A Method for Rapid Determination of Sugars in Lignocellulose Prehydrolyzate

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A simple and rapid dual-wavelength spectroscopic method is used for simultaneous determination of pentoses and hexoses in the prehydrolyzate from lignocellulosic biomass. The method is based on the following reaction mechanism: in the solution of hydrochloric acid, phloroglucinol gives color reaction with sugars or their degradation products, showing maximum absorbance at 553 nm and 410 nm. Based on dual-wavelength spectrophotometric measurement, the pentoses and hexoses can separately be quantified. It was found that the derivatives from these two different sugars have an isosbestic point at 425 nm. According to the validation results, high accuracy and reasonable recovery rate is shown with the present method (pentoses recovery 97.1 to 100.0%, hexoses recovery 97.2 to 102.0%). Additionally, the interferences from substances including lignin, furfural, 5-hydroxymethyl furfural (HMF), glucuronic acid, and galacturonic acid are insignificant. All of the above results illustrate the suitability of this method for analyzing sugars in the lignocelluloses prehydrolyzate, especially hardwoods or herbaceous plants, based on forest-related biorefinery research.

Keywords: Lignocellulose; Prehydrolyzate; Pentoses; Hexoses; Spectrophotometry; Biorefinery

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INTRODUCTION

Resource and energy shortages have become widespread issues for global development. As one of the most abundant renewable resources, lignocelluloses show great potential for future development, such as the production of transportation fuels and other value-added chemicals, while yielding lower greenhouse gas emissions. In the paper industry, lignocellulosic biomass is usually used for the production of chemical pulps, typically by alkaline pulping process. During the pulping process, a large amount of carbohydrates, mainly hemicelluloses, are released into the pulping liquor, which increases the loading in the spent liquor (black liquor) and provides few benefits (due to the lower heating value) to liquor combustion in the chemical recovery process. One of the key processes in the proposed "Integrated Forest Biorefinery" (van Heiningen 2006) is the pre-extraction of hemicelluloses for the production of fuel-based ethanol or biodiesel. In the prehydrolysis research, it is important to seek efficient methods and conditions for achieving a controlled sugar extraction yield (mainly from hemicelluloses) with minimum cellulose degradation. Data from the sugar content in the prehydrolyzate provides important information for better understanding the effects of the process

parameters, such as reaction temperature, time, acidity, alkalinity, *etc*., as they relate closely to the degree of hemicellulose hydrolysis and cellulose degradation.

Many traditional methods are available for sugar analysis. Colorimetric methods, including the dinitrosalicylic acid (DNS) assay (Miller 1959), the orcinol-sulfuric acid method (Scott and Melvin 1953), and the phenol-sulfuric acid method (Dubois *et al.* 1956), can just be used for quantifying total reducing sugars but not for the pentoses and hexoses separately, which will not provide the needed guidance relative to the subsequent sugar fermentation. The Douglas method (namely the phloroglucinol-glacial acetic acid method) (Douglas 1981), can only be applied for the determination of pentosans and/or pentoses. Among these colorimetric approaches, the Douglas method has the advantage of not only higher measurement accuracy, but also relative lower toxicity of its color reagent, phloroglucinol. Modern instruments, such as gas chromatography (GC), high performance liquid chromatography (HPLC), and high performance anion exchange chromatography (HPAEC), also have been applied for sugar analysis. The GC method involves conversion of sugars to volatile derivatives, a procedure that usually creates a large uncertainty in the sugar analysis (Li *et al.* 1987). Although HPLC and HPAEC are regarded as the best methods for sugars analysis, both qualitatively and quantitatively (Cheetham *et al.* 1981; Liang *et al.* 2006), they require high-cost analytical columns, effluent reagents, and instrument maintenance. Therefore, there is a need to develop a simple, rapid, and low-cost method for sugar analysis. In particular, for the forest biorefinery related research community, the quantification of the two sugars groups, *i.e.* the pentoses (5-carbon sugars) and hexoses (6-carbon sugars), which comprise the prehydrolyzate sugar monomers and oligomers from lignocellulosic hydrolysis, is more desired. It is these polysaccharides and oligosaccharides that are converted (enzymatically or through chemical hydrolysis) to monosaccharides, mainly xylose and glucose, which are the initial components for producing ethanol in the traditional fermentation process.

In this paper, a simple and low-cost spectroscopic method for simultaneous determination of pentoses and hexoses in the lignocellulose hydrolysates is introduced. Phloroglucinol is used as the color reagent in the experiment, just as it is in the Douglas method.

EXPERIMENTAL

Materials and Apparatus

All chemicals used in the experiments were analytical reagents. The eucalyptus chips (Eucalyptus ABL 12) were obtained from a pulp mill in Guangdong province, China.

Two UV-Vis spectrophotometers (SCINCO S-3100 & PerkinElmer Lambda XLS) equipped with 1 cm cuvettes were used in the spectroscopic measurements. A lab digester (M/K 609-2-10) was used in the wood autohydrolysis process.

Sample Preparation

The color reagent solution was prepared by dissolving 2 grams of phloroglucinol in 110 mL glacial acetic acid, followed by adding 10 mL of anhydrous alcohol and 2 mL of concentrated hydrochloric acid. Five sets of standard solutions of xylose, glucose, and

their mixtures were prepared. The concentration of monosaccharides ranged from 0 to 5.0 mmol/L. The total sugar concentration in the mixtures ranged from 1.10 to 1.55 mmol/L.

Lignin sample was separated from kraft black liquor by acid precipitation and then purified according to the method by Lundquist and Kirk (1980).

Spectral Analysis

One mL of standard sugar or wood prehydrolyzate was mixed with 10 mL of color reagent in a 30 mL test tube, which was then placed in a boiling water bath for exactly 10 minutes. After cooling down with tap water for exactly 5 minutes, spectral scanning was conducted in the wavelength range of 380 to 700 nm. The reference was the resultant solution by reacting distilled water with color reagent under the same conditions as above.

RESULTS AND DISCUSSION

Color Reaction and Spectroscopic Characterization

At high temperature in a strong acidic medium, pentoses and hexoses can be hydrolyzed into mono-sugars and their degradation products such as furfural and 5-hydroxymethyl furfural (HMF) (Hu *et al.* 2008), which may react with phloroglucinol to generate different colors for different sugar ratios (Browning 1967). Based on the absorbance at 553 nm originating from the derivatives of pentoses reacting with phloroglucinol, Douglas reported a method for the determination of pentosans in wheat flour (Douglas 1981).

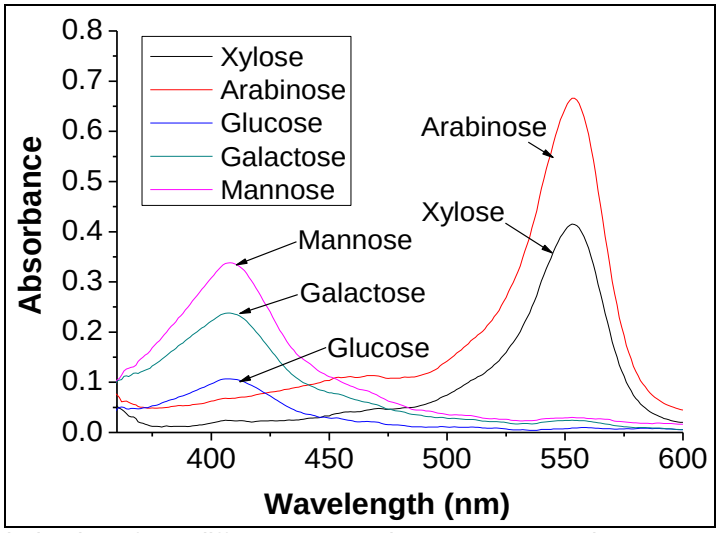

Fig. 1. Spectra of derivatives from different sugars (sugar concentration: 1.35 mmol/L)

In the present study, we found that the C6 sugars (glucose, *etc.*) can also react with phloroglucinol to yield a maximum absorption at 410 nm, as is shown in Fig. 1. It can be seen that the absorbance at 553 nm for hexoses was much lower than that of the same concentrations of pentoses. Meanwhile at around 410 nm the spectra of hexoses overlapped too much with those of pentoses. Additionally, derivatives from the same molar concentrations of glucose and xylose had the same absorbance at 425 nm, which was the isosbestic wavelength (Fig. 2).

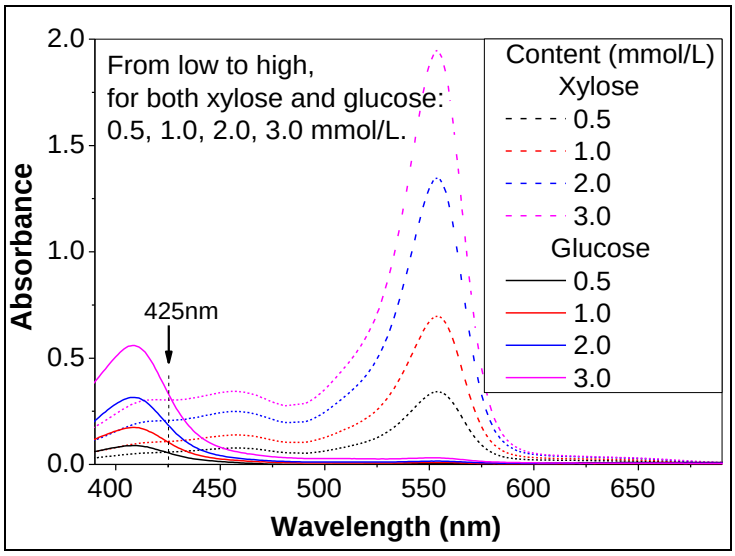

Fig. 2. Spectra of derivatives from various concentrations of xylose and glucose. Note: 425 nm is the isosbestic wavelength.

Conversion of Different Sugars

As is well known, any chemical reaction requires the participation of reactants and products. In the color reaction, sugars and phloroglucinol are the reactants, while the colored derivatives are the products. In order to know whether all of the sugars have been fully converted to colored derivatives under certain conditions, a series of experiments were conducted to test the sugar conversion trend, which can be illustrated by the characteristic absorbance at certain wavelengths (410 nm and 553 nm).

As can be seen from Figure 3(a), the conversion trend of xylose went up to a maximum at 10 minutes and then decreased afterwards. But for different concentrations of glucoses, a much longer time (25 minutes) was needed to attain the maximum absorbance at 410 nm (Fig. 3(b)). Furthermore, for various concentrations of glucose solutions (1.0, 2.0, and 4.0 mmol/L) at different reaction time, the absorbance at 410 nm was proportional (1:2:4) to the corresponding solution concentration. This indicated that the incomplete conversion of glucose at 10 minutes will not interfere with the measurement of hexoses based on the glucose standard curve. The above results can also explain why it is feasible and reasonable to apply the reaction time of 10 minutes in this experiment.

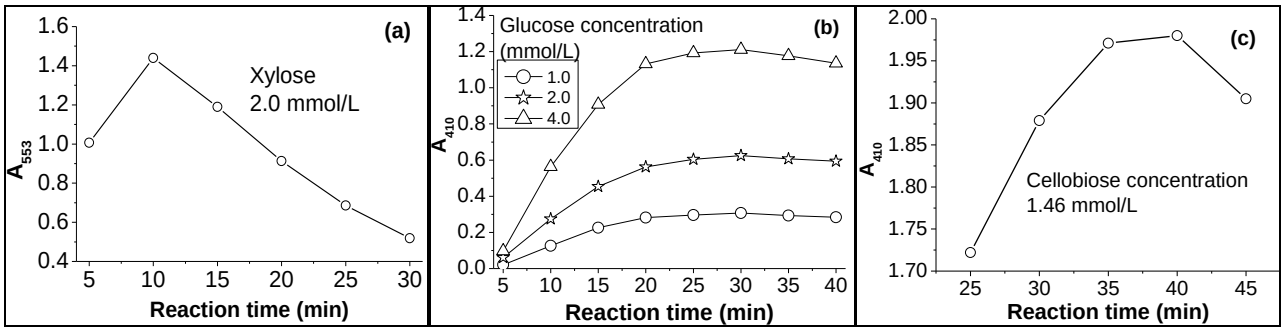

Fig. 3. Conversion trends of xylose, different concentration of glucoses, and cellobiose

We have also conducted experiments with cellobiose (Fig. 3(c)), and the results showed that a longer time (around 40 minutes) is needed to attain the maximum absorbance at 410 nm than glucose (around 25 minutes). Based on Douglas' results, boiling for 25 minutes for pentosans in wheat flour is optimal. The C5 sugars in the prehydrolyzate are mostly lower order oligmers based on the GPC results. Here, we just assume that all the C5 and C6 sugars exhibit the same general behavior as xylose and glucose, although there may be some deviation in the details.

Determination of Xylose and Glucose by Dual-Wavelength Spectrophotometry

Combined with the previous disscusion, the existence of glucose might interfere with the measurement of xylose only if the content of glucose is much higher than that of xylose. By calculation, for the same concentration of xylose and glucose, the absorbance at 553 nm for glucose was approximately 1% of that for xylose. Additionally, pentoses are the main sugars in the prehydrolyzates for hardwoods and herbaceous plants. By contrast for softwoods, the main hemicelluloses are galactoglucomannans, with minor amount of arabinoglucuronoxylans (around 25 to 50% of the former). So, the main sugars in the softwood prehydrolzate are hexoses, which are usually two to four times higher than that of pentoses, based on previous literature reports (Carvalheiro *et al.* 2008; Taherzadeh *et al.* 1999; Millati *et al.* 2002; Schell *et al.* 1999).

We can establish a spectophometric method for simultaneous quantification of pentoses and hexoses in lignocellulose prehydrolyzate. Based on the Beer-Lambert's Law, several linear relationships can be obtained by sets of standard sugars, and related to one another by molar absorption coefficient at different wavelenghts: $\varepsilon_{x553} = 0.662$, $\varepsilon_{x410} = 0.096$, and $\varepsilon_{g410} = 0.176$. Equations (1) to (4) can be used to calculate the content of xyloses (C_x), glucoses (C_g) and total sugars (C_t). As is shown in Fig. 2, 425 nm is the isosbestic wavelength for the derivatives from xylose and glucose. The relationship between C_t and A_{425} can be described by Equation (5), which can be used for the validation of data obtained Equations (1) to (4).

$$A_{x553} = A_{553} = \varepsilon_{x553} \times C_x \quad (1)$$

$$A_{410} = A_{g410} + A_{x410} \quad (2)$$

$$C_g = A_{g410} / \varepsilon_{g410} \quad (3)$$

$$C_x = A_{x410} / \varepsilon_{x410} = A_{x553} / \varepsilon_{x553} \quad (4)$$

$$C_t = C_x + C_g = A_{425} / \varepsilon_{425} \quad (5)$$

The parameters C_x, C_g, and C_t are the concentrations of xylose, glucose and total sugars (mmol/L), respectively, whereas ε_{x553}, ε_{x410}, and ε_{g410} are the molar absorption coefficient of xylose at 553 nm, xylose at 410 nm, and glucose at 410 nm. Equation parameters A_{553}, A_{410}, and A_{425} are the absorbances at 553 nm, 410 nm and 425 nm, whereas A_{x553}, A_{x410}, and A_{g410} are the absorbances of xylose at 553 nm, xylose at 410 nm, and glucose at 410 nm, respectively.

Interferences

In the process of lignocellulose autohydrolysis or acid hydrolysis, many other substances are released into the prehydrolyzate, of which sugars (hexoses and pentoses)

are the main components. In the spectrophotometric method for sugar analysis, those other substances that contain aldehyde groups can react with phloroglucinol to form colored products, which may interfere with the sugar quantification. Therefore, we investigated the interference from lignin, furfural, 5-hydroxymethyl furfural, glucuronic acid, and galacturonic acid. The results are shown in Figs. 4 to 6.

In order to investigate the impact of lignin interferences on sugar analysis, similar concentrations of alkali lignin, glucose, and xylose were prepared, and the spectra are shown in Fig. 4. It can be seen that no distinct absorbance of lignin derivatives appeared in the wavelength range of 425 to 600 nm (maximum 0.03 for 0.5g lignin/L). In addition, the lignin content in the prehydrolyzate is typically much lower compared to sugars, so the disturbance of lignin on the determination of C5 and C6 sugars should be negligible.

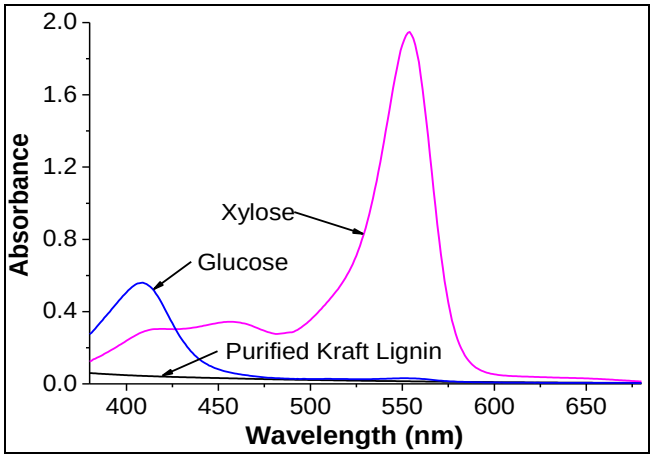

Fig. 4. Influence of lignin on sugar analysis. Note: the concentrations of xylose, glucose, and purified kraft lignin are all 0.5 g/L.

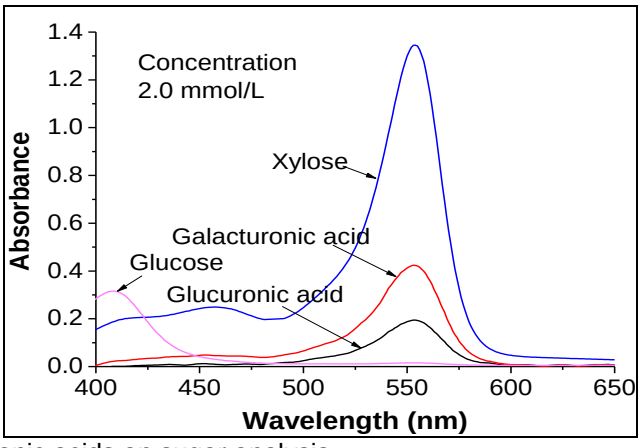

Fig. 5. Influence of uronic acids on sugar analysis

As can be seen from the spectra in Fig. 5, an absorption peak at *ca.* 553 nm for both uronic acids and xylose can be observed from after reacting with phloroglucinol. But the absorbances at 2 mmol/L for glucuronic acid (0.20) and galacturonic acid (0.42) were much lower than that for the same concentration of xylose (1.36). Hardwood xylans contain one 4-*O*-methylglucuronic acid group for every 9 to 10 xylose units (Chen and Yu 1989). Furthermore, based on the published data by Tunc and van Heiningen (2008), the uronic acids to xylose ratio in the prehydrolyzate is approximately 1 to 10, so their interference should be insignificant. Furthermore, under acidic conditions, the uronic

acids associated with hemicelluloses can be hydrolyzed to generate furfural derivatives, *e.g.*, 5-formyl-2-furoic acid (Scott 1979), which could form colored products by reacting with phloroglucinol and could overlap with the spectra derivatives from sugar-phloroglucinol reaction products. However, the glycosiduronic bonds in hemicelluloses were found to be quite resistant (Sjostrom 1981), *e.g.*, 2-*O*-(4-*O*-methylglucopyrano-syluronic acid)-xylopyranose is hydrolyzed 20 times more slowly than the corresponding xylitol (Timell 1964). Therefore, the amount of uronic acid in the prehydrolyzate should be much lower than that of the sugars, and their conversion to furfural derivatives under current hydrolysis pretreatment conditions (acetic acid medium, 5 minutes, 100°C) should be low. Based on this logical discussion, the interference from glucuronic acid and galacturonic acid should be negligible.

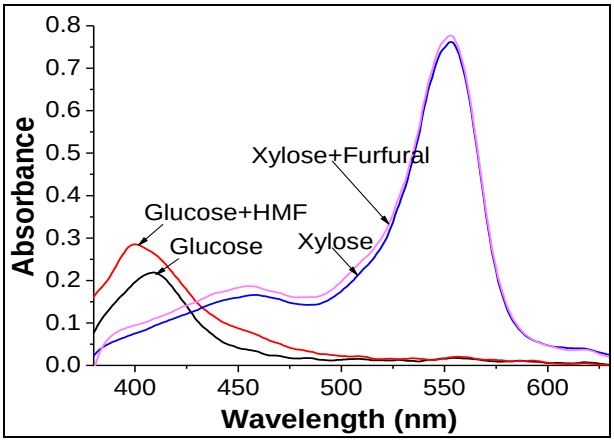

Fig. 6. Disturbance from HMF and furfural on sugar analysis. Note: The concentrations of glucose and xylose are both 1.35 mmol/L; the concentrations of furfural and HMF added are both 0.48 mmol/L.

According to the research results by Browning (1967), many phenols can react with carbohydrates or their degradation compounds to generate colored products in the presence of hydrochloric acid, phosphoric acid, or trichloroacetic acid (TCA). For example, phloroglucinol can react with aldopentose, ketose, and ketohexose to form different colors (Browning 1967). In order to explore the disturbance from furfural and HMF, certain amounts of them were added to glucose or xylose solutions, and the spectra of products are presented in Fig. 6. Based on our results and the literature references, a very small amount of furfural or HMF exists in the prehydrolyzate of wood chips. In our UV-Vis spectral experiments, the ratio of sugars to furfural/HMF is around 1:3, which is higher than that in the prehydrolyzate. Based on the results in Fig. 6, it is not difficult to draw the conclusion that furfural and 5-hydroxymethyl furfural in lignocellulose prehydrolyzate should not interfere with the proposed sugar quantification method.

Method Validation

Based on the measured absorbance data and Equations (1) to (4), the concentrations of C5 and C6 sugars can be calculated. The total sugars can be obtained by summing the values obtained from Equations (3) and (4). The summed value can be validated by Equation (5), which can also be used for calculating the total sugar concentration. Through statistical analysis, the relative standard deviation from five measurements was 1.6% for pentoses and 1.9% for hexoses. These deviations include the

experimental uncertainties in sample hydrolysis, derivatization, and spectroscopic measurement.

The recoveries of pentoses, hexoses, and total sugars ranged from 97.1% to 102.0% as listed in Table 1. This demonstrated that the method is suitable for quantitative analysis of sugars.

Table 1. Method Precision and Sugar Recovery

No.	Xylose			Glucose			Total sugars		
	Actual mmol/L	Measured mmol/L	Recovery %	Actual mmol/L	Measured mmol/L	Recovery %	Actual mmol/L	Measured mmol/L	Recovery %
1	0.704	0.718	98.1	0.580	0.597	97.2	1.280	1.315	97.3
2	0.700	0.708	98.8	0.800	0.812	98.5	1.500	1.521	98.6
3	0.600	0.600	100.0	0.750	0.735	102.0	1.350	1.335	101.1
4	0.600	0.612	98.1	0.950	0.962	98.8	1.550	1.574	98.5
5	0.600	0.618	97.1	0.500	0.510	98.1	1.100	1.127	97.6

In order to further validate the feasibility of this method for such analysis, we also chose several samples for sugar analysis by both IC chromatography and the spectroscopic method. The results are shown in Fig. 7. As shown below, a good linear relationship was demonstrated between the two methods for both pentose and total sugars, while the coefficient of determination (R^2) was just 0.753 for hexose. This indicates that the method is more suitable for samples containing relatively less C6 sugars.

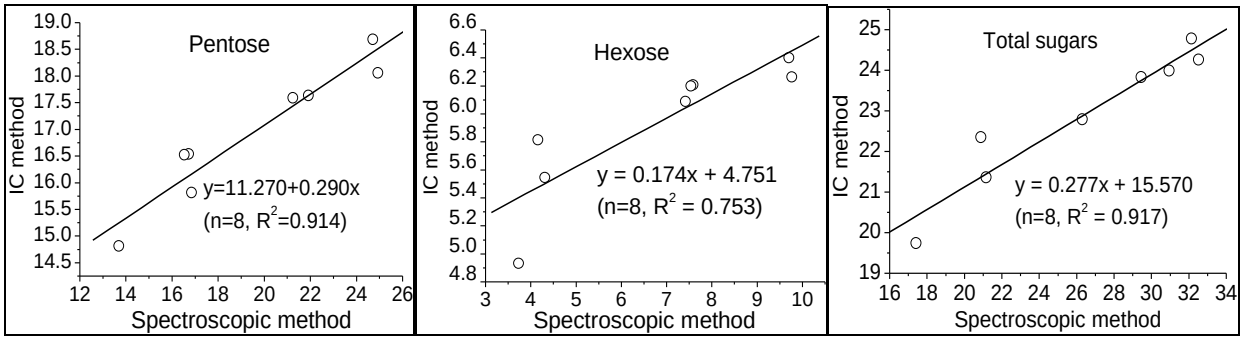

Fig. 7. Comparison of the sugar results by both spectrophotometry and IC method

CONCLUSIONS

A dual-wavelength spectrophotometric method for the determination of sugars in lignocellulose prehydrolyzate was developed. Based on the absorption measurement at the wavelengths of 553 nm and 410 nm, pentoses, hexoses, and total sugars can be simultaneously quantified. The present method is simple, rapid, and accurate. It is suitable to be applied for sugar analysis in forest-related biorefinery research.

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