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Short Communication

Evaluation of acid methanolysis for analysis of wood hemicelluloses and pectins

F. Bertaud, A. Sundberg*, B. Holmbom

Laboratory of Forest Products Chemistry, Process Chemistry Group, Åbo Akademi University, Porthansgatan 3, 20500 Turku/Åbo, Finland

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Abstract

The efficiency of acid methanolysis toward cleavage of glucuronosyl linkages in hemicelluloses and pectins was studied. Two aldo-uronic acids (a dimer and a tetramer) were submitted to methanolysis with different acidic strength and methanolysis time. The aldo-uronic acid tetramer was only affected by the acidity of the medium, whereas the dimer was sensitive towards both reaction time and acidity. It was concluded that methanolysis of 0.5–1 mg of model compounds with 2 ml of 2 M HCl/MeOH at 100°C for 3–5 h was the best compromise between good cleavage of the glucuronosyl bonds (≈ 70%, mol/mol) and little degradation of the uronic acid released (below 20%, mol/mol). The acid methanolysis of reference oligo- and polysaccharides verified the good accuracy of the method. The application of acid methanolysis on mechanical pulp fibres was tested on a thermomechanical pulp. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Methanolysis; Uronic acid; Polysaccharide; Hemicelluloses; Pectins; Glucuronosyl linkage; Fibres; Wood; Pulp

1. Introduction

A basic method for analysis of complex polysaccharides is the determination of their constituent sugar residues obtained by acid hydrolysis or methanolysis of the native polymer. Commonly used hydrolytic agents for cleavage of glycosidic bonds are sulphuric acid, trifluoroacetic acid and hydrochloric acid in water (Biermann, 1988; De Ruiter, Schols, Voragen & Rombouts, 1992). With all these methods, there is always a trade-off between incomplete cleavage of the glycosidic and glucuronosyl linkages under relatively mild conditions and decomposition of the liberated monosaccharides, especially uronic and aldonic acids, which can undergo decarboxylation reactions at severe conditions (Biermann, 1988). Acid methanolysis is superior to acid hydrolysis for analysis of hemicelluloses and pectins in wood and mechanical pulp fibres and has the advantage that it can be performed in one step (Sundberg, Sundberg, Lillandt & Holmbom, 1996). Acid methanolysis using hydrochloric acid in anhydrous methanol (1 M/85°C or 2 M/100°C) releases both neutral and acid sugar units from hemicelluloses and pectic substances (Quemener & Thibault, 1989; Quemener, Lahaye & Thibault, 1993; Marga, Freyssac & Morvan, 1995). The liberated monosaccharides are converted into corresponding methyl glycosides, and carboxyl groups of uronic acids are esterified with methyl groups (Huang, Indrarti, Azuma & Okumura, 1992). Thereby, methanolysis gives the advantage of a reasonable stability of released methyl glycosides, up to 24 h with 1 M or 2 M HCl/MeOH (Chambers & Clamp, 1971). This stability is explained by a complete consumption of HCl by methanol after 2–3 hours at elevated temperatures according to Preuss and Thiers (1982). In addition, methanolysis allows simultaneous analysis of acid and neutral sugars by capillary gas chromatography or liquid chromatography after suitable derivatisation (Ha & Thomas, 1988; Churms, 1990; Quemener et al., 1993; Quemener, Marot, Mouillet, Da Riz & Diris, 2000).

The purpose of this study was to determine the efficiency of acid methanolysis to cleave glycosidic and glucuronosyl linkages by performing analysis on reference carbohydrate oligomers and polymers, and a mechanical pulp.

2. Materials and methods

2.1. Samples

Acid methanolysis was performed on two aldo-uronic acids: a dimer (4-*O*-MGlcA(1 → 2)α-Xyl) and a tetramer (4-*O*-MGlcA(1 → 2)α-Xyl(1 → 4)β-Xyl(1 → 4)β-Xyl), both from Megazyme (USA). Some oligo- and polysaccharides

* Corresponding author. Tel.: +358-2-215-31; fax: +358-2-215-4868.
E-mail address: anna.sundberg@abo.fi (A. Sundberg).

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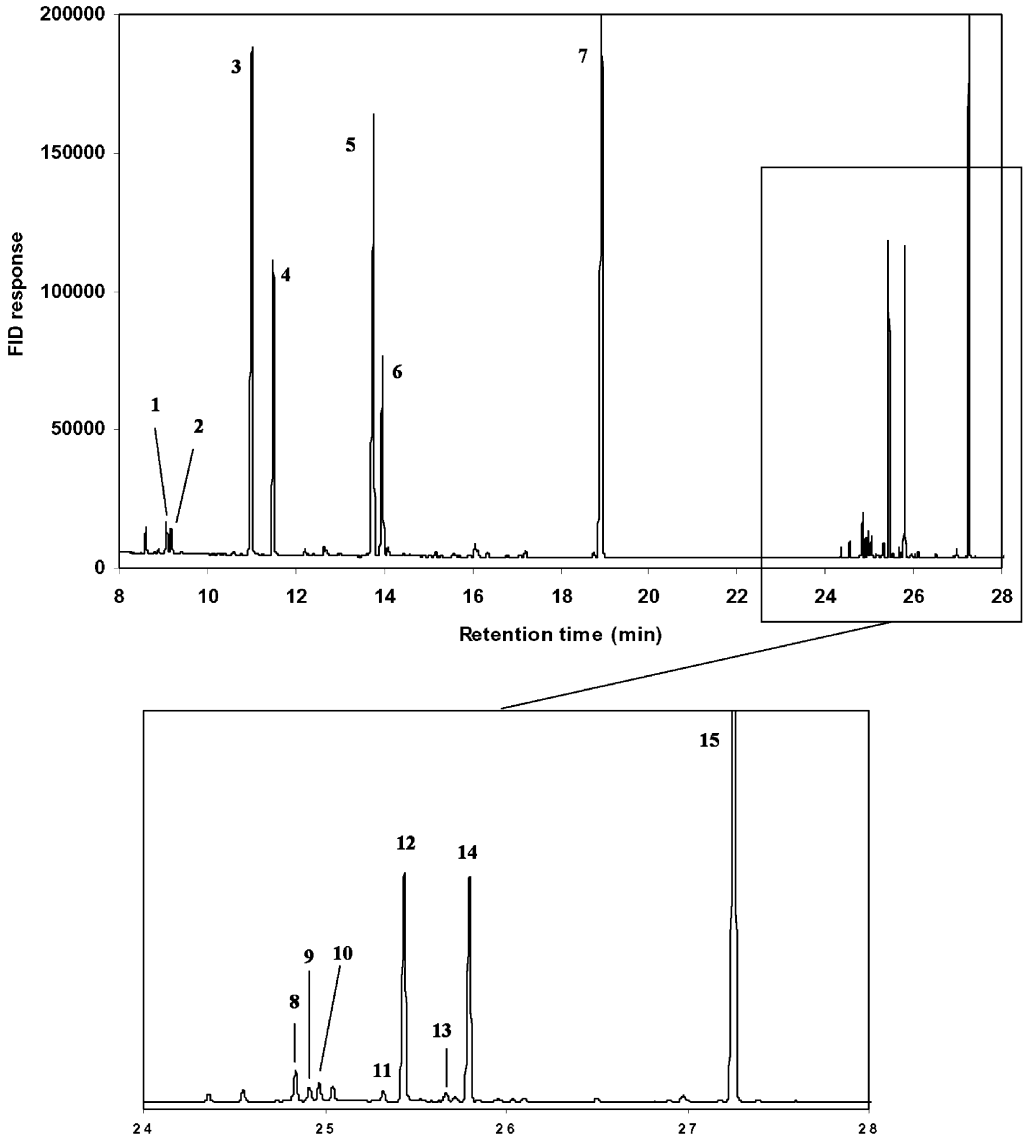

Fig. 1. GC-FID chromatogram of the silylated methanolysis products of aldo-uronic acid dimer. Peak identities: (1) (2) (3) (4) xylose, (5) (6) 4-*O*-methyl-glucuronic acid, (7) sorbitol (standard for monomer), (8) (9) (10) (11) (12) (13) (14) aldo-uronic acid dimer, (15) cellobiitol (standard for dimers).

were also tested: arabinohexaose ([αAra(1 → 5)αAra]$_3$), galactosylmannotriose ([αGal(1 → 6)βMan](1 → 4)βMan (1 → 4)βMan) and rhamnosylgalacturonotriose ([Rha (1 → 6)GalA](1 → 4)[GalA(1 → 4)GalA]) from Megazyme (USA), and 4-*O*-methyl-glucuronoxylan ([4-O-Me-αGlcA (1 → 2)βXyl](1 → 4)[βXyl(1 → 4)βXyl]$_n$) from Sigma. Analysis was performed also on a thermomechanical pulp (TMP) sample from Norway spruce, pre-extracted with hexane/acetone (90/10, v/v).

2.2. Methanolysis reagent

The acid methanolysis reagent was prepared by adding 14 ml acetyl chloride to 86 ml of dried methanol. This operation must be done carefully in an ice bath. The final concentration was determined by titration and adjusted to 2 M. The acid methanolysis reagent can be stored at −24°C for several weeks.

2.3. Methanolysis procedure and GC analysis

About 0.5–1 mg of model compound was freeze-dried and weighed into a pear-shaped flask. Acid methanolysis was performed with 2 ml of 2 M anhydrous HCl/MeOH at 100°C, applying various procedures. In the procedures A and B, 3 and 5 h of methanolysis time, respectively, were applied. Procedure C included a cooling step of 30 min after 2 h of heating time, followed by a second heating step for 1 h. The procedure D also included a cooling step and the addition of 2 ml of anhydrous HCl/MeOH before the second heating step. Neutralisation, derivatisation and gas chromatographic (GC) analysis were performed according

Table 1
Total yield of sugar monomers and aldo-uronic acid dimer, degree of cleavage of the glycuronosyl bond and degradation of the 4-*O*-methyl-glucuronic acid by acid methanolysis of the aldo-uronic acid dimer (4-*O*-MeGlcA (1 → 2)α-Xyl) under different conditions

Procedure	A 2 ml HCl/MeOH 3 h	B 2 ml HCl/MeOH 5 h	C 2 ml HCl/MeOH [2 h + 1 h][a]	D [2 + 2] ml HCl/MeOH [2 h + 1 h][b]
Sugar yield (w/w)				
Xylose (Xyl)	21 ± 2%	17 ± 2%	18 ± 1%	23 ± 1%
Methylglucuronic acid (MGlcA)	27 ± 4%	28 ± 2%	25 ± 1%	9 ± 4%
Aldo-uronic acid dimer (dimer)	24 ± 3%	21 ± 5%	23 ± 1%	20 ± 1%
Total	72 ± 5%	70 ± 5%	66 ± 2%	52 ± 5%
Cleavage (mol/mol dimer) (Xyl)/(Xyl + dimer)	66 ± 3%	75 ± 10%	61 ± 2%	45 ± 10%
MGlcA degradation (mol/mol dimer) (Xyl-MGlcA)/(Xyl)	22 ± 1%	72 ± 5%	3 ± 3%	71 ± 1%

[a] Heating during 2 h at 100°C, cooling 30 min and heating for 1 h at 100°C.
[b] An additional 2 ml HCl/MeOH was added after 2 h and the mixture was heated again for 1 h at 100°C.

to Sundberg and co-workers (1996). Sorbitol (i.e. D-Glucitol) was used as standard compound for neutral and acid sugar monomer determination, whereas cellobiitol (i.e. 4-*O*-β-D-Glucopyranosyl-D-Glucitol) was used as standard compound for the aldo-uronic acid dimer. The quantification of the dimer was based on the determination of the dimer isomers identified by GC (peaks 8–14, Fig. 1). GC calibration factors were determined by analysis of a mixture with equal amounts of cellobiitol and sugars. All experiments were made in triplicate, and were repeated five times.

TMP was freeze-dried and a 2–3 mg portion was weighed into a pear-shaped flask. The TMP sample was analysed according to the procedures B, E and F. In procedure E, acid methanolysis was performed for 3 h, followed by a cooling step of 30 min and a second heating step for 2 h. Procedure F was performed as procedure E, but included the addition of 2 ml 2 M HCl/MeOH before the second heating.

3. Results and discussion

3.1. Analysis of aldo-uronic dimer and tetramer

A GC-FID chromatogram of the acid methanolysis products of the aldo-uronic acid dimer is shown in Fig. 1. Four isomers/anomers of xylose and two of methylglucuronic acid were identified by mass spectroscopy (GC-MS). Several peaks of the dimer were also found. The cleavage of the glucuronosyl linkage and the stability of the released monomers were studied by examining the amounts of sugar monomers (Xyl and MGlcA) and the dimer (MGlcA-Xyl) (Tables 1 and 2). The residual dimer amount showed that the glucuronosyl linkage between methylglucuronic acid and xylose was cleaved only to the extent of 70–80% (w/w). After acid methanolysis of the aldo-uronic acid dimer, according to procedure A, 24% of the dimer (w/w) remained (Table 1). 16% of the dimer remained

Table 2
Total yield of sugar monomers and aldo-uronic acid dimer, degree of cleavage of the glycuronosyl bond and degradation of the 4-*O*-methyl-glucuronic acid by acid methanolysis of aldo-uronic acid tetramer (4-*O*-MGlcA(1 → 2)α-Xyl(1 → 4)β-Xyl(1 → 4)β-Xyl) under different conditions

Procedure	A 2 ml HCl/MeOH 3 h	B 2 ml HCl/MeOH 5 h	C 2 ml HCl/MeOH [2 h + 1 h][a]	D [2 + 2] ml HCl/MeOH [2 h + 1 h][b]
Sugar yield (w/w)				
Xylose (Xyl)	45 ± 1%	46 ± 3%	46 ± 4%	65 ± 2%
Methyl-glucuronic acid (MGlcA)	17 ± 1%	17 ± 1%	16 ± 1%	8 ± 1%
Aldo-uronic acid dimer (dimer)	16 ± 4%	16 ± 7%	15 ± 5%	6 ± 2 %
Total	78 ± 4%	79 ± 5%	77 ± 5%	79 ± 2%
Cleavage yield (mol/mol dimer) (Xyl/3)/(Xyl/3 + dimer)	73 ± 3%	70 ± 10%	70 ± 5%	89 ± 5%
MGlcA degradation (mol/mol dimer)(Xyl/3-MGlcA)/(Xyl/3)	20 ± 2%	19 ± 6%	25 ± 5%	74 ± 5%

[a] Heating during 2 h at 100°C, cooling 30 min and heating for 1 h at 100°C.
[b] An additional 2 ml HCl/MeOH was added after 2 h and the mixture was heated for 1 h at 100°C.

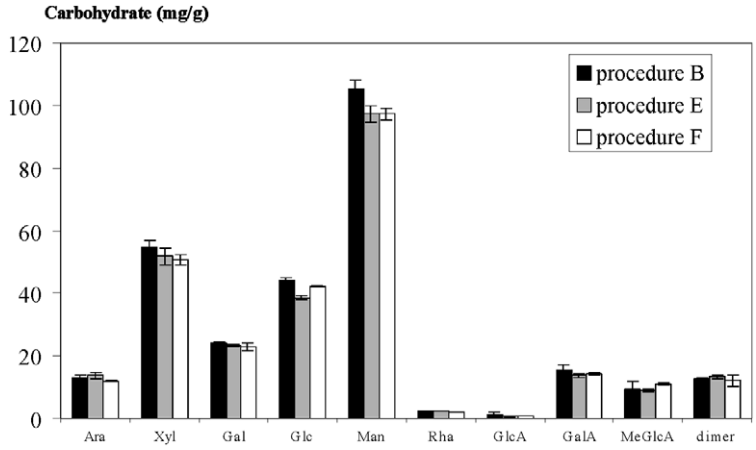

Fig. 2. Amounts of carbohydrate monomers and the aldo-uronic acid dimer (MGlcA-Xyl) obtained by acid methanolysis of a spruce thermomechanical pulp and GC analysis. Procedure B: 2 ml of 2 M HCl/MeOH for 5 h at 100°C. Procedure E: 2 ml of MHCl/MeOH for 3 h at 100°C with a cooling step of 30 min followed by a second heating at 100°C for 2 h. Procedure F: same as procedure E, but with the addition of 2 ml of 2 M HCl/MeOH before the second heating.

after acid methanolysis of the aldo-uronic acid tetramer (Table 2). Acid methanolyses by the procedures A and B were quite similar toward the aldo-uronic acid dimer and tetramer with a cleavage of about 70% (mol cleaved/mol aldo-uronic acid). The procedure C, which should be considered as a reference procedure for the procedure D, gave neither higher total amount nor better cleavage than the previous procedures for the two aldo-uronic acids studied. Concerning the degradation of MGlcA, cooling seemed to enhance uronic acid stability since the degradation rate was very low, only 3%, after methanolysis of the aldo-uronic acid dimer (Table 1). Acid methanolysis in stronger acidic medium (procedure D) did not give a higher total yield but, on the contrary, led to an extensive degradation, about 70%, of MGlcA. For the aldo-uronic acid dimer, the cleavage rate was lower using procedure D, 45%, whereas for the aldo-uronic acid tetramer (Table 2) the rate was higher, 89%. Huang and co-authors (1992) also obtained a maximum release of MGlcA from acid methanolysis of a beech xylan with 3.5–3.7N HCl/MeOH — 16 h/80°C. The use of a stronger acidic medium recommended by some authors (Huang et al., 1992; Quemener et al., 1993) would lead to MGlcA degradation, resulting in an erroneous ratio Xyl/MGlcA. The degradation of MGlcA during long reaction times (procedure B) was larger for the dimer, 72%, than for the tetramer, 19%. The tetramer contains, in addition to the glucuronosyl link, two glycosidic bonds between xylose units. A possible explanation for the higher stability of the tetramer is that the acidity of the reaction medium decreased faster in the presence of a tetramer than a dimer. This would lead to a higher stability of MGlcA during long methanolysis times with the tetramer than the dimer and, thereby, higher analysis yield of a polymer than a dimer. This is in agreement with the result as of Meier and Weissmann (1986). Free uronic acids, and uronic acid units in dimers and in polymers would be destroyed at a different rate (Quigley & Englyst, 1994). The studied aldo-uronic acid tetramer is a better representative of real samples than the aldo-uronic acid dimer. In procedure A (2 ml 2 M HCl/MeOH — 3 h/100°C), the degradation of MGlcA was low (20% in mol/initial mol of the aldo-uronic acid) (Tables 1 and 2), and it also gave a good yield of sugar monomer and dimers released from the aldo-uronic acid dimer or tetramer (70–80% in w/w). This is a reasonable yield considering the purity of the used aldo-uronic acids, which is about 80%.

3.2. Analysis of other oligo- and polysaccharide model compounds

Acid methanolysis was also performed on oligo- and polysaccharides, applying procedure A (2 ml 2 M HCl/MeOH — 3 h/100°C). Acid methanolysis was highly effective for arabinohexaose and galactosylmannotriose, and led to very good total yields of carbohydrates, 95 and 94%, respectively (w/w), considering the purity of the commercial product (i.e. 99 and 94%, respectively). The galactosylmannotriose gave a ratio of Gal/Man of 21/77 (mol/mol), well in agreement with the data of the supplier, i.e. 22/78. The amount of methylglucuronic acid, in 4-*O*-methyl-glucuronoxylan was lower (i.e. 5% in mol/mol total sugars) than the quantity given by the supplier (12.5% mol/mol total sugars, not warrant). The amount of galacturonic acid in rhamnosylgalacturonotriose was well in agreement with the data of the supplier, i.e. 52% compared to 54% (mol/mol total sugars). These results verified the very good accuracy of acid methanolysis and GC analysis, according to procedure A, for the determination of neutral (Ara, Xyl, Gal, Glc, Man, Rha) and acid sugar units (GalA, GlcA) in carbohydrate polymers. The determination of MGlcA was slightly less accurate, as previously pointed out.

3.3. Analysis of thermomechanical pulp

Procedure A could be extended to 5 h according to the

aldo-uronic acid tetramer results, without affecting either the total recovery yields or the degradation of uronic acids. This longer reaction time is recommended for treatment of wood and mechanical pulp to obtain a complete diffusion of sugar monomers residues out of the wood and mechanical pulp (Sundberg et al., 1996). The modifications in the acid methanolysis procedure concerned only the strength of the acidic medium (Procedure F: 4 ml of 2 M HCl/MeOH for 3 + 2 h at 100°C) and not the reaction time. The amount of the sugar units and the amount of the dimer after acid methanolysis are presented in Fig. 2.

The methanolysis (Procedure B: 2 ml of 2 M HCl/MeOH for 5 h at 100°C) gave a total sugar amount of 277 ± 10 mg/g of dry pre-extracted TMP. This was in good agreement with previous results (Sundberg et al., 1996). The relative standard deviations of the four series of analyses (performed in triplicate) for both neutral and acid sugars were below 5% (w/w), verifying the good repeatability of the procedure for mechanical pulp. The most abundant sugars released are mannose (Man), xylose (Xyl) and glucose (Glc) with relative contents 38, 19 and 17% (w/w of total sugar units), respectively. Uronic acids (GlcA, GalA and MGlcA) can be considered as minor compounds with a sum of 27 mg/g of dry pre-extracted TMP, and with a relative content equal to 12% (w/w) of the total monomers. Even small amounts of uronic acids units are important in pulp and paper chemistry, since they are responsible for the fibre charge and interact with cationic process chemicals and metal ions (Sundberg, Sundberg, Thornton & Holmbom, 1998). The dimer amount remaining after methanolysis (procedure B) was equal to 12 mg/g of dry pre-extracted TMP, representing less than 4% of the total yield (monomers and dimer). This low level verified the high efficiency of acid methanolysis towards the cleavage of the glucuronosyl linkage.

The other two procedures evaluated on TMP samples, i.e. stronger acidic conditions (procedure F: 4 ml of 2 M HCl/MeOH during 3 + 2 h at 100°C) and its reference procedure (procedure E: 2 ml of 2 M HCl/MeOH for 3 + 2 h at 100°C) showed that the cooling time only slightly affected the total yield of sugars. The total yield of sugars (255 ± 7 mg/g of dry sample) was indeed slightly smaller using procedure E compared to procedure B, indicating incomplete cleavage of glycosidic bonds during the cooling step. The amounts of arabinose (Ara) and xylose (Xyl) were however similar, indicating that they were released earlier than the other sugar units during acid methanolysis. This indicates a higher sensitivity of aldo-pentoses toward acid reagent underlined by Biermann (1988). The use of a stronger acidic medium with a cooling step (procedure F) increased neither the total amount of sugar monomers (253 ± 10 mg/g), nor decreased the residual aldo-uronic acid dimer content compared to procedure E. The stronger acidic medium did not affect the yield of uronic acids (GlcA + GalA + MGlcA = 26.5 ± 2 mg/g).

4. Conclusion

Acid methanolysis is a rapid and convenient technique for analysis of non-crystalline polysaccharides (hemicelluloses, pectins and pectic substances) in wood and mechanical pulp. However, the cleavage of the xylose–glucuronic acid bond is not complete and the released 4-*O*-methyl-glucuronic acid is partially degraded. Methanolysis results in a high yield of neutral sugar units, verifying its ability to cleave the glycosidic linkages. Methanolysis allows also determination of uronic acids (GalA, GlcA, MGlcA) which are recovered in good yields. Uronic acids are much more sensitive towards degradation in acidic conditions. Acid methanolysis with 2 M HCl/MeOH during 3–5 h at 100°C is the best compromise between efficient cleavage of glucuronosyl linkages and low degradation of the monomers released.

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