Carbohydrate Polymers 117 (2015) 903–909

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Quantification of xylooligomers in hot water wood extract by ^{1}H–^{13}C heteronuclear single quantum coherence NMR

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ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form
29 September 2014
Accepted 8 October 2014
Available online 23 October 2014

Keywords:
Hot water wood extract
Xylooligomer quantification
2D-HSQC

ABSTRACT

A new method that employs 2D-HSQCNMRwasdeveloped to determine xylooligomer concentrations in the hot water extracts of *Paulownia elongata*, aspen, sugar maple, southern hardwood mixture, and willow woodchips. Equations for computing oligomer concentrations calculation were developed based on HSQC corresponding resonance integrals of xylooligomer C_1H_1 and monomeric sugar standard curves. The degree of polymerization (*DP*) of xylooligomers in the hot water extract was computed by equation obtained from a series of xylooligomer standard solutions with *DP*s that ranged from 2 to 6. Another group of hot water wood extract that is served as a control group was hydrolyzed by 4% sulfuric acid at 121 °C for 60 min in order to convert all xylooligomer into xylose. As 2D-HSQC resonance response is different for acetylated xylo-units, as compared with non-acetylated units, proton NMR was used to calibrate the acetylated xylooligomer concentration. Xylooligomer concentrations determined from HSQC compared fairly well with data after hydrolysis.

1. Introduction

Hot water extraction is used as the pretreatment method to separate hemicellulose and extractives from woody biomass. With outstanding qualities (Wang & Liu, 2012), hot water extraction is a popular pretreatment method to extract useful components from woody biomass. During hot water extraction, most of hemicelluloses and extractives, as well as a small amount of cellulose and lignin, are extracted from woody biomass (Liu, 2012). Both monosaccharides (glucose, xylose, galactose, mannose, arabinose, and rhamnose) and oligosaccharides (xylooligomer, etc.) are obtained during hot water extraction. Usually, mineral acids are used to convert oligosaccharides into monosaccharides (Biermann, 1988; Bose et al., 2009; Cara, Ruiz, Oliva, Sáez, & Castro, 2008; Hu, Lin, Liu, & Liu, 2010; Mittal, Scott, Amidon, Kiemle, & Stipanovic, 2009; Yat, Berger, & Shonnard, 2008) before determination of the extraction liquor contents.

The monomeric sugars are usually quantified using High Pressure Liquid Chromatography (Sjöström & Alen, 1999), Gas Chromatography/Mass Spectrometry (Sjöström & Alen, 1999), Proton NMR (1D NMR)(Kiemle, Stipanovic, & Mayo, 2004), or Heteronuclear Single Quantum Coherence NMR (2D-HSQC) (Shupe, Kiemle, & Liu, 2012). Compared to the other methods, 2D-HSQC technique has unique merits, such as easy sample preparation, fast determination, and most importantly provide a second dimension to the spectrum which enables the data easier to be interpreted. HSQC has become an attractive method for the determination of sugar concentrations in complex mixtures (Kiemle et al., 2004; Shupe et al., 2012).

While quantification of monomeric sugars has been shown to be effective and formed many clear applications (Kiemle et al., 2004; Mittal et al., 2009; Shupe et al., 2012), there has been no data published on the direct quantification of oligomers with NMR. The objective of this paper is to develop a method to quantify xylooligomer concentration in the hot water extract by using ^{1}H–^{13}C Heteronuclear Single Quantum Coherence NMR (2D-HSQC).

2. Materials and methods

2.1. Materials

In this study, *Paulownia elongata*, aspen, sugar maple, southern hardwood mixture and willow were acquired from various sources. They were chipped with bark and screened to a uniform size of 2.5 cm × 2.0 cm × 0.5 cm. Each species of chips were well mixed and

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http://dx.doi.org/10.1016/j.carbpol.2014.10.031
0144-8617/

stored in the sealed drum, separately. In the wood chip preparation process, the Carthage chipper and vibratory screener were used.

2.2. Xylooligomer solutions

Hot water extraction was carried out in a 1.84 m^3 digester, as well as in a 4.7-liter M/K digester with external steam heating and liquor circulation system. Extracts of the liquor samples (or xylooligomer solutions) were taken from the sample line built in the middle of the digester and connected to the liquor circulation system. A condenser was used to cool the liquor samples before taken out of the digester system. The concentration of the xylooligomer was low especially at the early stages of hot water extraction process. Moderately "higher" concentration was desired for 2D-HSQC analysis. In order to increase the xylooligomer concentrations, the hot water extract were concentrated in a vacuum rotary evaporator at 70 °C. The concentrated samples were divided into two groups. One group of the samples was used to conduct 2D-HSQC analyses directly. The other group of samples was hydrolyzed with 4% H_2SO_4 at 121 °C for 60 min in an autoclave in order to convert the oligosaccharides into monosaccharides and to determine the sugar concentration.

2.3. NMR analysis

All spectra were acquired at 30 °C with a Bruker AVANCE III 600 spectrometer (600 MHz 1H frequency) equipped with a 5 mm triple resonance z-gradient probe. All ge-HSQC were recorded with standard pulse sequences (hsqcetgpsp) using Echo/Antiecho-TPPI gradient selection, and shaped pulse for the uniform inversion of the f2 channel (13C). The recycle delay was 2.0 s and the acquisition time was 0.125 s. Spectral widths of 8270 Hz and 2414 Hz with 2094 × 200 data points were collected for 1H and 13C spectra, respectively. The 8 transients (scans per FID) were acquired for a total experimental time of ~1 h. Data were acquired in TOPSPIN v3.2 from Bruker BioSpin. A coupling constant 1JC-H of 165 Hz was used as that is the expected value or JCH for the anomeric C1-H1 coupling constant. The 2D data set was processed with 1 K × 1 K data points using Qsine function in both directions. All 1D 1H spectra were acquired with a recycle delay was 10.0 s and the acquisition time 1.7 s. Spectral widths of 9615 Hz with 32K data points were used, with a total experimental time of ~7 min. Data were processed using MestReNova version 6.0.3-5604 from Mestrelab Research S.L. To avoid the effect of lignin and aromatic compounds during 2D-HSQC determination, samples were purified using a centrifuge before the preparation of samples.1.5 ml of concentrated sample was transferred to a 2 ml microcentrifuge tube and centrifuged at 12,000 rpm for 5 min. This treatment also effectively eliminated macromolecules from the xylooligomer solutions, thus only short-chain xylooligomers were present alongside monomeric sugars and other small molecular components. The NMR samples were prepared by taking 400 μl of supernatant and adding 100 μl of standard solution, along with 500 μl of D_2O, which resulted a total volume of one ml. D_2O was used to reduce residual water peak in the NMR spectrum. The standard solution used for 2D-HSQC and proton NMR determination contained D_2O, 3-(trimethylsilys) propionic-2,2,3,3-d4 acid (TSP) (0.0530%), and glucosamine (4.187%). Glucosamine served as a calibration standard while TSP was used as a chemical shift reference (0.0 ppm). The solution was added into the 5 mm NMR tube.

Table 1 and Fig. 1 are summarized based on the studies of Shupe et al. (2012) and Teleman, Lundqvist, Tjerneld, Stålbrand, and Dahlman (2000) which assisted in locating monosaccharides and oligosaccharides in the 2D-HSQC spectrum.

For the monosaccharide quantification in 2D-HSQC, pure monosaccharides' standard solutions were used to build calibration curves for each of the six monosaccharides. Glucosamine was spiked at the same concentration into each sample and was used as the internal standard. The alpha-anomeric (C_1H_1) peak of glucosamine was set to 100 and response factors for each of the alpha and beta anomeric for all standards were calculated. A linear relationship was found between sugar 2D-HSQC resonances and sugar mole concentrations. It's important that either the α-anomeric peak or the β-anomeric peak C_1H_1 could be used to calculate the sugar concentrations as long as the correct response factor was used (Shupe et al., 2012). The NMR signal was directly proportional to the mole concentration of the anomeric proton or carbon of interest (C_1H_1). In general, 2D-HSQC resonance integral can be expected to follow

$$C_i = \frac{A_{ij}}{\alpha_{ij}} \quad (1)$$

where C_i is the mole concentration of species i and j designations the functional group (C_1H_1) identified by NMR belonging to species i. A_{ij} is the 2D-HSQC resonance integral for the j functional group of species i, and α_{ij} is the corresponding resonance constant.

A series of standard solutions were prepared to relate the 2D-HSQC resonance integral with mole concentration. Standard monomer sugar concentrations were varied from 0.0063 mol/L to 0.1 mol/L. The mole concentrations of sugars were correlated to their C_1H_1 2D-HSQC resonances integral which were shown in Fig. 2 and Table 2.

Fig. 2 shows the relationship between the 2D-HSQC resonance integral as a function of the concentration for common sugars. One can observe a clear linear relationship. Glucosamine served as the internal standard and the integral of α-C_1H_1 was normalized uniformly to 100. All the other peak integrals are normalized to α-C_1H_1 for glucosamine.

Table 1
2D-HSQC chemical shifts of monosaccharides and oligosaccharides in hot water extract and hydrolysate (Shupe et al., 2012; Teleman et al., 2000).

Peak	Chemical shifts in (ppm)[a]	
	H-1	C-1
α-Glucosamine	5.45	92.00
β-Glucosamine	4.94	95.61
α-Glucose	5.23	94.91
β-Glucose	4.64	98.74
α-Arabinose	5.24	95.40
β-Arabinose	4.51	99.60
α-Xylose	5.20	95.06
β-Xylose	4.57	99.47
α-Mannose	5.18	96.88
β-Mannose	4.89	96.50
α-Rhamnose	5.11	96.82
β-Rhamnose	4.87	96.34
α-Galactose	5.27	95.12
β-Galactose	4.58	99.26
α-Xylooligomer reducing end	5.19	94.92
β-Xylooligomer reducing end	4.59	99.38
Internal xylose unit, I	4.47	104.55
Terminal xylose unit, I	4.45	104.71
Internal xylose unit, II	4.41	105.55
Terminal xylose unit, II	4.40	105.70
2-O-acetylated internal xylose unit	4.67	102.75
2-O-acetylated terminal xylose units	4.65	102.95
3-O-acetylated internal xylose units	4.56	104.33
3-O-acetylated terminal xylose units	4.55	104.40
2,3-O-acetylated xylose units	4.78	102.36

[a] All the chemical shifts are relative to TSP at 0.00 ppm.

3. Results and discussion

The corresponding areas for the sugars of samples obtained in the process of hot water extraction were shown in Table 3.

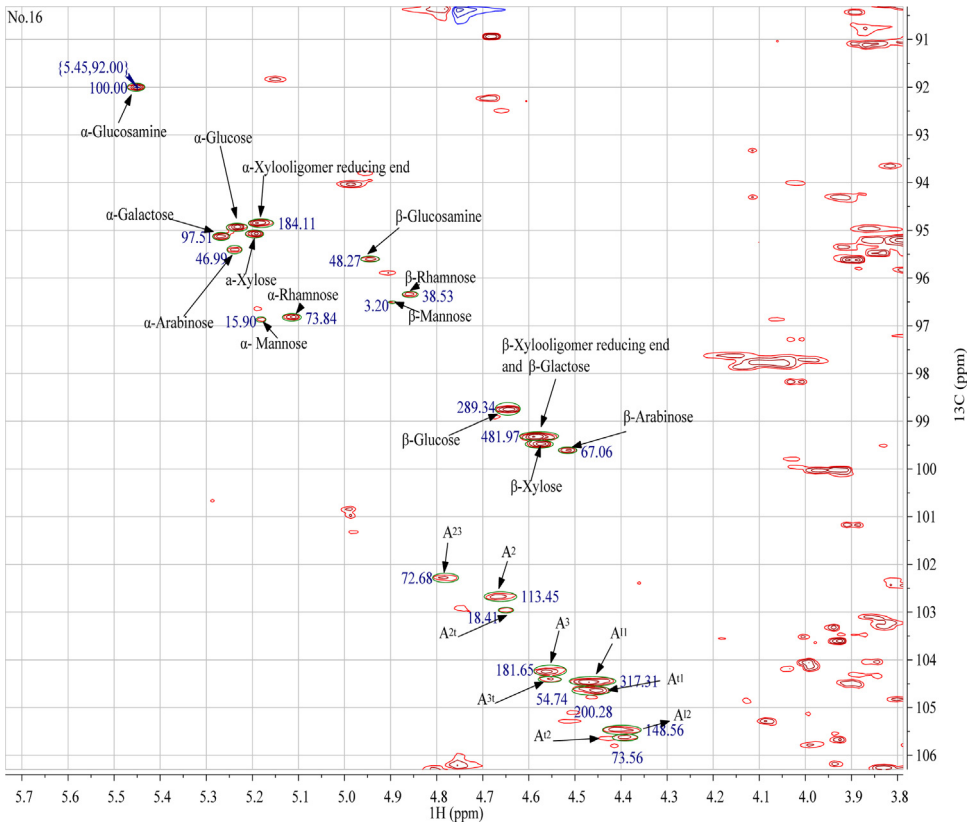

Fig. 1. 2D-HSQC chemical shifts of sugars in a hot water wood extract. A_{t1} and A_{t2} are the peaks corresponding to non-acetylated xylooligomer terminal end C_1H_1. A_{l1} and A_{l2} are the peaks corresponding to non-acetylated xylooligomer internal C_1H_1. $A_{23}$is the peak corresponding to 2, 3-di-O-acetylated xylooligomer unit C_1H_1. A_2 is the peak corresponding to 2-O-acetylated internal xylooligomer unit C_1H_1. A_{2t} is the peak corresponding to 2-O-acetylated terminal xylooligomer unit C_1H_1. A_3 is the peak corresponding to 3-O-acetylated internal xylooligomer unit C_1H_1. A_{3t} is the peak corresponding to 3-O-acetylated terminal xylooligomer unit C_1H_1. All the peak integrals are normalized to α-C_1H_1 for glucosamine.

Table 3 showed the peaks corresponding to C_1H_1 of xylooligomer and galactose in hot water extract. $A_{\alpha\text{-}red}$ and $A_{\alpha\text{-}gal}$ are the peak intensity of α-xylan reducing end C_1H_1 and α-galactose C_1H_1, respectively. A_s is the sum of peak intensity of β-xylan reducing end C_1H_1 and β-galactose C_1H_1.

The quantification results are shown in Table 4. Standard curves are employed to calculate the mole concentration of xylooligomer and xylose. For the mole concentration of bound acetylated groups, they are calculated using Eq. (2) which is obtained from proton NMR determination.

Table 4 showed the quantification results. One can observe that both of reducing end and non-reducing end of xylooligomer are used for the calculation. Most of the results agree with each other very well. And xylose units' concentration obtained from HSQC and acid hydrolysis match with each other very well. The comparison between C_{xm} and C_{XH} is shown in Fig. 6.

One may expect that the 2D-HSQC resonance response is different for acetylated xylo-units, as compared with non-acetylated units. Therefore, proton NMR was employed to investigate the relationship between the acetylated xylo-unit concentration and the HSQC resonance integral. The acetyl group concentration can be quantified accurately with proton NMR, as depicted by Eq. (2). Chemical shifts from 2.25 ppm to 2.00 ppm in ^{1}H NMR were integrated that represented bound acetylated groups in the hot water extract (Bose et al., 2009). The same ingredients of internal standard solution are used in the proton NMR and in the 2D-HSQC. Glucosamine served as the internal standard and the integral of α-C_1H_1 was normalized uniformly to 100. All the other peak integrals are normalized to α-C_1H_1 for glucosamine. The mole concentration of reference standard (glucosamine) was 2.2×10^{-5} mol/L.

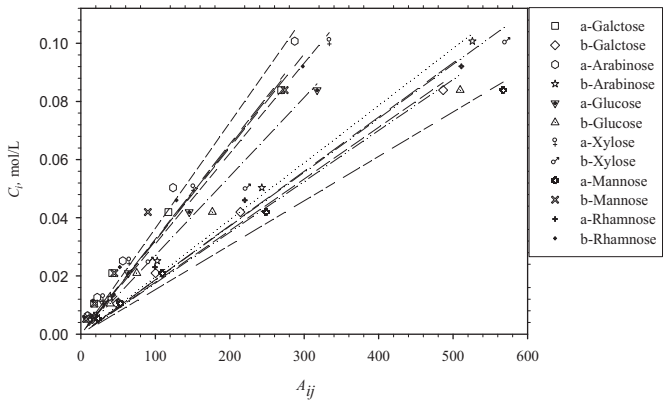

Fig. 2. Standard curves for 2D-HSQC.

Table 2
2D-HSQC resonance constant for monomeric sugar standard solutions.

Monomeric sugar, *i*	α_{ij}	
	α-C_1H_1	β-C_1H_1
Xylose	3207	5314
Glucose	3672	5565
Arabinose	2731	5060
Mannose	6505	2975
Rhamnose	5325	3079
Galactose	3050	5599

Table 3
NMR data of xylooligomer and galactose in hot water extract.

Species	No.	HSQC Corresponding resonance[a]											
		$A_{\alpha\text{-}red}$	$A_{\alpha\text{-}gal}$	A_S	A_{t1}	A_{t2}	A_{I1}	A_{I2}	A_{23}	A_2	A_3	A_{2t}	A_{3t}
Paulownia elongata	1	140.4	100.1	421.2	203.9	154.0	304.3	178.0	95.7	150.9	179.2	63.4	23.8
	2	140.5	69.2	397.2	201.4	126.2	286.3	164.2	96.5	139.3	178.4	63.5	28.9
	3	102.1	58.8	278.7	118.0	91.4	196.4	108.2	42.9	93.9	122.3	44.1	23.3
	4	184.1	97.5	482.0	200.3	73.6	317.3	148.6	72.7	113.5	181.7	54.7	18.4
	5	93.2	49.7	254.0	105.7	52.4	157.8	77.3	37.8	68.3	87.7	30.5	12.9
	6	211.1	50.5	504.5	257.6	199.8	436.0	271.5	7.9	207.8	333.4	111.1	68.0
	7	269.3	73.8	541.1	274.5	173.3	365.8	230.3	79.5	184.5	302.9	97.8	83.8
	8	116.1	33.9	234.5	123.8	80.4	164.9	103.4	52.3	83.7	118.2	51.6	16.9
	9	248.6	56.3	479.6	250.1	131.6	343.2	181.2	58.7	158.9	232.7	91.0	69.5
	10	200.6	44.6	482.1	161.4	90.4	250.7	109.0	58.5	104.4	158.5	67.8	39.8
	11	63.1	14.5	129.0	34.5	16.8	53.1	14.0	31.2	16.9	30.7	21.0	0.0
	12	97.9	26.9	230.8	60.0	16.6	71.6	18.1	37.0	27.2	46.0	34.8	25.1
	13	60.5	13.5	122.1	34.9	12.2	45.2	6.8	8.3	18.2	26.4	24.0	0.0
	14	63.7	19.0	154.4	37.5	9.0	34.0	4.8	5.5	15.8	17.2	23.6	0.0
	15	98.6	26.0	250.9	48.9	12.7	42.6	5.0	34.2	25.3	19.3	35.0	0.0
Aspen	16	152.1	11.1	261.0	274.6	107.4	461.5	152.3	50.0	90.7	148.2	9.8	26.1
	17	153.9	8.5	257.1	252.3	113.4	476.6	148.7	8.7	95.9	157.5	7.1	27.4
	18	138.2	6.9	262.1	258.0	109.7	419.9	153.6	40.6	98.2	134.2	11.2	22.4
	19	154.5	7.9	245.6	237.7	53.8	371.3	109.7	22.1	70.2	114.2	11.6	25.4
	20	66.0	3.0	103.4	83.8	20.6	149.9	39.6	6.6	26.2	42.3	6.4	7.4
Southern hardwood mixture	21	133.7	15.6	205.6	231.5	168.8	518.3	216.8	6.9	105.9	191.1	0.0	56.4
	22	70.3	8.4	108.5	107.9	69.7	192.6	75.8	29.5	82.6	60.7	9.5	25.6
	23	141.5	17.0	207.3	178.7	83.3	362.3	153.2	27.0	96.8	121.7	19.1	45.6
	24	204.7	18.1	280.9	280.9	138.4	414.3	159.3	31.3	98.5	166.6	26.7	57.6
	25	87.0	7.7	124.6	107.9	32.1	147.8	55.4	12.4	55.9	55.8	14.5	17.0
Sugar maple	26	10.8	0.0	14.5	7.4	3.3	20.5	16.5	0.0	0.0	17.2	0.0	0.0
	27	23.8	0.0	42.3	37.3	15.1	39.5	22.5	0.0	0.0	24.7	0.0	13.2
	28	33.9	0.0	54.0	30.5	16.2	49.1	25.6	0.0	0.0	25.6	0.0	8.4
	29	19.5	0.0	38.3	13.6	8.1	17.4	8.8	0.0	0.0	19.2	0.0	3.1
	30	39.1	0.0	79.0	41.7	4.8	55.8	14.8	0.0	0.0	24.8	0.0	15.0
Willow	31	6.6	0.0	17.5	22.8	1.3	38.0	10.5	0.0	0.0	17.8	0.0	2.0
	32	12.4	0.6	15.9	24.1	8.9	36.9	13.8	0.0	0.0	13.8	0.0	0.0
	33	15.7	0.0	37.1	37.5	24.8	64.8	35.3	0.0	0.0	33.0	0.0	1.8
	34	22.4	2.4	39.0	56.4	12.9	77.4	39.8	0.0	0.0	27.6	0.0	13.5
	35	25.6	0.0	38.2	62.7	22.2	52.9	32.8	0.0	0.0	15.2	0.0	0.0

[a] See the explanation of the designations used in Fig. 1.

The concentration of bound acetylated groups can be calculated by,

$$C_{BA} = \frac{C_I \times P_{BA}}{3P_I} \quad (2)$$

where C_{BA} is the mole concentration of bound acetylated groups; C_I is the mole concentration of internal standard; P_{BA} is the integrated corresponding area of bound acetylated groups; P_I is the integrated corresponding area (sum of α-C_1H_1 and β-C_1H_1) of internal standard. Since acetyl groups contain three protons per molecule, the integrated corresponding area for acetyl groups was divided by 3 to obtain the corresponding area for each mole of protons.

Subsequently, the concentration of acetylated xylo-units is known based on the acetyl group concentration. Thus,

$$C_{BA} = \frac{\alpha_{BA}(2A_{23} + A_2 + A_3 + A_{2t} + A_{3t})}{\alpha_{\text{xylose-β-C}_1\text{H}_1}} \quad (3)$$

where α_{BA} is a proportionality constant. $\alpha_{\text{xylose-β-C}_1\text{H}_1}$ is the corresponding resonance constant for xylose which can be obtained from Table 2. The explanations of the other designations used in Eq. (3) were mentioned in Fig. 1.

Correlating the data obtained with Eq. (2) and shown in Table 4, the proportion constant was obtained, α_{BA} = 1.6. Therefore, the corresponding of xylo-unit concentration can be computed by,

$$C_{ax} = \frac{1.6(2A_{23} + A_2 + A_3 + A_{2t} + A_{3t})}{\alpha_{\text{xylose-β-C}_1\text{H}_1}} \quad (4)$$

where C_{ax} is the acetylated xylose unit mole concentration after calibration. The explanations of the designations used in Eq. (4) were mentioned in Fig. 1.

In order to show the accuracy and consistence of quantifying xylooligomers, a series of standard xylooligomer solution with *DP* from 2 to 6 was prepared. 2D-HSQC NMR results were listed in Table 5.

A linear relationship was found between *DP* and ratio of HSQC peak integral of β-proton internal unit C_1H_1 and β-proton terminal unit C_1H_1 that was shown in Fig. 2. The linear relationship was given by,

$$DP = \frac{A_{\text{internal}}}{A_{\text{ter min al}}} + 2 \quad (5)$$

Based on Eq. (5) and Fig. 3, one can infer that the HSQC resonance peak integral for the non-reducing terminal unit C_1H_1 and internal unitC_1H_1 share the same proportionality constant with respect to their molar unit concentrations. This increased the reliability in using HSQC integral to quantify the xylooligomers.

Eqs. (3) and (5), the *DP* of hot water extract could be calculated by,

$$DP = \frac{A_{11} + A_{12} + 1.6(A_{23} + A_2 + A_3)}{A_{t1} + A_{t2} + 1.6(A_{2t} + A_{3t})} + 2 \quad (6)$$

The explanations of the designations used in Eq. (6) were mentioned in Fig. 1.

Table 4
Quantification results.

Species	No.	Concentration (mol/L)					
		C_{xo1} [a]	C_{xo2} [b]	C_{xo3} [c]	C_{xm} [d]	C_{XH} [e]	C_{BA} [f]
Paulownia elongata	1	0.008	0.008	0.010	0.045	0.047	0.023
	2	0.011	0.012	0.012	0.057	0.058	0.031
	3	0.007	0.007	0.007	0.032	0.049	0.028
	4	0.013	0.013	0.009	0.046	0.062	0.034
	5	0.010	0.010	0.008	0.038	0.037	0.029
	6	0.012	0.014	0.015	0.065	0.051	0.039
	7	0.014	0.014	0.013	0.057	0.050	0.026
	8	0.010	0.009	0.009	0.039	0.051	0.027
	9	0.016	0.015	0.014	0.058	0.047	0.037
	10	0.017	0.018	0.012	0.051	0.048	0.042
	11	0.009	0.009	0.004	0.018	0.033	0.019
	12	0.011	0.012	0.006	0.025	0.030	0.021
	13	0.008	0.008	0.004	0.015	0.026	0.015
	14	0.008	0.009	0.004	0.013	0.023	0.016
	15	0.009	0.010	0.004	0.013	0.021	0.019
Aspen	16	0.012	0.012	0.012	0.055	0.064	0.040
	17	0.013	0.013	0.012	0.059	0.065	0.043
	18	0.016	0.017	0.016	0.078	0.067	0.046
	19	0.018	0.017	0.014	0.064	0.068	0.047
	20	0.018	0.018	0.012	0.057	0.073	0.043
Southern hardwood mixture	21	0.013	0.012	0.017	0.080	0.072	0.010
	22	0.016	0.014	0.018	0.082	0.089	0.056
	23	0.022	0.019	0.019	0.090	0.109	0.055
	24	0.030	0.026	0.027	0.113	0.108	0.080
	25	0.026	0.023	0.019	0.083	0.089	0.070
Sugar maple	26	0.007	0.006	0.002	0.019	n.d.[g]	0.035
	27	0.015	0.015	0.015	0.056	n.d.	0.061
	28	0.021	0.021	0.013	0.053	n.d.	0.056
	29	0.012	0.013	0.006	0.025	n.d.	0.026
	30	0.024	0.027	0.015	0.056	n.d.	0.044
Willow	31	0.003	0.004	0.005	0.025	0.024	0.033
	32	0.006	0.006	0.006	0.026	0.039	0.035
	33	0.008	0.010	0.011	0.053	0.039	0.039
	34	0.012	0.011	0.016	0.064	0.050	0.031
	35	0.013	0.013	0.015	0.052	0.049	0.048

[a] Concentration of xylooligomers (C_{xo}) are calculated by Eq. (9) and employing the peak integral of xylose α-reducing end C_1H_1.
[b] Concentration of xylooligomers (C_{xo}) are calculated by Eq. (10) and employing the peak integral of xylose α-reducing end C_1H_1 and β-reducing end C_1H_1.
[c] Concentration of xylooligomers (C_{xo}) are calculated by Eq. (11) and employing the resonance integral of non-reducing end C_1H_1.
[d] The xylose units' concentration in the xylooligomers (C_{xm}) are calculated based on C_1H_1 HSQC peak integral of internal unit and terminal.
[e] Xylose units' concentrations in the xylooligomers (C_{XH}) are obtained by acid hydrolysis.
[f] The mole concentration of bound acetylated groups.
[g] not detected.

A liner relationship was obtained between peak intensity of α-galactose C_1H_1($A_{\alpha\text{-}gal}$) and β-galactose C_1H_1($A_{\beta\text{-}red}$) based on Table 1, which was shown by,

$$A_{\beta\text{-}gal} = 1.84 \times A_{\alpha\text{-}gal} \quad (7)$$

Table 5
HSQC integral for soluble xylooligomers normalized by the peak of α-reducing end C_1H_1.

DP	A_I	$A_{N\text{-}red}$	$A_{\alpha\text{-}red}$	$A_{\beta\text{-}red}$	$\frac{A_I}{A_{N\text{-}red}}$	$\frac{A_{\alpha\text{-}red}}{A_{N\text{-}red}}$	$\frac{A_{\beta\text{-}red}}{A_{N\text{-}red}}$	$\frac{A_{\alpha\text{-}red}}{A_{\beta\text{-}red}}$
2	0	328.5	100	179.2	0	0.304	0.545	0.558
3	293	318.2	100	185.6	0.92	0.314	0.583	0.539
4	635.5	314.9	100	185.8	2.02	0.318	0.590	0.538
5	993.8	342.4	100	189.6	2.9	0.292	0.553	0.528
6	1373.1	326.0	100	197.5	4.21	0.306	0.606	0.506
Avg	326	100	187.5	2.01	0.307	0.576	0.534	
Stdev	10	0	6.7	1.65	0.0099	0.0254	0.0189	

$A_{\alpha\text{-}red}$ is the peak integral of α-reducing end which is normalized uniformly to 100. A_I is the peak integral of internal unit C_1H_1. $A_{\beta\text{-}red}$ is the peak integral of β-reducing end C_1H_1. $A_{N\text{-}red}$ is the peak integral of non-reducing end C_1H_1.

The peak intensity of β-xylan-reducing end ($A_{\beta\text{-}red}$) could be calculated by,

$$A_{\beta\text{-}red} = A_s - 1.84 \times A_{\alpha\text{-}gal} \quad (8)$$

where A_s is the sum of peak intensity of β-xylan reducing end C_1H_1 and β-galactose C_1H_1.

Figs. 4 and 5 showed HSQC resonance peak integral comparisons between non-reducing terminal end C_1H_1 and reducing end C_1H_1 in hot water extract. Based on the linear trend line, the ratio between α-reducing end C_1H_1 and non-reducing end C_1H_1 was 0.32. And the peak integral ratio between β-reducing end C_1H_1 and non-reducing end C_1H_1 was 0.5. The ratios were very close to that obtained from standard xylooligomer solutions.

Employing Eq. (1), the mole concentration of xylooligomers (C_{xo}) could be calculated by the following equations:

$$C_{xo} = \frac{A_{\alpha\text{-}red}}{\alpha_{\text{xylose-}\alpha\text{-}C_1H_1}} \quad (9)$$

or

$$C_{xo} = \frac{(A_{\alpha\text{-}red}/\alpha_{\text{xylose-}\alpha\text{-}C_1H_1}) + (A_{\beta\text{-}red}/\alpha_{\text{xylose-}\beta\text{-}C_1H_1})}{2} \quad (10)$$

where $A_{\alpha\text{-}red}$ is the peak integral of xylose α-reducing end C_1H_1. $A_{\beta\text{-}red}$ is the peak integral of xylose β-reducing end C_1H_1.

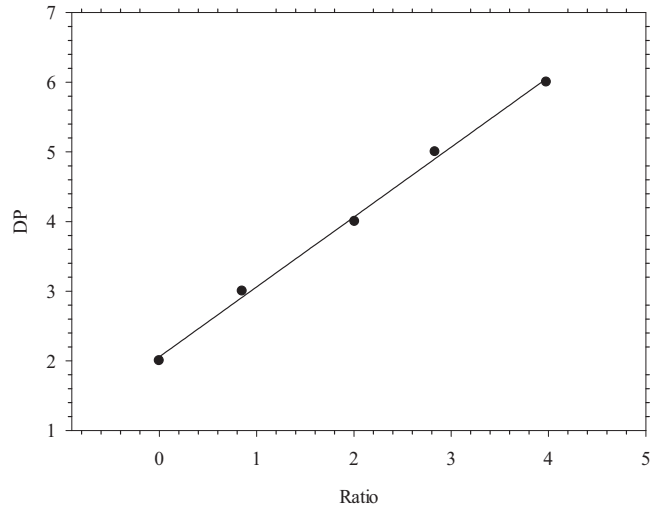

Fig. 3. Variations of *DP* with ratio of HSQC resonance C_1H_1 peak integral of internal unit and terminal unit.

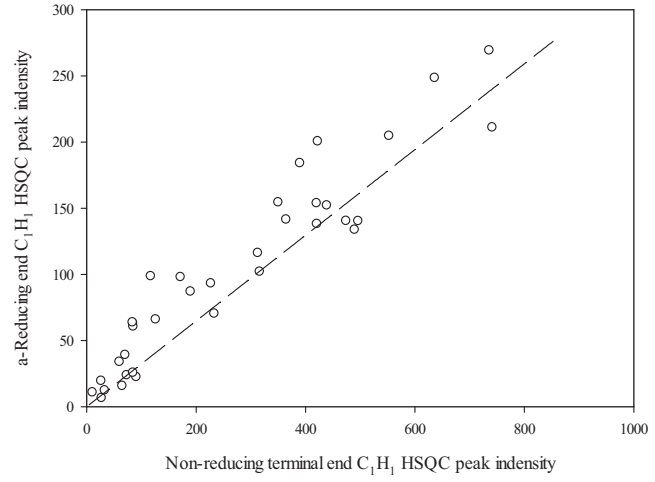

Fig. 4. HSQC resonance peak integral comparisons between non-reducing terminal end C_1H_1 and α-reducing end C_1H_1. Non-reducing terminal end integral was calculated by using the data from Table 3 and proportion constant from Eq. (4). Reducing integral was calculated based on the data from Table 3 and Eq. (8).

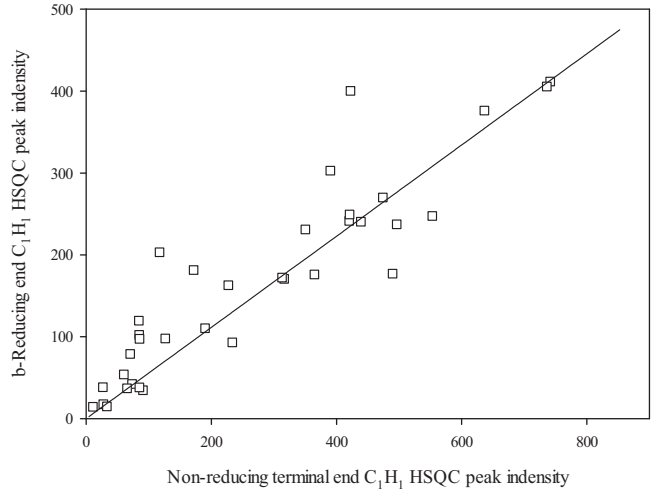

Fig. 5. HSQC resonance peak integral comparisons between non-reducing terminal end C_1H_1 and β-reducing end C_1H_1. Non-reducing terminal end integral was calculated by using data from Table 3 and proportion constant from Eq. (4). Reducing integral was calculated based on data from Table 3 and Eq. (8).

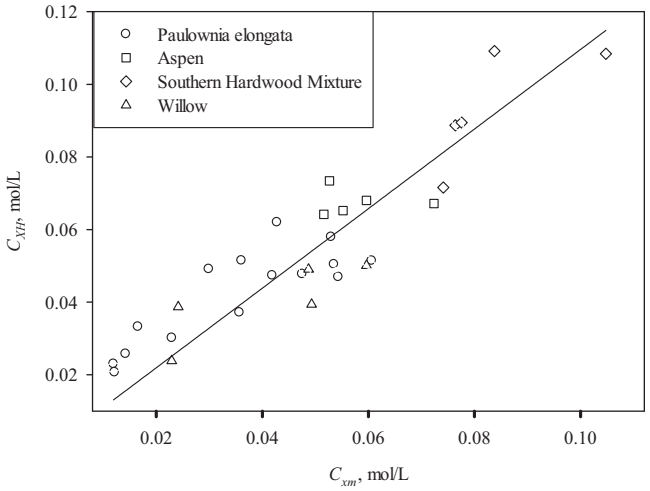

Fig. 6. Results comparison between C_{xm} and C_{XH}.

$\alpha_{\text{xylose-}\alpha\text{-}C_1H_1}$ and $\alpha_{\text{xylose-}\beta\text{-}C_1H_1}$ are the corresponding resonance constant for xylose which can be obtained from Table 2. The calculation results are shown in Table 4.

The mole concentration of xylooligomers (C_{xo}) could also be obtained by using the resonance integral of non-reducing end C_1H_1 and data obtained from Eq. (10),

$$C_{xo3} = 0.000105 \times [A_{t1} + A_{t2} + 1.6 \times (A_{2t} + A_{3t})] \quad (11)$$

The explanations of the designations used in Eq. (11) were mentioned in designation of Fig. 1. The calculation results are shown in Table 4.

Control group of hot water wood extract was hydrolyzed with 4% sulfuric acid at 121 °C for 1 h. And the xylose concentrations were determined by 2D-HSQC. By comparing the xylose concentrations before and after acid hydrolysis, xylose units' concentrations in the xylooligomers (C_{XH}) were obtained which were shown in Fig. 6. Applying Microsoft solver, the xylose units' concentration in the xylooligomers (C_{xm}) based on C_1H_1 HSQC peak integral of internal unit and terminal unit could be calculated by,

$$C_{xm} = 0.000105 \times \{2 \times [A_{t1} + A_{t2} + 1.6 \times (A_{2t} + A_{3t})] + A_{I1} + A_{I2} + 1.6 \times (A_{23} + A_2 + A_3)\} \quad (12)$$

The explanations of the designations used in Eq. (12) were mentioned in designation of Fig. 1. The calculation results are shown in Table 4.

Fig. 6 shows a linear correlation relationship between the xylo-unit concentrations contained in xylooligomer (C_{xm}) and hot water wood extract acid hydrolysate (C_{XH}). One can observe that C_{xm} fit well with C_{XH}. But differences between C_{xm} and C_{XH} exist. The possible reason is that when hot water extract contain large amount of insoluble xylooligomers which cannot be detected by 2D-HSQC NMR, C_{xm} is lower than C_{XH}. When hot water extract contain few insoluble xylooligomers xylose degradation exist during hydrolysis, C_{xm} is higher than C_{XH}. One can conclude that most of hot water extract samples contain considerable insoluble xylooligomers.

4. Conclusions

Xylooligomer concentration can be determined by using 2D-HSQC NMR together with monomer sugar standard curve. The results based on this new method fit well with that from acid

hydrolysis. One should notice that only soluble xylooligomers are determined by 2D-HSQC NMR. Analysis of hot water extract without hydrolysis could give more accurate results for soluble xylooligomer concentration than that obtained from the traditional acid hydrolysis method as part of xylooligomers degrade into some small molecules during the process of hydrolysis.

2D-HSQC resonance response is different for acetylated xylo-units, as compared with non-acetylated units. ^{1}H NMR calibration could help to obtain proportion constant for the acetylated xylo-units C_1H_1 in 2D-HSQC. Besides, internal xylo-units C_1H_1 and non-reducing end units C_1H_1 have the same proportion constant. Therefore, the *DP* of hot water extract could be calculated by using the liner relationship between the *DP* and the 2D-HSQC resonance C_1H_1 peak integral of internal unit and terminal unit from the soluble xylooligomer solution.

One should also pay attention to integration of 2D NMR peaks especially for the integration of 2,3-di-O-acetylated xylooligomer unit and 2-O-acetylated xylooligomer unit because they were very close to the water peak and may overlap with the water proton signal.

Acknowledgements

The authors appreciate the support from Bio-refinery and Bioprocess Engineering Research group at SUNY ESF. In addition, the authors are grateful to the help from Mr. Yipeng Xie and Christopher D. Wood.

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