Development of Method to Determine the Concentration of Alkali-Soluble Lignin using Coomassie Brilliant Blue G-250

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A new method involving the non-covalent binding of Coomassie Brilliant Blue G-250 (CBBG) to alkali-soluble lignin was developed. The binding of the dye to alkali-soluble lignin caused an increase in visible absorption at the maximum wavelength of 630 nm or 640 nm. A linear correlation of the absorbance at their maximum absorbing peak with alkali-soluble lignin concentration was observed. Lignin estimation in black liquor showed that the result of the new method and the gravimetric methods after acidification were closer to quantitative information than that obtained from UV spectroscopy. The isothermal titration calorimetric experiments, and Fourier Transform Infrared (FTIR) spectroscopy comparative analysis of precipitates washed by water, 4% ethanol, and 95% ethanol indicated that CBBG was bound to alkali-soluble lignin, and the binding was non-covalent. This potential method is reproducible, rapid, and cheap, and there is little or no inference from carbohydrate degradation products.

Keywords: Format; Alkali-soluble lignin; Coomassie Brilliant Blue G-250; Black liquor

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INTRODUCTION

Lignin is the second most abundant natural polymer next to cellulose and hemicellulose and the most abundant aromatic polymer in terrestrial ecosystems, accounting for 15 to 30% by weights of dry biomass (Hall *et al.* 2015; Klein *et al.* 2015). As the global effort increases to find a high-valued biorefinery route, the importance of lignin quantity determination has proven to be paramount (Lupoi *et al.* 2015). In pulping and paper-making, and the first generation cellulosic projects, most of the lignin is combusted (Mohamad Ibrahim *et al.* 2011; Ragauskas *et al.* 2014). The heterogenic complexity of the lignin provides a barrier to its fractionation and economical use for higher-valued purposes. Imprecision of information about lignin structure also tends to limit both the research and utilization of lignin (Eastwood *et al.* 2011).

The quantitative determination of lignin in solutions is indispensable to lignin research and utilization. The widely used gravimetric method after acidification and spectroscopic methods including Pearl-Benson method (Barnes *et al.* 1963), ultraviolet (UV) spectroscopy (Alen and Hartus 1988), fluorescence spectroscopy (Bublitz 1981), FTIR spectroscopy (Faix *et al.* 1988), nuclear magnetic resonance (NMR) spectroscopy (Akim *et al.* 2001; Heikkinen *et al.* 2003), and electron spin resonance (ESR) spectroscopy are used in determining lignin content in solution (Lin *et al.* 1992). The gravimetric method after acidification requires a longer time in achieving the quantification. The principal

drawback of the Pearl-Benson method is the interference and error from phenolic components. Fluorescence, FT-IR, NMR, and ESR spectroscopic methods require the use of costly instruments, special reagents, and special sample preparation techniques. Although sample preparation and spectral measurements in UV spectroscopy are simpler, carbohydrate degradation products that remain in lignin samples may have the strong absorbance in ultraviolet regions and thus can interfere with the accuracy of results (Hatfield and Fukushima 2005).

In our research, a color change in the reaction between the alkali-soluble lignin and Coomassie Brilliant Blue G-250 (CBBG) reagent was discovered. Specifically, when the alkali-soluble lignin and CBBG reagent were mixed, the original color became deep. The visible spectroscopy of alkali-soluble lignin mixed with CBBG reagent was scanned, and a linear correlation of the absorbance of the mixture at the maximum absorbing wavelength with alkali-soluble lignin concentration was established. In order to verify the validity of the new method (*i.e.* the CBBG method), parallel tests of matched specimens were carried out with quantitative preparation, UV spectroscopy, and a gravimetric method after acidification, by quantifying lignin in black liquor from aspen kraft pulping. Isothermal titration calorimetric experiments and FT-IR spectroscopy were used to investigate the interaction between CBBG reagent and alkali-soluble lignin.

EXPERIMENTAL

Reagents and Materials

All solvents used in the study were of A.R. grade unless otherwise stated. Microcrystalline cellulose (USP) and guaiacol ((≥98%) were purchased from Sigma-Aldrich (Shanghai, China). Furaldehyde and 5-hydromethyl furaldehyde were obtained from Aladdin Industrial Inc. (Shanghai, China).

Aspen cellulolytic enzyme lignin (CEL) was isolated according to Chang *et al*. (1975) and Hage *et al*. (2009). The average molecular weight of CEL measured was about 15075 g/mol, using a Waters 1515-2414 gel permeation chromatograph (Waters, Milford, MA, USA) with the Styragel® HT THF column. The alkali lignin ($M_w \approx 10000$ g/mol) and sodium lignosulfonate ($M_w \approx 10000$ g/mol) were purchased from Sigma-Aldrich (Shanghai, China) and TCI Development Co., Ltd (Shanghai, China), respectively.

Black liquor of aspen kraft pulping was provided by the Key Lab of Pulp and Paper Science and Technology of the Ministry of Education in Qilu University of Technology.

Alkali-soluble Lignin Preparation

An accurately weighed 10 mg sample of aspen CEL, alkali lignin, or sodium lignosulfonate was dissolved in 10 mL of 0.1 M (or 1 M) NaOH solution. The resulting solution was centrifuged. The supernatant liquids were diluted to 100 μg/mL, 200 μg/mL, 300 μg/mL, 400 μg/mL, 500 μg/mL, 600 μg/mL, 700 μg/mL, 800 μg/mL, and 900 μg/mL and then 100 μg/mL lignin solutions were diluted to 10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, 50 μg/mL, 60 μg/mL, 70 μg/mL, 80 μg/mL, and 90 μg/mL. Likewise, 10 mg/mL sodium lignosulfonate was prepared by 0.1 M (or 1 M) NaOH solution and was diluted to 1 mg/mL to 9 mg/mL.

Coomassie Brilliant Blue G-250 Reagent

Coomassie Brilliant Blue G-250 reagent (CBBG reagent) was prepared according to Bradford (1976) except that the final concentration of CBBG was kept at 0.005 (w/v). The CBBG reagent blank (CBBG blank) contained the same components as that in CBBG reagent except that CBBG component was omitted.

Visible Light Spectroscopy of Alkali-soluble Lignin Mixed with CBBG Reagent

A mixed solution of 900 μL of CBBG reagent (or CBBG blank) and 45 μL of 1 mg/mL lignin dissolved in 0.1 M NaOH (or 0.1 M) was prepared. Then the mixture was added to a 1 mL of cuvette and scanned from 400 nm to 700 nm against double distilled water, using an UV−VIS spectrometer (UV-3100, Shimadzu Co., Ltd, Kyoto, Japan).

Alkali-soluble Lignin Assay by CBBG

About 10 μL (or 5 μL) of the certain concentration of alkali-soluble lignin (in 0 to 100 μg/mL, or 0 to 1000 μg/mL, or 1 to 10 mg/mL) and 200 μL of CBBG reagent were added to the hole of a 96-well plate. The alkali-soluble lignin was pipetted into a row of holes on a 96-well plate in accordance with the concentration gradient. In 30 min, the absorbance at maximum absorbing wavelength was measured using Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Every lignin concentration had four replicates. The concentration of lignin was plotted against the corresponding absorbance, resulting in a standard curve, which was used to determine the concentration of lignin in unknown samples.

Interference by Non-lignin Components

Microcrystalline cellulose, cellobiose, glucose, xylose, guaiacol, furfural, and 5-hydroxymethyl furfural were dissolved 0.1 M NaOH to obtain a 0.1% concentration, respectively. The CBBG reagent (900 μL) and 0.1% non-lignin component (45 μL) were mixed in 1 mL of UV-cuvette. Then, the absorbance of the mixture was measured at the same wavelength as alkali-soluble lignin determination.

Assay of Lignin in Black Liquor

Quantitative preparation

A lignin sample from 3 mL of black liquor from aspen kraft pulping was prepared by acid precipitation according to a previous report (Rautiainen and Alén 2010). The lignin precipitate was washed by 5 mM H_2SO_4 twice and then with water for three times, respectively. After that, the lignin sample was transferred into a weighed weighing bottle that was dried to constant weight beforehand, and then was dried under vacuum at 30 °C. Polysaccharides content in the prepared lignin sample was calculated based on the amount of hexose and pentose present after hydrolysis, using an anhydro correction of 0.88 for C-5 sugars and a correction of 0.90 for C-6 sugars. The amounts of hexose and pentose sugars were determined at 490 nm and 480 nm, respectively, using the phenol-sulphuric acid assay (Dubois *et al.* 1956). The accurate concentration of lignin in black liquor was then calculated after subtracting the polysaccharide content.

UV spectroscopy

Lignin concentration in black liquor was determined using the absorbance at 280 nm according to the method of Rautiainen and Alén (2010).

CBBG method

Lignin prepared from black liquor as an accurately known mass was dissolved in 0.1 M NaOH to a solution of concentration of 1mg/mL. The lignin solution was used to make a standard curve for CBBG method, which was used to determine the lignin content in black liquor. The absorbance at 630 nm of the black liquor after diluting was determined by CBBG reagent, until the absorbance was in the range from 0.4 to 0.7. Using the standard curve, the absorbance and the dilution factor, the lignin content in black liquor was obtained.

Gravimetric method after acidification

Klason lignin in black liquor after evaporation to dryness was determined according to TAPPI T 222om-02.

Every kinds of lignin determination as described above had four replicates.

Isothermal Titration Calorimetry and FT-IR Analysis of Alkali-Soluble Lignin and CBBG Mixture

Isothermal titration calorimetric (ITC) experiments were performed using an iTC200 system (MicroCal, Inc., Northampton, MA, USA). The sample cell contained a solution of dissolved 0.003 mM lignin in 0.1 M NaOH (The control sample only contained 0.1 M NaOH in the sample cell). The syringe contained a mixture of 0.029275 mM CBBG, 713.5 mM phosphate acid, and 1.03 M ethanol. The control cell contained 0.1 M NaOH. The temperature in the titration cell was 25 °C and the solution was stirred at 1000 rpm. Each injection lasted 4 s and 2 μL solutions were injected except that the first injection lasted 0.8 s and 0.4 μL solution was injected. And there was an interval of 150 s between successive injections.

A volume of 100 mL and 50 mL of CBBG reagent were added to 2.5 mL of 1 mg/mL alkali-soluble alkali lignin and aspen CEL, respectively, in triplicates. After 24 h at 25 °C, a precipitate was produced as the lignin-dye complex has a tendency to aggregate with time. After centrifugation, one of the triplicate precipitates were washed three times with double distilled water, the other with 4% ethanol, and another with 95% ethanol. The CBBG blank was added to an alkali-soluble lignin and the produced precipitate was used as the control. All precipitates were dried under vacuum at 30 °C.

Infrared spectra of the dried sample were obtained by using NEXUS 670 Transform Infrared Spectrometer (Thermo Nicolet Company, Madison, Wisconsin, USA).

Data Analysis

All data were the mean value of four times determination, and the results were expressed as the mean value ± standard deviation except for the results of the visible spectroscopy, ITC experiments, and FT-IR. A linear regression was performed with Microsoft Excel 2007. The ITC experimental data was analyzed by Origin 7.0.

RESULTS AND DISCUSSION

Visible Light Spectra of Alkali-Soluble Lignin mixed with CBBG Reagent

Figure 1(a) shows the spectra of 0.1 M NaOH, 1 mg/mL alkali-soluble aspen CEL, 1 mg/mL alkali-soluble alkali lignin, and 1 mg/mL alkali-soluble lignosulfonate, each mixed with CBBG reagent in the visible light region. Compared with the visible spectra of 0.1 M NaOH mixed with CBBG reagent (the black curve in Fig. 1(a)), the increase of the maximum absorbance in alkali-soluble alkali lignin (the navy curve in Fig. 1(a)) and alkali-soluble aspen CEL (the red curve in Fig. 1(a)) was significant, whereas the increase in alkali-soluble sodium lignosulfonate (the green curve in Fig. 1(a)) was less. Alkali-soluble alkali lignin and alkali-soluble aspen CEL had the maximum absorption around 630 nm, and the alkali-soluble sodium lignosulfonate showed maximum absorption around 640 nm in Fig. 1(a).

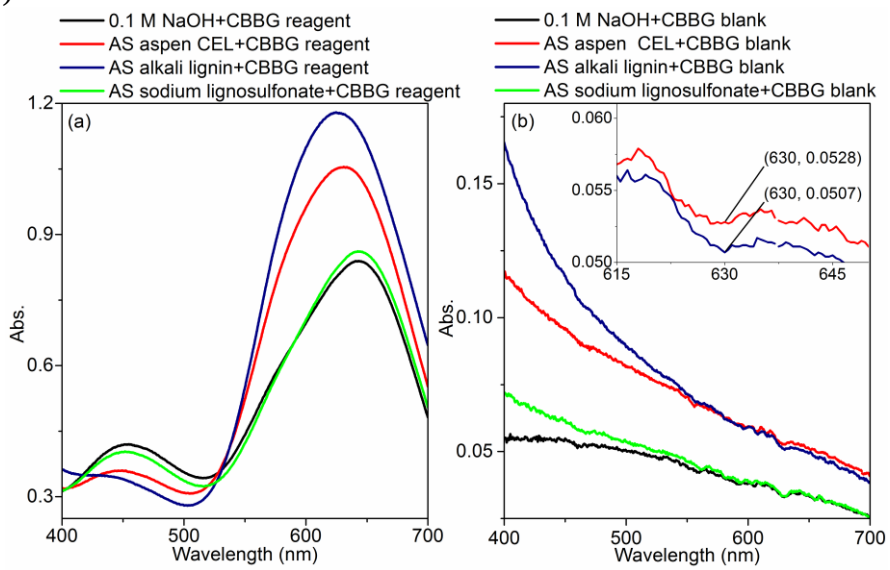

Fig. 1. (a) Visible light spectra of 1 mg/mL alkali-soluble lignin dissolved in 0.1 M NaOH mixed with CBBG reagent and 0.1 M NaOH mixed with CBBG reagent (V(CBBG reagent)/V(lignin solution), 20:1); (b) Visible spectra of 1 mg/mL alkali-soluble lignin dissolved in 0.1 M NaOH mixed with CBBG blank and 0.1 M NaOH mixed with CBBG blank (V(CBBG blank)/V(lignin solution), 20:1); AS, alkali-soluble.

Figure 1(b) showed that the absorbance of alkali-soluble lignin mixed with CBBG blank at 630 or 640 nm was less than 0.053. It also indicated that the absorbance of alkali-soluble lignin by itself at 630 nm or 640 nm did not contribute to the increase of the absorbance at the maximum wavelength.

Linearity and Reproducibility of Alkali-soluble Lignin Assay by CBBG

Figure 2 shows the linear regression of the absorbance of alkali-soluble aspen CEL mixed with CBBG reagent at 630 nm and its concentration. The high regression coefficient R^2-value during 10 to 1000 μg/mL concentrations of aspen CEL was observed (Figs. 2(a) and (b)). However, the regression of the 10 to 100 μg/mL alkali-soluble aspen CEL had the higher standard deviation than the 100 to 1000 μg/mL alkali-soluble aspen CEL. The

results indicated that it was more suitable to determine the 100 to 1000 µg/mL alkali-soluble aspen CEL by the new CBBG method.

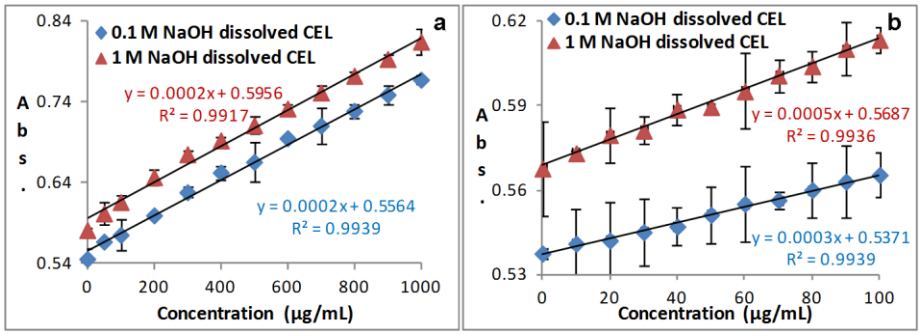

Fig. 2. Linear regression of the absorbance of alkali-soluble aspen CEL mixed with CBBG reagent at 630 nm and its concentration (V(CBBG reagent)/V(lignin solution), 20:1). (a) 100 to 1000 µg/mL alkali-soluble aspen CEL; (b) 10 to 100 µg/mL alkali-soluble aspen CEL.

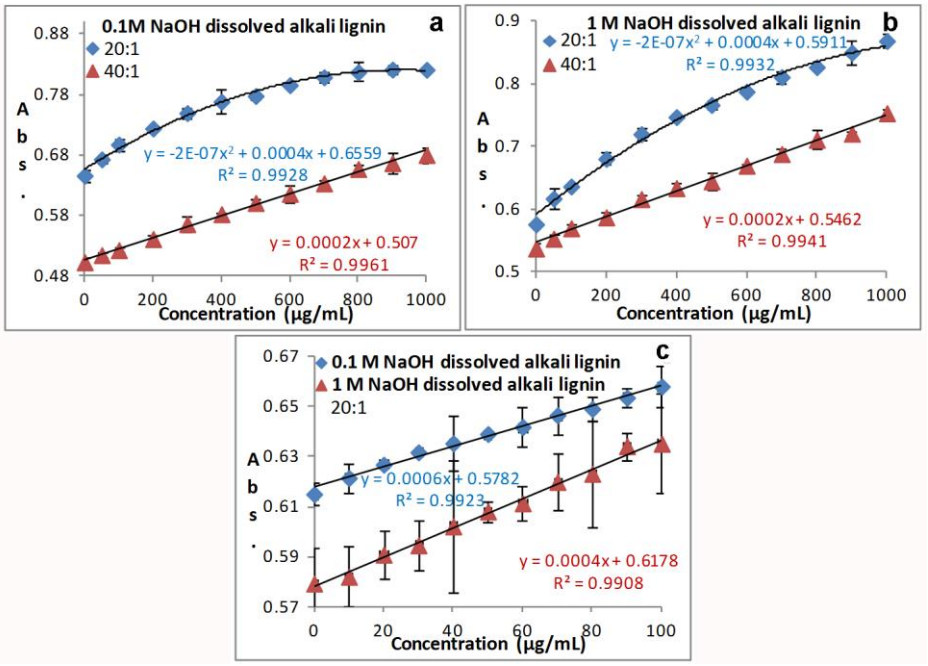

Fig. 3. Linear regression of the absorbance of alkali-soluble alkali lignin mixed with CBBG reagent at 630 nm and its concentration (20:1 and 40:1,V(CBBG reagent)/V(lignin solution)). (a) 100 to 1000 µg/mL alkali lignin dissolved in 0.1 M NaOH; (b) 100 to 1000 µg/mL alkali lignin dissolved in 1 M NaOH. (c) 10 to 100 µg/mL alkali-soluble alkali lignin.

Figure 3 shows the linear regression of the absorbance of alkali-soluble alkali lignin mixed with CBBG reagent at 630 nm and its concentration. When the alkali lignin concentration was 100 to 1000 µg/mL and the volume ratio of CBBG reagent/lignin solution was 20:1 (Figs. 3(a) and (b)), the absorbance of 800 to 1000 µg/mL alkali-soluble alkali lignin solution increased slowly and the R^2-value of the linear fitting was found to be low. But in the same volume ratio, 10 to 100 µg/mL alkali-soluble alkali lignin solution had the good linear regression, but the data errors were higher than 100 to 1000 µg/mL

alkali-soluble alkali lignin (Fig. 3(c)). These findings indicated it was more suitable to determine the 100-1000 µg/mL alkali-soluble alkali lignin by the new CBBG method. Figures 3(a) and (b) also displayed that when the volume ratio increased to 40:1, the absorbances of 800 to 1000 µg/mL alkali-soluble alkali lignin solutions increased more rapidly, and the R^2-value also increased accordingly. This indicated that for 100 to 1000 µg/mL alkali-soluble alkali lignin, the amount of CBBG added in the low volume ratio may be inadequate to fully bind to the lignin.

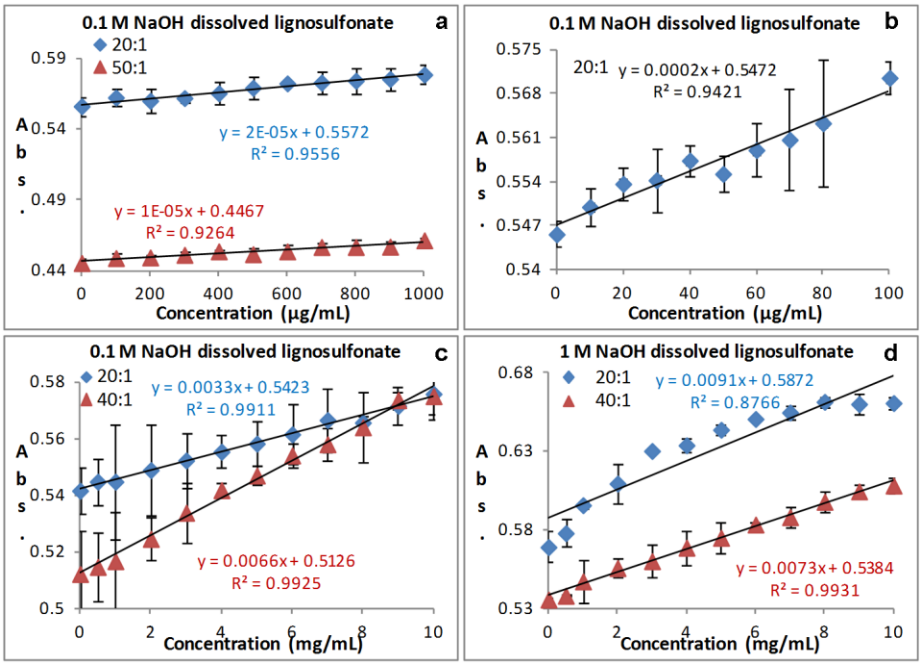

Fig. 4. Linear regression of the absorbance at 640 nm of alkali-soluble sodium lignosulfonate mixed with CBBG reagent (20:1, 50:1, and 40:1, V(CBBG reagent) : V(lignin solution)). (a) 100 to 1000 µg/mL alkali-soluble lignosulfonate; (b) 10 to 100 µg/mL alkali-soluble lignosulfonate; (c) 1 to 10 mg/mL lignosulfonate dissolved in 0.1 M NaOH; and (d) 1 to 10 mg/mL lignosulfonate dissolved in 1 M NaOH.

Figure 4 shows the linear regression of the absorbance of alkali-soluble lignosulfonate mixed with CBBG reagent at 640 nm and its concentration. For 10 to 1000 µg/mL alkali-soluble sodium lignosulfonate, even if the volume ratio increased from 20:1 to 50:1 (Figs. 4(a) and (b)), the linear regression appeared not as good as the relationship shown in Figs (c) and (d). This may be due to the interaction of alkali-soluble sodium lignosulfonate with CBBG reagent, which caused the low-wavelength absorption to increase (Fig. 1). When the concentration of alkali-soluble sodium lignosulfonate increased from 1 to 10 mg/mL and the volume ratio was 40:1, a good linear regression was obtained (Figs. 4(c) and (d)). So, the new CBBG method is a suitable method to determine the range from 1 to 10 mg/mL alkali soluble lignosulfonate

Statistical analysis of triplicate standard assays of CEL, alkali lignin, and sodium lignosulfonate gave a standard deviation of 2.0%, 1.9%, and 1.7%, respectively, for the mean value of the assay. It also showed a highly reproducible response pattern.

Figures 2, 3, and 4 show that the new CBBG method is a good way to determine the 100 to 1000 μg/mL alkali-soluble aspen CEL, the 100 to 1000 μg/mL alkali-soluble alkali lignin, and the 1 to 10 mg/mL alkali-soluble lignosulfonate. And the absorbance of the 100 to 1000 μg/mL alkali-soluble aspen CEL, the 100 to 1000 μg/mL alkali-soluble alkali lignin, and the 1 to 10 mg/mL alkali-soluble lignosulfonate mixed with CBBG reagent ranged from 0.5 to 0.8. As far as the sample to be determined, if its absorbance mixed with CBBG reagent was not in the range from 0.5 to 0.8, the sample needed to be diluted or prepared at higher concentration until the absorbance was within the selected range.

Interference by Non-lignin Components

Table 1 shows that the absorbance values at 630 nm or 640 nm of 0.1% alkali-soluble interferences mixed with CBBG reagent (the data from the absorbance at 630 nm or 640 nm of 0.1% alkali-soluble interferences mixed with CBBG reagent minus the absorbance of 0.1 M NaOH mixed with CBBG reagent) were all around zero. This indicated that the alkali-soluble interferences measured did not affect the enhancement of the absorbance of alkali-soluble lignin mixed with CBBG reagent at the maximum absorbing wavelength. So, microcrystalline cellulose, cellobiose, glucose, xylose, guaiacol, furaldehyde, and 5-hydromethyl furaldehyde had no interference to the concentration assay of the alkali-soluble lignin by CBBG reagent. Especially, furaldehyde and 5-hydroxymethyl furaldehyde are the sources that interfere with lignin determination by UV spectroscopy at 280 nm (Hatfield and Fukushima 2005).

Table 1. Absorbance of Non-Lignin Components at 630 nm and 640 nm.

Wavelength	630 nm	640 nm
Microcrystalline Cellulose	-0.0131(0.002)	-0.0101(0.003)
Cellobiose	-0.013325(0.007)	-0.011325(0.004)
Glucose	-0.01(0.002)	-0.008(0.002)
Xylose	-0.00665(0.008)	-0.00625(0.005)
Guaiacol	-0.0044(0.004)	-0.0048(0.005)
Furaldehyde	-0.00412(0.003)	-0.00406(0.003)
5-(Hydroxymethyl)furfural	-0.00402(0.003)	-0.00398 (0.003)

Lignin Content Assay in Black Liquor

Lignin content in black liquor from aspen kraft pulping was determined by quantitative preparation (after subtracting the quantity of carbohydrates), UV spectroscopy, CBBG reagent, and gravimetric methods after acidification. Table 2 shows that every liter of black liquor contained 0.037 g lignin according to quantitative preparation. Lignin content determined by UV spectroscopy was much higher than quantitative preparation. The amount of lignin determined using CBBG reagent and Klason lignin were close to the level of quantitative preparation. These findings indicated that the assaying lignin content in black liquor by CBBG reagent was reliable and the complicated carbohydrate degradation products in black liquor had much less interference on the absorbance of lignin at 280 nm, but they had almost no effect on the determination of lignin by CBBG reagent.

Table 2. Lignin Content in Black Liquor

Determination Methods	Lignin (g/L)
Quantitatively prepared lignin	0.037 (0.0019)
UV spectroscopy	0.069 (1.4173)
CBBG reagent method	0.035 (0.0058)
Klason lignin	0.034 (0.0017)

Isothermal Titration Calorimetry and FT-IR Analysis of Combination between Alkali-Soluble Lignin and CBBG

Table 3 showed the thermodynamic parameters of CBBG binding to alkali-soluble lignin in isothermal titration calorimetric experiments. The C value between 10 and 500 indicated that the results of ITC experiments were reliable (Turnbull and Daranas 2003). Among three kinds of lignins, the alkali-soluble alkali lignin had the most CBBG binding sites, but its K_a value was lower than that of CEL. This indicated that the affinity of CBBG binding to alkali lignin was not strong. It also explains why only the high volume ratio of CBBG reagent/alkali lignin solution exhibited a good linear correlation when the concentrations of alkali-soluble alkali lignin were 100 to 1000 μg/mL (Fig. 3). The K_a of alkali-soluble aspen CEL was the highest, which indicated that CBBG more easily bounds to alkali-soluble aspen CEL. Therefore, although alkali-soluble aspen CEL did not have a high amount of CBBG binding sites, its high concentration appeared to have good linear regression under the low volume ratio of CBBG reagent/lignin solution (Fig. 2). For alkali-soluble sodium lignosulfonate, the ITC experiment showed its less CBBG binding sites and weak affinity for CBBG reagent.

Table 3. Thermodynamic Parameters of CBBG Binding to Alkali-Soluble Lignin

	Alkali-soluble alkali lignin	Alkali-soluble aspen CEL	Alkali-soluble sodium lignosulfonate
N	1.08	0.732	0.674
K_a (M^{-1})	2.90e7	4.12e7	2.17e7
Δ*H* (cal/mol)	-5.67e7	-9.05e7	-9.52e7
*T*Δ*S*(cal/mol)	-5.66e7	-9.03e7	-9.51e7
C value	87.00	123.60	65.10
N, the binding sites number; K_a, the affinity constant; Δ*H*, the enthalpy; *T*Δ*S*, the entropy; *C* value=the initial concentration of alkali-soluble lignin / the dissociation constant (K_d)			

As a consequence, there was less increase in the absorbance at 640 nm (Fig. 1). The observed result may be due to the steric hindrance of the sulfonic group in sodium lignosulfonate (Fig. 5). The values of enthalpy change (Δ*H*) and entropy change (*T*Δ*S*) showed that enthalpy change was favorable to the binding of CBBG to alkali-soluble lignin, but entropy change was disadvantageous to the binding process.

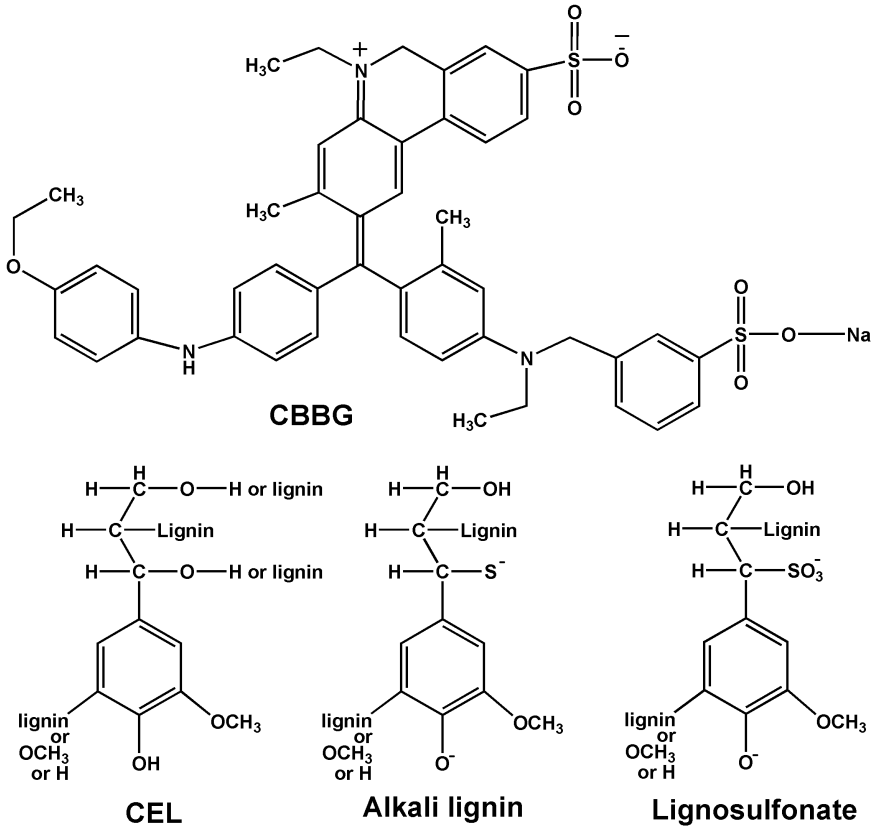

Fig. 5. Structure of CBBG, CEL, alkali lignin, and sodium lignosulfonate

Figure 6 shows FT-IR spectra of the precipitate washed by water, 4% ethanol, and 95% ethanol, respectively. Compared with the control sample, the change of infrared spectra of alkali lignin and aspen CEL precipitates after washing with water and 4% ethanol was similar (Figs. 6(a) and (b)). New peaks located at wavelengths 719 cm^{-1}, 1160 cm^{-1}, 1338 cm^{-1}, and 1396 cm^{-1} appeared. A different peak at 1652 cm^{-1} appeared on FT-IR spectra of alkali lignin precipitates with water and 4% ethanol washings. The peaks at 719 cm^{-1}, 1160 cm^{-1}, 1396 cm^{-1}, and at 1650 cm^{-1} were assigned to the S-H bending, the S=O symmetric stretch, the C-N stretch, and the C=N absorption, respectively (Ishida *et al.* 1980; Maugé *et al.* 2001; Pavia *et al.* 2008). The S-H, S=O, C-N, and C=N bonds exist in CBBG rather than in alkali lignin and aspen CEL (Fig. 5). So, CBBG was bound to lignin and was not washed away by water and 4% ethanol. On the other hand, the FT-IR spectra of 95% ethanol washed alkali lignin and aspen CEL precipitates changed little (Figs. 6(a) and (b)). This indicated that the binding of CBBG and alkali-soluble lignin can be dissociated by 95% ethanol. Therefore, it appears that the bonding between alkali-soluble lignin and CBBG was non-covalent.

CBBG under the condition of testing (pH≈0.98 to 0.99, V(0.1 M NaOH):V(1.463 M H_3PO_4), 1:40 or 1:20, K_{a1} of H_3PO_4 =7.5×10^{-3}) was a neutral form that had the maximum absorbance around 640 nm (Fig. 1). It was similar to the spectrum of CBBG under pH=1, and its maximum absorbance was around 650 nm (Georgiou *et al.* 2008). Under the conditions of testing, CEL, lignosulfonate, and alkali lignin were also neutral

(Fig. 5). So, the electrostatic interaction was not the main interaction between alkali-soluble lignin and CBBG. Non-covalent interaction-hydrogen bond, Van der Waals forces, and hydrophobic interaction may play important role in interaction between them.

Under the condition of testing in the experiment, the concentrations of 100 to 1000 μg/mL alkali-soluble CEL, 100 to 1000 μg/mL alkali-soluble alkali lignin, and 1 to 10 mg/mL alkali-soluble lignosulfonate can be determined by CBBG reagent. Notably, the interferences assayed were restricted to microcrystalline cellulose, cellobiose, glucose, xylose, guaiacol, furaldehyde, and 5-hydromethyl furaldehyde. If other contaminants were present in the alkali-soluble lignin, then the method needs to be used carefully. However, at least the contaminants in the black liquor from aspen kraft pulping did not affect the determination of alkali-soluble lignin concentration by the CBBG method.

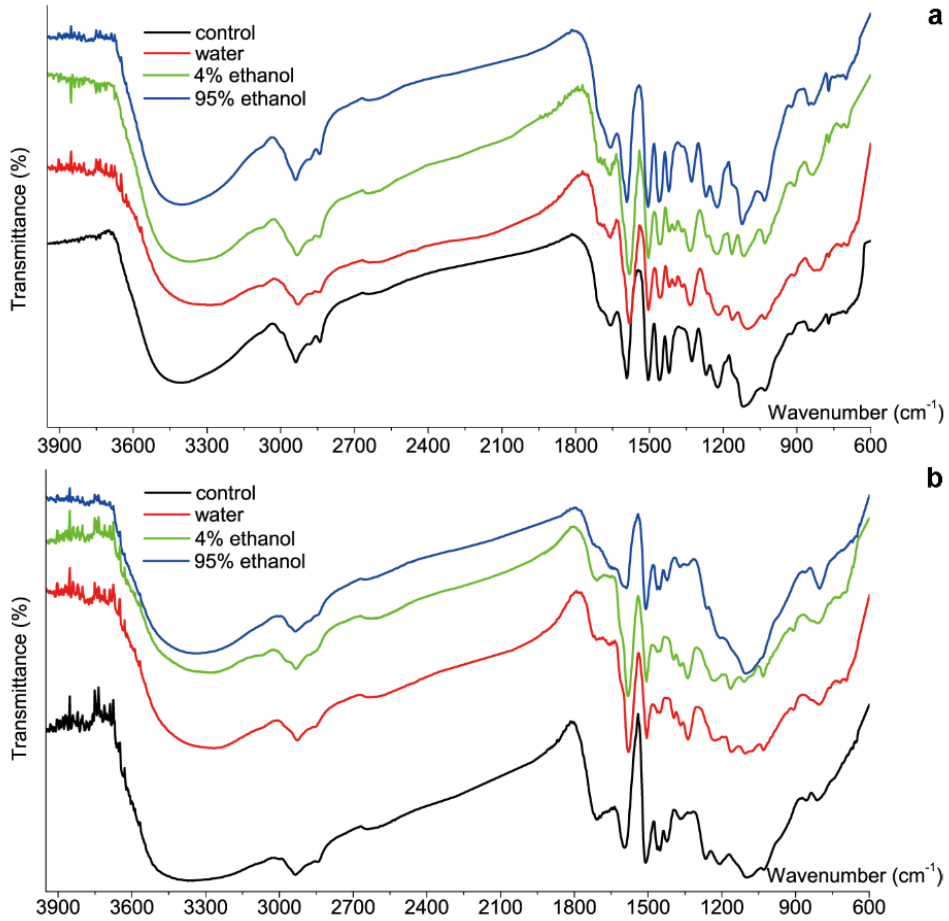

Fig. 6. FT-IR spectra of the precipitates after washing with water, 4% ethanol, and 95% ethanol (The precipitates of the mixture of alkali-soluble lignin and CBBG blank were used as the control). (a) The precipitate from alkali-soluble alkali lignin; and (b) the precipitate from alkali-soluble aspen CEL.

CONCLUSIONS

1. The concentrations of alkali-soluble aspen CEL and alkali lignin were more suitable to be determined with 0.005% CBBG reagent. In the case of alkali-soluble sodium lignosulfonate only a high concentration could be determined by CBBG reagent because of the weak binding and less available CBBG binding sites.
2. The CBBG reagent is a suitable way to determine the lignin content in pulping black liquor, which will be convenient for subsequent utilization of the black liquor.
3. Compared with UV-Vis spectroscopic and the gravimetric methods after acidification, the determination of the alkali-soluble lignin content by CBBG reagent can avoid the inference of carbohydrate degradation products and shorten the determining time.
4. The CBBG method is a cheap, simple, quick, and accurate spectroscopic method for determining the concentration of alkali-soluble lignin in visible light region. It should be of great utility in paper making, the biofuel and bioethanol industry, and other biomass related research or industry.

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