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An improved methodology for the quantification of uronic acid units in xylans and other polysaccharides

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Abstract—Uronic acids can be quantified either by a colorimetric determination after treatment with concentrated sulfuric acid and carbazole or by gas chromatography after methanolysis and subsequent acetylation. Both methods suffer from incomplete hydrolysis, an unavoidable degradation of the products to be analysed, and an inability to separate and quantify different types of uronic acids. In the present work, the fundamental chemistry involved in the two methods has been evaluated, and some modifications to increase their accuracy are suggested. By combining the two methods, a complete quantification of all individual types of uronic acids present in a sample can be achieved.

Keywords: Uronic acid; Carbazole; Methanolysis; 4-*O*-Methylglucuronic acid; Galacturonic acid; Glucuronic acid; Hexenuronic acid; Xylan

1. Introduction

Hemicelluloses are major components of lignocellulosic material. Their amounts and structures are of importance in studies of the chemistry of food, wood and other natural biomass resources. In analytical work on hemicellulose structures, identification and quantification of uronic acid groups are often required since such groups frequently occur in many types of hemicelluloses. For example, in xylans, which contribute some 7–10% and 5–30%, respectively, by weight in softwoods and hardwoods, the xylan chain is substituted with 4-*O*-methyl D-glucuronic acid (4-*O*-Me-α-D-Glc*p*A) (4-Me-GlcA) side groups to a considerable extent.[1] A quantification of these is often desirable, for example, in order to identify chemical changes occurring during kraft pulping. In other polysaccharides containing uronic acid groups, a quantification is also often important since these groups may exert a dominant influence on the chemical, physical and physiological properties of the polymer.

The most commonly applied methods for the quantification of uronic acid groups in xylans, for example, are the colorimetric uronic acid assay[2] and the methanolysis method.[3,4] Both these methods have advantages over, for example, enzymatic methods in terms of simplicity, rapidity and lack of any need for special instruments or chemicals. The methods are, however, strictly dependent on the operating conditions and therefore very empirical. In addition, chemical changes in the uronic acid structure encountered during different types of chemical processing, storage or biochemical conversion of a polysaccharide may mean that it escapes detection by either of the two methods. Hexenuronic acid (HexA), derived from the native 4-*O*-methyl-D-glucuronic acid (4-MeGlcA) in wood xylans and formed during kraft pulping,[5–7] for example, cannot be directly detected by either the colorimetric or the methanolysis method. The presence of HexA and other uronic acids in pulps is, however, of great technical interest since such groups exert a pronounced influence on the bleaching process and on properties such as paper strength[8] and brightness reversion.[9] In fact, the failure of the colorimetric method

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to distinguish between HexA and its precursor 4-MeGlcA may be one of the explanations for the long delay between the first report on HexA[5] and its actual confirmation in kraft pulps.[7] Even today, there are misconceptions as to whether or not the data from the colorimetric uronic acid group analysis include HexA.[10] The fact that softwood xylan has been found to be chemically linked to a linear type of lignin, whereas glucomannan is connected to a branched and cross-linked lignin,[11] indicates that the presence of different types of hemicellulose, acidic or neutral, can play an important role in the biosynthesis of lignin. In the continuation of such studies, an accurate analysis of the amounts and types of uronic acid groups present in the wood xylan is an essential prerequisite.

In the present work, the details of the carbazole–sulfuric acid method have been clarified, and the method has been further improved by the application of an external standard, commercial D-galacturonic acid (GalA), which permits a total uronic acid (4-MeGlcA + HexA + GalA + Glucuronic acid (GlcA) + other uronic acids) determination in xylans. Furthermore, the methanolysis–acetylation–GC method has been modified by applying a commercial xylan, free from HexA and almost free from GalA and other uronic acids, as an external standard based on its content of 4-MeGlcA, the latter being independently determined by the carbazole–sulfuric acid method. This procedure enabled an accurate quantification of 4-MeGlcA, xylose, GalA and GlcA to be achieved. By combining the two methods, a complete quantification of all types of uronic acid groups present in a kraft pulp can be achieved.

2. Materials and methods

2.1. Materials

D-Galacturonic acid, D-glucuronic acid, xylose, xylan from birchwood (X-0502), erythritol, furfural, hydroxymethylfurfural (HMF) and 2-furancarboxylic acid (FA) were commercial products obtained from Sigma Chemical Co. 5-Formyl-2-furancarboxylic acid (FFA) was obtained from TCI Europe. All the chemical reagents used were of analytical grade.

2.2. Kraft pulping and pulp samples

Industrial birch (*Betula pubescens*/*pendula*) chips were air dried and laboratory screened, and the 2–8 mm fraction was retained and used. Chips with knots and bark were removed by hand. The selected birch chips were presteamed at 1.5 MPa for 5 min in a forced circulation digester, and the pulping liquor was then pumped into the digester. A high liquor-to-wood ratio of 75:1 L/kg was employed with the following initial concentrations: $[OH^-] = 0.4$ mol/L and $[HS^-] = 0.2$ mol/L. NaCl was added to achieve a sodium ion concentration of 1.0 mol/L and thus to simulate the ionic strength in an industrial cook. The temperature was controlled by heat exchanging steam with the circulating pulping liquor. The temperature was kept at 100 °C for 30 min and increased by around 10 °C/min from 100 °C to final temperature of 140 and 160 °C. The time at the maximum temperature was adjusted to obtain similar final kappa numbers of 16.4 and 16.0, respectively.

2.3. Analytical methods

The carbazole–sulfuric acid method was performed in a test tube on 50–400 nmol of uronic acids or 1 mg of dried pulp sample in 0.4 mL of water.[2] To the sample, 40 μL of 4 mol/L sulfamic acid–potassium sulfamate was added followed by 2.4 mL of concd H_2SO_4. When the mixture reached room temperature, 100 μL of the carbazole reagent (0.1% w/v of carbazole in EtOH) was added. The tube was placed in a boiling water bath for 20 min, followed by cooling in an ice-water bath until room temperature was reached. The light absorbance of the solution at 525 nm was measured.

Methanolysis was conducted in a sealed glass tube on 1–20 mg of dried sample as described in Ref. 3 by adding 1.2 mL of ~2.6 mol/L methanolic HCl (prepared by mixing 0.2 mL of CH_3COCl with 1 mL of dried MeOH containing 2 mg of erythritol as internal standard), and the reaction was kept for 16 h at 80 °C. After cooling, the solvent was evaporated in a stream of N_2 at room temperature. The residue was acetylated for 30 min at room temperature with 300 μL of 1:1 pyridine–acetic anhydride before analysis by gas chromatography (GC) or GC in combination with mass spectrometry (MS). GC and GC–MS analyses were carried out using a DB-5MS and an Rtx-5MS column, respectively, with helium as carrier gas. The initial temperature was 150 °C for 2 min, and it was then increased at 5 °C/min to 230 °C, followed by an increase at 25 °C/min to 330 °C. Thermo Finnigan Trace 2000 Series GC–MS was applied with Xcalibur software and NIST library. The mass spectrum was obtained at 70 eV.

High-performance liquid chromatography (HPLC) was performed using a Waters system consisting of two 510 pumps, a 717 plus autosampler, a model 996 photodiode array detector and MILLENIUM 32 software for operation control and data processing. Separations were carried out with 5 μL of solution on an ODS column (HICHROM H5ODS-3519) with a size of 4.6×150 mm using UV-detection and an isocratic mobile 7:3 H_2O–CH_3CN phase with a flow rate of 1 mL/min.

The HexA groups in the pulp samples were quantified using an HPLC method.[12] The pulp was pretreated with $Hg(OAc)_2$ and then subjected to the carbazole–sulfuric

acid colorimetric method by stirring 1 g of pulp in 25 mL of aq $Hg(OAc)_2$ (60 mmol/L), adjusted to pH 5.3,[12] at room temperature for 30 min, followed by filtration and thorough washing with water, 0.2% aq EDTA and water.

The kappa number was determined automatically according to the standard ISO 302 (second edition 2004-07-01) method by using a 'Kappamat' model K9701 instrument produced by Kappa Electronics AB in Sweden.

3. Results and discussion

3.1. The carbazole–sulfuric acid method

Uronic acid analysis by the carbazole–sulfuric acid method (the carbazole method) is still widely applied in the fields of lignocellulosics and other biopolymers. The method was first described by Dische in 1947,[13] was further improved by Galambos in 1967[14] and was extensively reviewed in 1991.[2] The basic element of the method is the addition of concentrated sulfuric acid to the test sample, followed by the addition of carbazole and heating to form a red-coloured solution with an intensity that can be related to the content of uronic acids in the sample.

The addition of concentrated sulfuric acid to a polysaccharide, followed by heating, should result in dehydration and hydrolysis reactions resulting in the formation of various furan structures as depicted in Figure 1. Of these, furfural and hydroxymethylfurfural (HMF) are known to be formed from neutral pentoses and hexoses, respectively, whereas 2-furancarboxylic acid (FA) and 5-formyl-2-furancarboxylic acid (FFA) are formed during the acid hydrolysis of HexA.[15] 5-Formyl-2-furancarboxylic acid (FFA) is also known to be formed from various other uronic acids.[16]

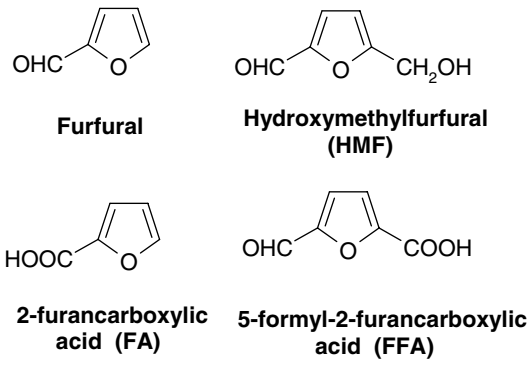

Figure 1. Furan derivatives formed through acid hydrolysis of carbohydrates; furfural and hydroxymethylfurfural (HMF) from pentoses and hexoses, respectively, 2-furancarboxylic acid (FA) and 5-formyl-2-furancarboxylic acid (FFA) from hexenuronic acid (HexA) and other uronic acids.

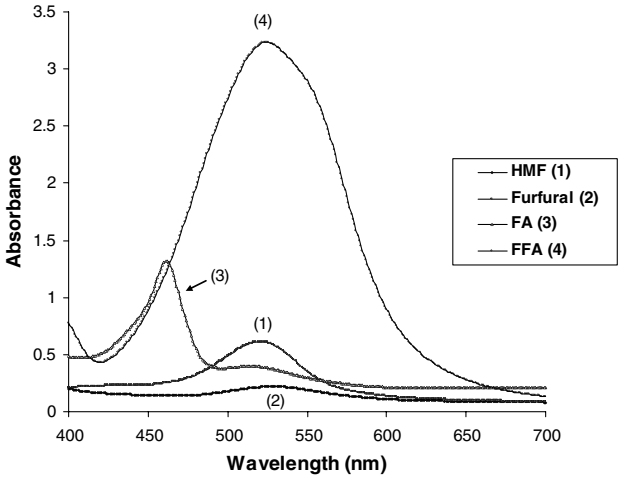

Figure 2. Light absorption spectra in the 400–700 nm range of solutions of hydroxymethylfurfural (HMF), furfural, 2-furancarboxylic acid (FA) and 5-formyl-2-furancarboxylic acid (FFA) after heating with carbazole–sulfuric acid at 100 °C for 20 min.

In order to obtain chemical information about the carbazole method, each of the furan structures was reacted with carbazole at an elevated temperature. With FFA, a red colour was rapidly formed with a maximum absorption at about 525 nm (Fig. 2), that is, at the same wavelength as that used in the analytical method.[2] Thus, in agreement with previous work,[16] the formation of FFA constitutes the qualitative foundation of the carbazole method. All the other furan structures, however, also gave rise to light absorption at about 525 nm, indicating that there is interference from both neutral sugars and other uronic acid degradation products such as FA. As shown in Figure 2, HMF from hexoses or cellulose is the most interfering structure, whereas the others are of less importance.[2]

When the carbazole method was applied on different concentrations of FFA, a linear relationship was found between the absorbance at 525 nm and the amount of FFA present (50–250 nmol) as shown in Figure 3. A similar analysis, carried out on D-galacturonic acid (GalA) also gave a straight line but with a different slope. The large differences in absorbance intensity thus mean that FFA is inappropriate as a direct calibration substance in the carbazole method for uronic acid quantification. The reason for the large discrepancy must be an incomplete conversion of GalA to FFA or, alternatively, the formation of other products from GalA in addition to FFA. In earlier work, it has been reported that the amount of FFA formed is strongly dependent on the amount of sulfuric acid added, and also that the speed of mixing of sulfuric acid with the sample plays an important role in the determination.[16] It has also been observed that the rate and extent of the formation of FFA from various uronic acids are dependent on the structural configuration of the uronic acid. The rate

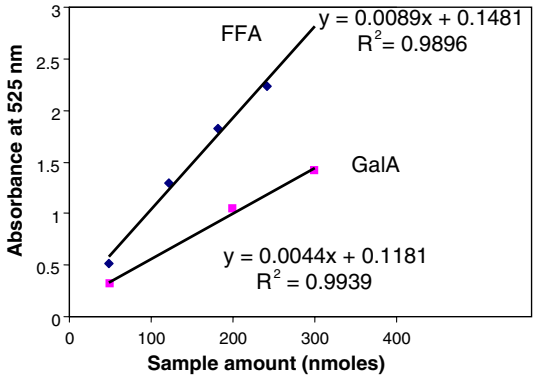

Figure 3. Light absorbance at 525 nm as a function of the amount of sample after treatment of 5-formyl-2-furancarboxylic acid (FFA) and D-galacturonic acid (GalA), respectively, with carbazole–sulfuric acid at 100 °C for 20 min.

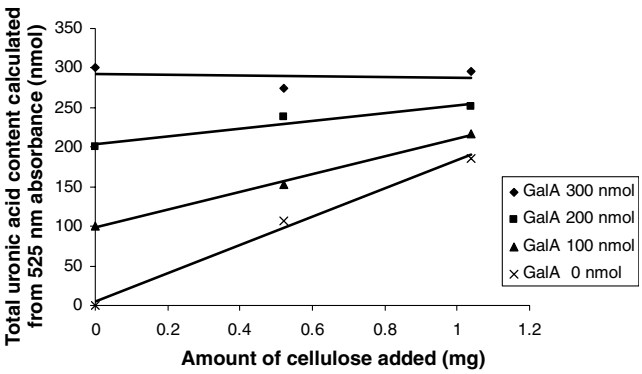

Figure 5. Uronic acids obtained from D-galacturonic acid (GalA) (0–300 nmol) by the carbazole method in the presence of cellulose (0–1.2 mg). Detection at 525 nm.

of FFA formation from either GalA or 4-MeGlcA (whether as a monomer or present in a glucosidic structure) has, however, been found to be virtually identical, which means that GalA is suitable as a calibration substance for the 4-MeGlcA groups present in xylans.[16]

To obtain further confirmation, HPLC analysis was used to monitor the formation of FFA from GalA and the stability of FFA when heated in concentrated sulfuric acid. As shown in Figure 4, GalA gave rise to a rapid formation of FFA (even without heating), reaching a maximum after about 10 min, but with the total conversion not exceeding ~60%.[16] In parallel, a slow decomposition of FFA was observed, but even after 20 min (the heating time applied in the carbazole method[2]) the original concentration had declined by only less than 5%. In a series of preliminary experiments with GalA in the concentration range of 100–400 nmol, the carbazole method was found to be sensitive to the experimental conditions such as the ratio of uronic acid to sulfuric acid, the manner and speed of mixing, the type of reaction vessel, and the manner and speed of heating. It is therefore suggested that GalA be used as an external reference, that is, treated in parallel with the sample under investigation using identical reaction conditions and equipment. Furthermore, a new calibration curve should be created on each occasion together with the sample analysis.

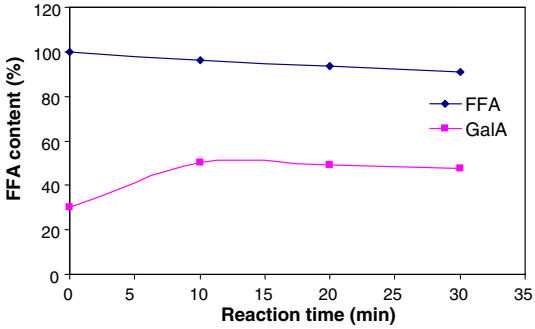

Figure 4. Yield of 5-formyl-2-furancarboxylic acid (FFA) from D-galacturonic acid (GalA) (0.25 nmol) on treatment with sulfuric acid at 100 °C for different times in comparison with the stability of FFA (0.25 nmol) under identical conditions.

The fact that HMF together with carbazole also yields a chromophore with a noticeable light absorption around 525 nm (Fig. 2) results in an inferior quantification of FFA at this wavelength.[2] In model experiments with GalA together with microcrystalline cellulose, it was, however, demonstrated that the influence of cellulose was dependent on the cellulose/GalA ratio. At a ratio of 20 or lower (in the nanomoles range), the influence of cellulose was negligible, but at higher ratios a correction factor had to be applied based on the ratio (Fig. 5). For example, in the analysis of birch kraft pulps (further described below) containing ~80% cellulose with apparent values of 150–200 μmol/g pulp of uronic acids obtained by the carbazole method, 75 μmol had to be subtracted as shown in Figure 5, to give a more accurate value as shown in Table 1.

3.2. The methanolysis method

In methanolysis, hydrochloric acid in methanol is employed to obtain a fast hydrolytic cleavage of the glycosidic linkages in a polysaccharide. The liberated monosaccharides are present as the corresponding methyl glycosides, whereas the carboxyl groups are esterified in the reaction medium. After subsequent acetylation, the products can be analysed by GC and GC–MS.

By methanolysis of a birch kraft pulp sample, the chromatogram shown in Figure 6 was obtained. Here, the 4-*O*-methylglucuronic acid (4-MeGlcA) was eluted as two major peaks, the α and β anomers, as well as two minor peaks, representing the α and β anomers with free end groups before the acetylation, that is, incompletely methanolysed products, as reported in Ref. 17

Table 1. Results from a complete uronic acid analysis of two birch kraft pulps by integrations of the carbazole–sulfuric acid and methanolysis methods in comparison with verification results by independent methods as described in the text

Sample/method	Carbazole	Methanolysis				By difference[a]
	Uronic acids, total (μmol/g)	4-MeGlcA (μmol/g)	GalA (μmol/g)	GlcA (μmol/g)	Xylan (% by weight)	HexA (μmol/g)
Pulp 1 (kappa no. 16.4)	129	60.4	14.5	9.7	17.1	44.4
Pulp 2 (kappa no. 16.0)	135	64.2	11.6	10.6	18.8	48.7
Verification by independent methods						
Pulp 1			81[b]		16.5[c]	48[d]
Pulp 2			80[b]		16.0[c]	55[d]

4-MeGlcA: 4-*O*-methyl-D-glucuronic acid; GalA: galacturonic acid; GlcA: glucuronic acid and HexA: hexenuronic acid.
[a] The difference between the result obtained by the carbazole method and the total amount of uronic acids from the methanolysis method.
[b] Carbazole method after pretreatment with $Hg(OAc)_2$ to remove all the HexA.[12]
[c] Acid hydrolysis followed by GC analysis.[18]
[d] HPLC method for HexA content.[12]

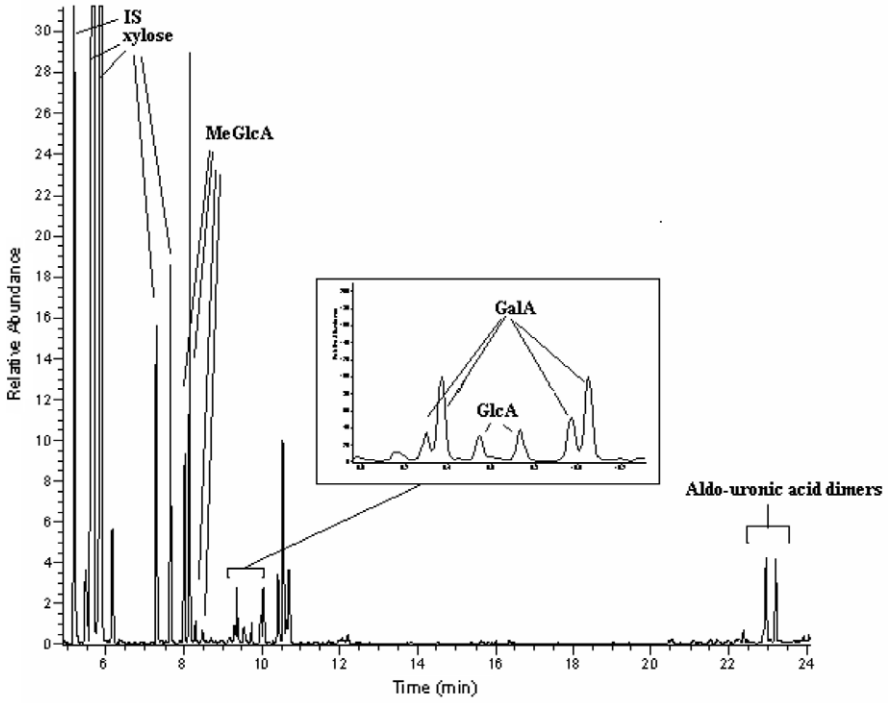

Figure 6. GC separation (ion chromatogram) of acetylated products after methanolysis (2.6 mol/L HCl/MeOH, 80 °C, 16 h) of a birch kraft pulp, with peaks assigned by the mass spectra (MS) and comparison with authentic compounds.

and the literature therein and assigned by their mass spectra. At the same time, xylose gave four peaks originating from two complete and two incomplete methanolysed isomers with a pyran ring, as assigned by the MS. In addition, substantial amounts of aldo-uronic acid dimers were found by GC–MS analysis.[3,4] From a chemical point of view, the glycosidic linkage between 4-MeGlcA and a xylose unit is much more stable than the linkage between two xylose units. The presence of aldo-uronic acid dimers, therefore, demonstrates that a large error in the analysis of both xylose and 4-MeGlcA is unavoidable using the published method. It has been demonstrated that the conditions employed in the methanolysis reaction, viz. the concentration of hydrochloric acid, the reaction time and the temperature, are critical for the hydrolysis yield and hence for the final quantification. After a comprehensive comparison of different reaction conditions, the best compromise between cleavage of the glucuronosyl bonds and degradation of the uronic acid released from the aldo-uronic acid dimer was found in Ref. 4 to be about 70% for the cleavage of the glucuronosyl linkages, whereas, at the same time, ca. 20% of the released uronic acid had been degraded.

The only possible way to achieve quantification by a reaction that suffers from both an incomplete reaction and the simultaneous degradation of a major reaction product would be by the introduction of an external reference having a behaviour virtually identical to that of

the unknown sample. Such a reference, 4-*O*-methylglucuronic acid, has been suggested,[3] but it is, in our opinion, less suitable since it has no glucosidic linkage and can therefore be expected to suffer from an even greater degree of degradation in the methanolysis reaction than a uronic acid attached to a xylan chain. As an external standard, a commercial xylan having a known content of uronic acid groups would be suitable. For this purpose, the modified carbazole method described above, employing GalA as an external standard, should be first applied on the xylan.

In our experiments, a commercial birchwood xylan from Sigma (X-0502) was subjected to methanolysis under the optimal conditions described as those giving the most complete hydrolysis of both xylan and 4-MeGlcA from beech xylan.[3] For quantification, erythritol was used as an internal standard. In a series of 11 experiments employing different amounts of xylan in the range of 11–44 mg, a linear relationship was found between the four-peak intensity from 4-MeGlcA and the amount of xylan as shown in Figure 7. The applied method can, therefore, be used, at least in this weight range, and related to the amount of 4-MeGlcA obtained by the carbazole method.

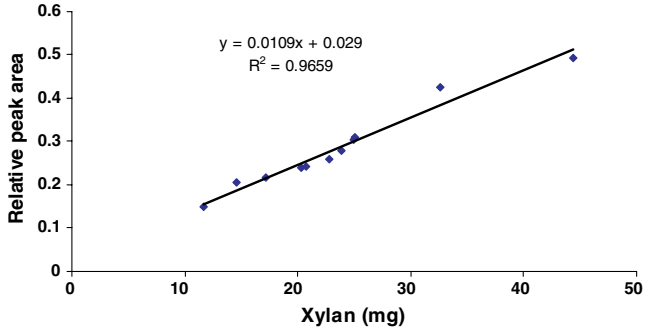

Figure 7. Total area of the four peaks originating from 4-*O*-methylglucuronic acid (4-MeGlcA) on methanolysis (2.6 mol/L HCl/MeOH, 80 °C, 16 h) of a commercial xylan using erythritol as internal standard.

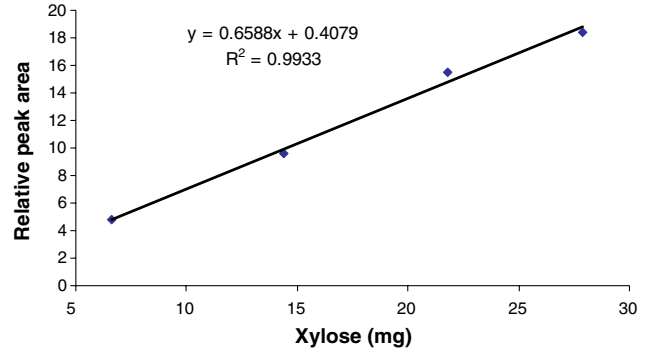

Figure 8. Total area of the four peaks obtained in the methanolysis (2.6 mol/L HCl/MeOH, 80 °C, 16 h) of xylose using erythritol as internal standard.

Data from the methanolysis of the commercial xylan were further compared to those obtained by methanolysis of pure xylose employing identical reaction conditions. Again, a straight-line relationship was obtained when the four-peak intensity from xylose was plotted against the charge (Fig. 8). From these data, an amount of xylose of 4005 (±6.8%) μmol/g of xylan was found, giving a molar ratio of 4-MeGlcA:xylan of 0.126, that is, close to the commonly accepted value of 0.10 for birch xylan.

3.3. An integrated analytical methodology for uronic acid quantification

In principle, the carbazole method should give the total amount of all uronic acid groups present in a sample that can be dehydrated to FFA by treatment with concentrated sulfuric acid. After the addition of concentrated sulfuric acid to a eucalypt kraft pulp containing ~50 μmol/g of HexA and heating, HPLC analysis of the resulting liquor revealed the presence of FFA, but no FA could be detected. Obviously, the addition of water to the aldehyde group (Fig. 9), which is required to form FA in addition to FFA, is completely suppressed in the strongly dehydrating sulfuric acid solution. This implies that a common intermediate giving rise to FFA is formed not only from GalA and 4-MeGlcA (as above) but also from HexA. It must, therefore, be assumed that the analysis of uronic acid groups by the carbazole method includes the contribution from HexA, when present—a fact that has not been reported before.[10] On methanolysis, on the other hand, HexA is so sensitive to acid decomposition that it cannot be directly methanolysed and methylated, but it is instead degraded and withdrawn from the analysis, whereas other types of uronic acid should still be detectable. Methanolysis of birch kraft pulps confirmed that FFA and FA are the degradation products from HexA. Consequently, a combination of the carbazole and the methanolysis methods should be able to provide a complete analysis of all types of uronic acids in a sample.

In order to evaluate this combination, two laboratory-prepared birch kraft pulps were analysed. Since the carbazole method includes a significant contribution from HMF formed from cellulose and other hexose-containing polysaccharides, a correction factor was applied as described above. The total amount of uronic acids was thus obtained as shown in Table 1. From the methanolysis method, the content of 4-MeGlcA was obtained directly by integration of the corresponding GC peaks using a commercial xylan as external standard, as described above (Table 1). In addition to the four peaks originating from 4-MeGlcA itself, six other peaks were, however, also found in the chromatogram. Based on

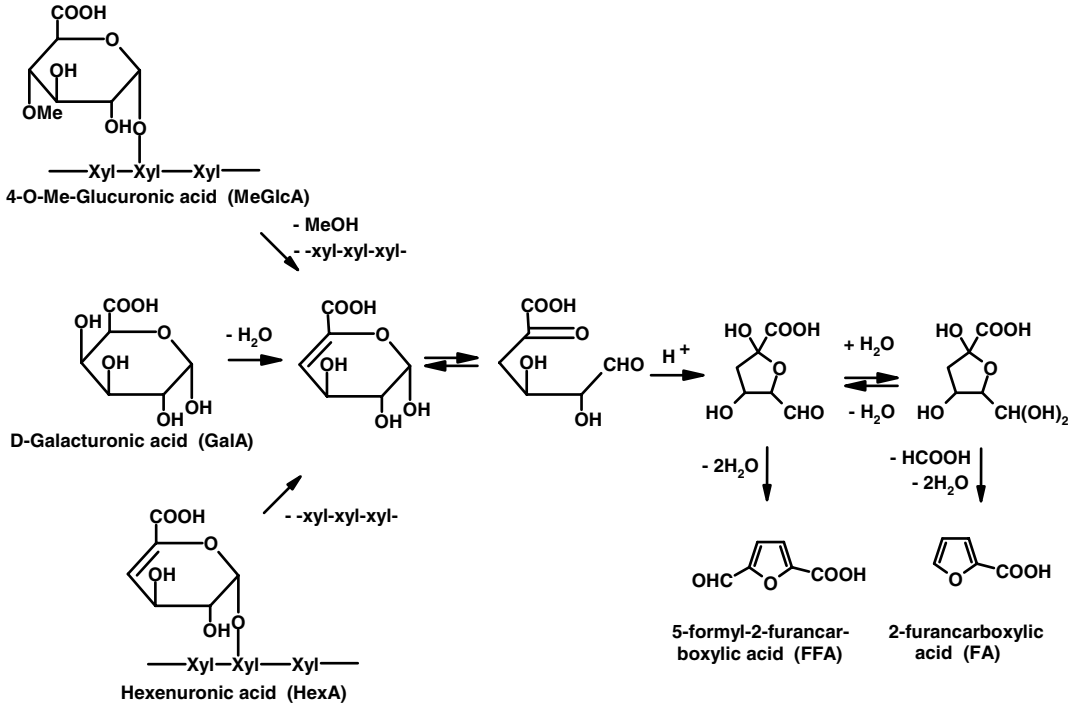

Figure 9. Structures of 4-*O*-methyl-D-glucuronic acid (4-MeGlcA), D-galacturonic acid (GalA) and hexenuronic acid (HexA), their conversion into a common intermediate on treatment under acidic conditions and further reactions to 5-formyl-2-furancarboxylic acid (FFA) and 2-furancarboxylic acid (FA).

their similar MS fragmentation patterns as from commercial D-glucuronic acid, especially with a fragment at *m*/*z* 229, which is a characteristic peak in distinguishing uronic acids from neutral sugars (Fig. 10), these were assigned to uronic acid groups. By comparing the methanolysis products of commercial GalA and GlcA, four of the peaks were assigned to originate from GalA and two from GlcA (Fig. 6). By applying the same response factors as for 4-MeGlcA, the total amounts of GalA and GlcA could be calculated for the two pulp samples (Table 1). By subtracting the sum of uronic acids obtained by methanolysis from the total amount obtained by

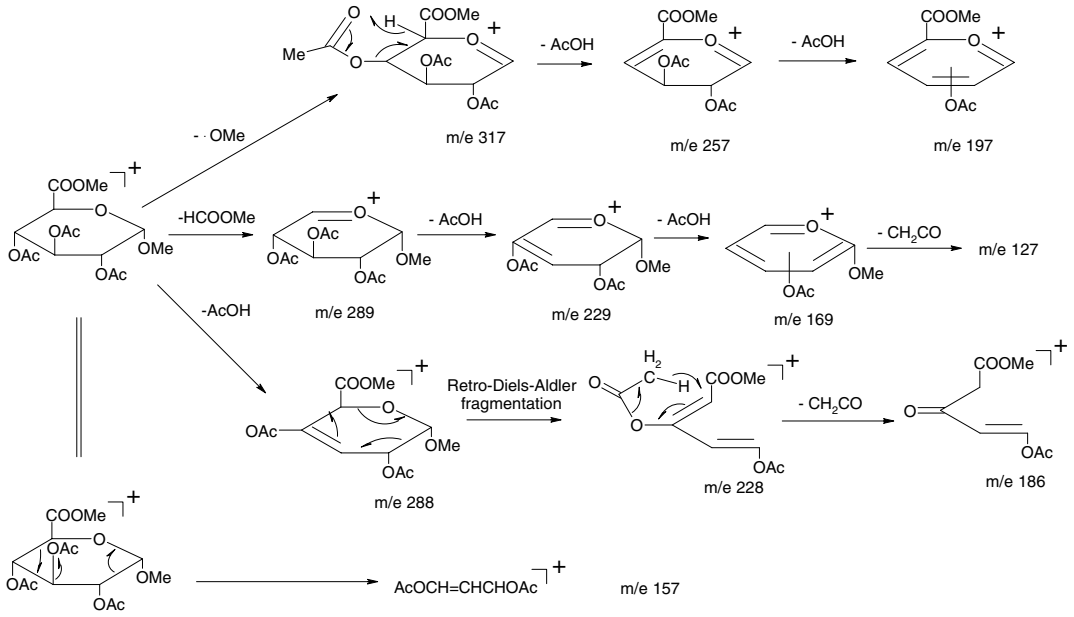

Figure 10. Mass fragmentation scheme for methyl (methyl tri-*O*-acetyl-D-glucopyranosid)uronate.

the carbazole method, a value for the HexA groups could be calculated (Table 1).

As mentioned above, the methanolysis data could also be used to calculate the xylan content in the pulps after external calibration with xylose. From these data (Table 1), the ratio of uronic acids to xylose in the two pulps was calculated, and a value of about 0.11 was obtained for the two pulps, that is, a value close to that calculated for the commercial birch xylan, as given above.

The analytical data obtained by the carbazole and methanolysis methods were also verified by analysing the two pulps with independent methods for the presence of xylan, and for HexA and 4-MeGlcA, together with GalA and GlcA, respectively. Acid hydrolysis of the pulps afforded the content of xylan,[18] HexA was analysed by a published method,[12] and all other uronic acids except HexA were determined by a modified carbazole method in which all the HexA was first selectively eliminated by treatment with mercury(II)acetate.[12] In the latter analysis, it was also necessary to correct the data for the hexoses/cellulose present as HMF, as described above. In all cases, a reasonable agreement of the data with those given by the carbazole and methanolysis methods was obtained (Table 1).

4. Conclusions

Neither the carbazole–sulfuric acid nor the methanolysis method can be used alone to directly quantify the various types of uronic acids present in the xylan portion of kraft pulps. Both analytical methods suffer from several weaknesses, and these can only be handled by a detailed knowledge of the chemistry involved in the various analyses. By combining both methods and by a careful choice of external standards, a complete uronic acid analysis can be achieved with an acceptable degree of accuracy.

Acknowledgement

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