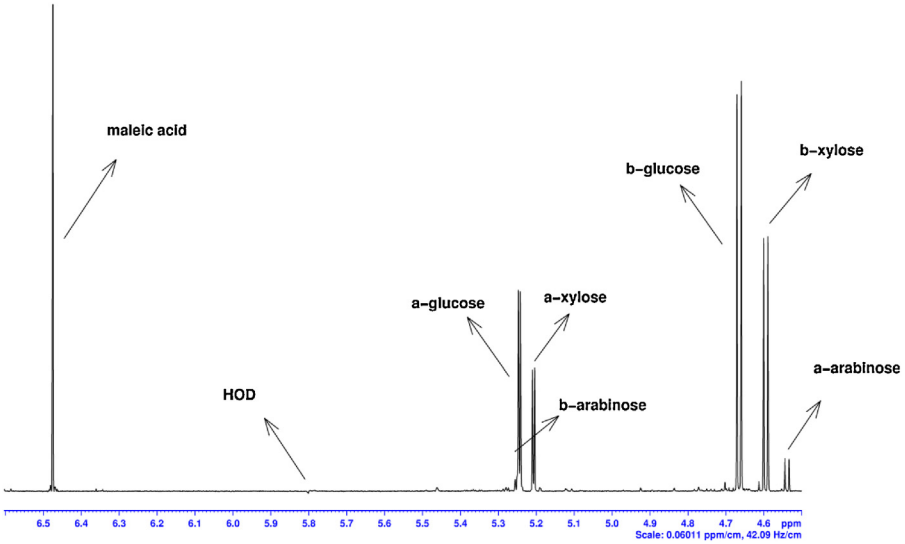

Fig. 4. 600 MHz ^{1}H NMR spectrum of the anomeric region of acid pretreated wheat straw after hydrolysis after hydrolysis.

Table 6
Carbohydrate content in agricultural wastes (w/w %). It should be noted that the carbohydrates were expresses in w/w% of the polysaccharide. Each monomeric unit of the polymer has a molecular weight, which is 18 Da lower than the molecular weight of the measured free monosaccharide. Thus, MWx in Eq. (1) must be 162 for hexoses (glucose and mannose), 161 for hexoamines (glucosamine), and 132 for pentoses (arabinose and xylose).

Sample	Glucan	Xylan	Arabinan	Galactan	Sum
Wheat bran	39.6	11.2	6.1	1.6	58.5
Washed wheat brand	36.3	13.9	7.5	0.6	58.3
Corn fiber	28.3	20.9	12.1	3.3	64.6
Washed corn fiber	24.0	24.8	14.2	2.4	65.4
Acid pretreated wheat straw	35.6	14.2	2.0	<0.1	51.8
Washed acid pretreated wheat straw	54.6	2.6	<0.1	<0.1	57.2
Acid pretreated corn stover	34.3	18.2	2.4	<0.1	54.9
Washed acid pretreated corn stover	55.2	5.4	<0.1	<0.1	60.6

could be explained. In the case of Rnase B, where only a small amount of the weight composition corresponds to carbohydrate, we were still able to determine the carbohydrate content. The mannose/glucosamine ratio in the sample corresponds to an average of 6.8/2 per glycan. This result is in excellent agreement with the values reported in literature for Rnase B (Plummer & Hirs, 1964).

Table 6 shows the results obtained for the investigated agricultural wastes. Wheat bran and corn fiber were analyzed directly after milling. Other samples were pretreated in the following way: First, the samples were washed with hot water (70 °C), then freeze-dried and finally, the residue was milled before the hydrolysis according to the optmized method. From Table 6 it appears that the washing procedure removes glucan from the wheat bran and corn fiber. This reduction might be explained by the loss of residual starch in the samples. The industrial mild acid pretreatments results in the complete hydrolysis of arabinoxylan to corresponding monosaccharides and the degradation products such as furfural, HMF, formic, levulinic and acetic acids. In these cases, a strong reduction of the xylan and arabinan fraction was observed when the samples were washed. These water soluble carbohydrates and degradation products are washed away, which results in a higher glucan/cellulose concentration in analyzed sample. These results show that the optimized method is very versatile and can be applied to a large variety of polysaccharides.

Finally, the reproducibility of the method was tested with both washed untreated agricultural wastes, washed acid pretreated wheat straw. To this end, each sample was analyzed in 5-folds at three consecutive days. The relative deviations in all cases were lower than 4.8%, and the day-to-day variation is lower then 5% (supplementary material). This means that when an experiment is performed on a random day, the average of a duplo measured and a sample of the duplo on a 95% confidence interval must be within 2 times the standard deviation (Fig. 4).

4. Conclusions

A universally applicable method for the hydrolysis of polysaccharides was presented. The method gives excellent results for such different products as: cellulose, arabinoxylan, glucan, xylan and glycosylated proteins like mannoproteins and RNase B. The conditions of this method, which is based on the Saeman hydrolysis, are as follows: First a presolubilization for 60 min at 30 °C in 72% deuterated sulfuric acid. Next, the solution is diluted to 10% and hydrolysis is performed for 90 min at 100 °C.

Hydrolysis of even the most stable polysaccharides such as cellulose and glucan is complete, while at the same time degradation of monosaccharides under these conditions is generally limited to 4–11%, with exception of fructose, uronic acids and sialic acid (data not shown). A reference solution of monomers provides the precise correction factors for this degradation, which improves the accuracy of the method.

Quantitative detection of the monosaccharides is achieved by 360 MHz or higher field ^{1}H NMR. Due to the high ionic strength of the sample, the 90 °C pulse at high fields are very long. Therefore, to reduce the 90 °C pulses during measurement to normal ranges we advise to use 3-mm tubes.

Finally, the experimental design demonstrated in this report is a strong methodology to optimize the hydrolysis parameters set for a more dedicated analysis. The optimization can be performed

within three days resulting in a parameter set fine-tuned for very special polysaccharides.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.02.036.

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