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Determination of surface-accessible acidic hydroxyls and surface area of lignin by cationic dye adsorption

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Abstract

A new colorimetric method for determining the surface-accessible acidic lignin hydroxyl groups in lignocellulose solid fractions was developed. The method is based on selective adsorption of Azure B, a basic dye, onto acidic hydroxyl groups of lignin. Selectivity of adsorption of Azure B on lignin was demonstrated using lignin and cellulose materials as adsorbents. Adsorption isotherms of Azure B on wheat straw (WS), sugarcane bagasse (SGB), oat husk, and isolated lignin materials were determined. The maximum adsorption capacities predicted by the Langmuir isotherms were used to calculate the amounts of surface-accessible acidic hydroxyl groups. WS contained 1.7-times more acidic hydroxyls (0.21 mmol/g) and higher surface area of lignin (84 m^2/g) than SGB or oat husk materials. Equations for determining the amount of surface-accessible acidic hydroxyls in solid fractions of the three plant materials by a single point measurement were developed. A method for high-throughput characterization of lignocellulosic materials is now available.

Keywords: Surface area, lignin, acidic, hydroxyl, adsorption

1 Introduction

Lignins are aromatic polymers synthesized mainly from three phenolic monomers called monolignols at proportions varying between plant species. The monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol contain zero, one, and two methoxy groups in the aromatic ring, and form *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) phenylpropanoid units of lignin, respectively (Grabber et al., 1997). Among other biological functions, lignin provides plants with protection against microbial degradation, as evidenced by negative linear correlation between lignin content and carbohydrate digestibility of forages and herbaceous plants (Jung, 1989; Sewalt et al., 1997a).

Synthetic H, G, and S lignin polymers have similar inhibitory effects on cell wall degradability (Grabber et al., 1997), suggesting that inhibition might not be related to the number of methoxyl groups. Instead, phenolic hydroxyl groups of lignin preparations have appeared detrimental to cellulolytic enzymes (Sewalt et al., 1997b; Berlin et al., 2006). Most of the studies investigating the effect of lignin structure on enzymatic hydrolysis have been made using model compounds or isolated lignins. However, synthetic lignins might not adequately represent native lignin, as structure of isolated lignins depends on their origin in the plant tissues and the method used for their isolation (Sipponen et al., 2013).

Porosity increases the total surface area and heterogeneity of plant materials, and lignin in the pores may have a negative effect on hydrolysis of the plant material (Mooney et al., 1998). At least water conducting tissues are enriched in lignin (Laschimke, 1989), and it would thus be valuable to be able to measure non-degradatively the amount of surface-

accessible lignin in plant solid materials. This could be achieved by determining acidic hydroxyls such as phenolic groups on surfaces. However, the known techniques are either degradative such as the chlorine dioxide titration, or, as in the case of reflectance UV-Raman spectroscopy, limited to measuring lignin on external surfaces only (Eshkiki et al., 2007; Lähdetie et al., 2009). Furthermore, the above mentioned methods have not been aimed at providing information of the surface area covered by lignin.

The objective of the current study was to develop a method for determining surface-accessible acidic hydroxyl groups of lignin in solid materials. The adapted approach is based on adsorption of Azure B onto lignin in aqueous suspension of the plant material. Like methylene blue, Azure B is a basic dye that carries a cationic charge in water, inducing binding to anionic acidic groups (Graham, 1955). Previously, Azure B has been used to study lignification of wood (Kutscha and Gray, 1972), but not in quantitative characterization of lignin. The current work was organized in three parts. First, specificity of adsorption of Azure B on isolated lignins in comparison to acetylated lignin and pure cellulose materials was elucidated. Second, the amount of surface-accessible acidic hydroxyl groups and the corresponding surface area of lignin were determined in wheat straw, sugarcane bagasse, and oat husk materials, based on the maximum equilibrium adsorption capacities predicted by the Langmuir isotherms. Finally, equations were developed for a single-point procedure for quantifying surface-accessible acidic hydroxyl groups in the three plant materials, varying for instance as a function of growth stage, pretreatment, or hydrolysis of associated carbohydrates.

2 Materials and methods

2.1 Materials

Wheat straw (WS), sugarcane bagasse (SGB) was obtained from Danisco, and oat husk materials were used as test materials for comparative characterization of surface-accessible acidic hydroxyl groups. These agricultural residues were obtained from Finland (WS and oat husks) or from Brazil (SGB). Oat husks and WS were milled to pass a 1 mm sieve, suspended in cold water, wet-sieved and air dried. SGB was milled to pass a 1 mm sieve, and used as such. Wheat straw soda lignin (hereafter referred to as GreenValue lignin) originated from an industrial soda pulping process (Lora, 2008), and was purchased from GreenValue SA (Switzerland). Whatman I filter paper (Whatman, USA), was defibrillated by milling to pass a 1 mm sieve. Emcocel 50M microcrystalline cellulose (Penwest Pharmaceuticals, England) was used as such. Azure B was reagent grade and prepared by direct synthesis (Lot# MKBH6990V, CAS: 531-55-5, Aldrich, USA). Other chemicals used in this work were of analytical grade.

2.2 Preparation of extractive-free wheat straw and preparation of holocellulose

Extractive-free wheat straw (WS-EF) was prepared by Soxhlet extraction of WS (16.9 g) with distilled water, and then with ethanol (96%, v:v). WS-EF was air-dried and recovered at 65% yield. Preparation of holocellulose from WS-EF was carried out by sodium chlorite pulping in presence of acetic acid (Hallac et al., 2009). Briefly, 4.8 g of WS-EF was continuously stirred in 400 mL water containing 3.2 mL glacial acetic acid and 3.2 g

sodium chlorite at 70 ºC. After 2 h, similar amounts of acetic acid and sodium chlorite were supplemented and the treatment continued for 2 h. The solid fraction was recovered by filtration, washed with deionized water, and air-dried.

2.3 Preparation of WS lignin and acetylation of lignin

WS lignin was prepared from black liquor obtained from soda delignification of pre-extracted wheat straw (Pihlajaniemi et al., 2013). The black liquor (1500 g, 8.4% solids) was adjusted to pH 9.8, and after centrifugation the supernatant was acidified to pH 5 using 6 M sulfuric acid. The suspension was supplemented with 3 mL of commercial xylanase preparation GC 140 (Genencor), in order to remove insoluble xylan, and the continuously stirred 72 h enzymatic reaction was carried out at 50 ºC. The solid and liquid phases were separated by centrifugation, and the solid fraction was washed three consecutive times with acidified water (pH 3, HCl) and lyophilized. Elemental analysis (CHN) showed that the obtained WS lignin contained 0.6% nitrogen as compared to 1.0% in GreenValue lignin. The two lignins were acetylated in pyridine:acetic anhydride (1:1, v:v) mixture, and purified following the literature procedure (Gosselink et al., 2004).

2.4 Compositional analysis

Lignin contents and carbohydrate compositions of the lignocellulosic materials were determined by the two-stage sulfuric acid hydrolysis procedure (Sluiter et al., 2010). Acid insoluble residue separated after the second stage hydrolysis by filtration on Whatman

GF/F membrane was corrected for its ash content and termed Klason lignin. Ash content of the materials without any prior hydrolysis treatment was also determined by gravimetric method after ignition of the samples at 650 ºC for 10 h. Carbohydrates in the lignin preparations (100 mg) were released by hydrolysis in 4% sulfuric acid (5 mL, 121 °C, 1 h). Monosaccharides were analyzed by high-performance liquid chromatography (HPLC) (Pihlajaniemi et al., 2014), the system comprising a liquid chromatography pump LC-6A, an autosampler SIL-20A, a column compartment CTO-20A, a refractive index detector RID-10A, and a System controller SCL-10A VP, all from Shimadzu (Japan). Analyses were carried out in duplicates and the mean values were calculated.

2.5 Analysis of lignin by ^{31}P NMR spectroscopy

Quantification of different hydroxyl groups in lignin was carried out using ^{31}P nuclear magnetic resonance (NMR) spectroscopy (Granata and Argyropoulos, 1995). The analysis was started by fully dissolving GreenValue lignin or WS lignin in 0.15 mL of dimethylformamide. Then, 0.1 mL of pyridine and 0.2 mL of internal standard endo-N-Hydroxy-5-norbornene-2,3-dicarboximide (9.26 g L^{-1} or 8.97 g L^{-1}) in pyridine:deuterated chloroform solvent mixture (1.6:1, v:v) were added. Chromium(III) acetylacetonate (0.05 mL, 0.58 mg in the above solvent mixture) was added as a relaxation agent. Tetramethylphospholane (0.15 mL) was added slowly to start the phosphitylation reaction. Finally, 0.3 mL deuterated chloroform was added, and the solution analyzed with a Bruker Avance III 400 MHz spectrometer, using 512 scans with 5 s relaxation delay and 30° pulse angle. Analyses were carried out in triplicates and the mean values were calculated.

2.6 Adsorption experiments

Adsorption experiments were carried out in borosilicate glassware, either in conical flasks or in screw cap tubes. In a typical experiment, 100 mg of plant material was weighed into a 250 mL conical flask, and 50 mL of Azure B solution (0.01…0.8 g L^{-1}) in aqueous 0.05 M Na-phosphate buffer (pH 7) was added to start the experiment. The flask was agitated at 150 rpm in a thermostatic incubator. In the end of the experiment 1 mL sample was withdrawn from the suspension and filtered through 0.45 µm poly(tetrafluoroethylene) disc filter (VWR) to remove the solid particles. The concentration of Azure B in the permeate was determined relative to the concentration of the Azure B solution agitated without adsorbent by measuring the absorbance at 647 nm. The equilibrium adsorption capacity q_e (mg g^{-1}) was calculated from Eq. (1):

$$q_e = \frac{V_0(C_0 - C_e)}{m_{\text{adsorbent}}} \quad (1)$$

Here, V_0 (mL) is the volume of Azure B solution, C_0 and C_e are the respective concentrations (mg mL^{-1}) of Azure B in the solution at time zero and at the end of the experiment, and $m_{\text{adsorbent}}$ (g) is the dry weight of the adsorbent. The effect of pH on adsorption of Azure B was studied with 24 h residence time at 25 °C. The buffer solutions (0.05 M) used were phosphate-citrate (pH 3), Na-citrate (pH 4), Na-phosphate (pH 5…7), and Tris-HCl (pH 8). Adsorption kinetics and the effect of temperature were studied at pH 7 by measuring the equilibrium adsorption capacity of the adsorbent agitated in 0.1 g L^{-1}

Azure B solution (50 mL) for 10 min to 24 h. The effect of particle size was studied by measuring the equilibrium adsorption capacity of wheat straw milled through 1 mm or 0.2 mm sieve under similar conditions: 100 mg straw in 50 mL of 0.1 g L^{-1} Azure B, 24 h at 25 °C. These conditions were used to study comparatively the effect of extractives by measuring q_e of WS and WS-EF relative to proportion of lignin in the two adsorbents. Adsorption experiments were carried out in duplicates and the mean values were calculated.

2.7 Equilibrium isotherms

Equilibrium isotherms were fitted to Langmuir (1916) and Redlich-Peterson (1959) models. Langmuir isotherm is shown:

$$q_e = \frac{X_m a_L C_e}{1+a_L C_e} \quad (2)$$

Here, q_e (mg g^{-1}) is the equilibrium adsorption capacity, X_m (mg g^{-1}) is the maximum adsorption capacity, a_L (L mg^{-1}) is the Langmuir equilibrium constant, and C_e (mg L^{-1}) is the equilibrium concentration. The Redlich-Peterson isotherm incorporates features of the Langmuir and the Freundlich (1907) isotherms. The equation can be described as follows:

$$q_e = \frac{K_R C_e}{1+a_R {C_e}^{br}} \quad (3)$$

Here, q_e (mg g^{-1}) is the equilibrium adsorption capacity, K_R (L g^{-1}), a_R (L mg^{-1}), and b_r (dimensionless), are the empirical 'best fit' constants obtained from the linearization of the equation (Ho and McKay, 1998), and C_e (mg L^{-1}) is the equilibrium concentration.

2.8 Determination of the amount of surface-accessible acidic hydroxyl groups and the corresponding surface area of lignin

The amount of surface-accessible hydroxyl groups on WS, SGB, and oat husk materials was estimated from the maximum adsorption capacity (X_m) predicted by the Langmuir isotherms and calculated from Eq. (2). The calculations thus assumed monolayer adsorption. The surface-accessible acidic hydroxyls can be expressed relative to the whole adsorbent or proportion of lignin in the adsorbent:

$$\text{Surface-accessible acidic OH } \left(\frac{\text{mmol}}{\text{g material}}\right) = b \cdot \frac{X_m}{305.83} \quad (4)$$

$$\text{Surface-accessible acidic OH } \left(\frac{\text{mmol}}{\text{g lignin}}\right) = b \cdot \frac{X'_m}{305.83} \quad (5)$$

$$b = 1 - \frac{X_{m\ (\text{Emcocel 50M})}(1 - \text{proportion of lignin})}{X_{m\ (\text{plant material})}}$$

Here, 305.83 is the molar mass of Azure B (g/mol), X'_m is X_m divided by the proportion of lignin in the adsorbent, b is the correction factor for non-specific binding of Azure B on non-lignin components, $X_{m\ (\text{Emcocel 50M})}$ and $X_{m\ (\text{plant material})}$ are the maximum adsorption capacities of the microcrystalline cellulose and the plant material, respectively. Azure B

was assumed to have spherical molecular geometry and cover surface area of 0.66 nm^2. This area is one fourth of the Connolly (1983) molecular surface area of the protonated Azure B (2.63 nm^2), obtained using ChemBioDraw software (CambridgeSoft). The surface area of lignin is obtained as the product of the maximum adsorption capacity (X_m or X'_m) and the area covered by mass unit of Azure B (1.297 m^2/mg), corrected by the non-specific binding (*b*):

$$SA = \text{Surface area of lignin } (\frac{m^2}{\text{g material}}) = b \cdot 1.297 \cdot X_m \quad (6)$$

$$SSA = \text{Specific surface area of lignin } (\frac{m^2}{\text{g lignin}}) = b \cdot 1.297 \cdot X'_m \quad (7)$$

2.9 Single-point procedure for quantitative measurement of surface-accessible acidic hydroxyls and corresponding surface area of lignin

A single point procedure for determining surface-accessible acidic hydroxyls in solid lignocellulose was developed. In the proposed procedure, adsorbent weight (dry weight) and volume of Azure B solution were 100 mg and 50 mL (flask) or 20 mg and 10 mL (tube), for reactions in flasks or tubes, respectively. Into the tube or flask containing the adsorbent, 0.1 g L^{-1} Azure B solution in 0.05 M Na-phosphate buffer at pH 7 was added. The mixture was agitated at 25 °C for 24 h and absorbance at 647 nm was measured from the liquid phase diluted to give absorbance within the range of 0.2 to 0.7. Based on decrease in absorbance compared to the 0.1 g L^{-1} Azure B solution incubated at similar conditions without adsorbent, the amount of Azure B removed from the solution was

calculated, and the q_e was obtained from Eq. 1. The effect of differing proportion of lignin in the adsorbent was taken into account by the correction factor $F = q_{e1}/q_{e2}$. Here, q_{e1} is the equilibrium adsorption capacity obtained when the ratio of lignin to Azure B was 4 (assuming 20% total lignin content in the sample), and q_{e2} was the equilibrium adsorption capacity obtained when the ratio of lignin to Azure B was varied from 1.9 to 6.8. The amount of surface-accessible acidic hydroxyls in the sample was based on the q_e measured by the single-point procedure:

$$\text{Surface-accessible acidic OH } \left(\frac{mmol}{g \text{ material}}\right) = bF \cdot \frac{\frac{X_m}{q_e'} \cdot q_e}{305.83} \quad (8)$$

Here, $\frac{X_m}{q_e'}$ is the ratio of the maximum equilibrium adsorption capacity (X_m) to the predetermined equilibrium adsorption capacity of the test material (q_e') in conditions similar to the single-point procedure.

3 Results and discussion

3.1 Composition of materials

Various cellulose and lignin materials selected for the adsorption tests presumably contained low and high proportion of lignin. In addition to the cellulose and lignin materials, WS, SGB, and oat husks were characterized for their composition in order to

elucidate their adsorbent properties. The results in Table 1 show that Whatman I and Emcocel 50M turned out being almost pure cellulose materials containing 96%…97% glucan and only negligible amounts of lignin (1% in Whatman I and 0.2% in Emcocel 50M). WS holocellulose contained 83% carbohydrates, but also 4.1% KL and 4.6% acid-soluble lignin (ASL), despite the acidic sodium chlorite delignification used in its isolation. There are indications that prolonged treatment time or the use of peracetic acid could decrease the KL content further (Kumar et al., 2013). WS and SGB contained more glucan (39% and 41%) than oat husks (31%), while Klason lignin (KL) content of the materials was between 20% and 22%. Oat husks also contained more arabinoxylan (35%) compared to 25% in WS and SGB. The two alkali lignins had different compositions, stemming from their different isolation conditions. WS lignin contained 83% total lignin and only 1% carbohydrates, as compared to 92% total lignin and 4% carbohydrates in GreenValue lignin. These materials were then used to study the effects of different parameters on adsorption of Azure B.

3.2 Effect of pH and cellulose on adsorption of Azure B on hydroxyl groups of lignin

Adsorption of Azure B on WS, cellulose, GreenValue lignin and acetylated GreenValue lignin materials was studied at a pH range from 3 to 8 to determine the binding specificity to acidic hydroxyl groups of lignin. The results in Fig. 1 show that equilibrium adsorption capacity (q_e) of wheat straw increased from 12.6 mg/g to 24.5 mg/g when pH increased from 3 to 7, and remained at the same level at pH 8. Increasing alkalinity from pH 3 to pH 6 increased adsorption of Azure B on GreenValue lignin from 58.9 mg/g to 105 mg/g, but

thereafter lower q_e was obtained at pH 7 and pH 8, possibly due to partial dissolution of lignin. Many guaiacyl and syringyl phenolic compounds have been shown to have pKa -values between 7 and 10 (Ragnar et al., 2000). Increasing pH would thus make more phenoxide groups available and facilitate binding of Azure B to lignin. On the contrary, adsorption on Whatman I decreased slightly with increasing pH, and the q_e was at most 2.5 mg/g. At pH 7 Whatman I showed q_e of 2.0 mg/g as compared to q_e of GreenValue lignin, 102.4 mg/g, at similar conditions.

The specificity of binding of Azure B for hydroxyl groups of lignin was studied by using acetylated GreenValue lignin, in which phenolic hydroxyls had been completely blocked by acetylation, as revealed by ^{31}P NMR (Table 2). Very low adsorption occurred on acetylated lignin as a function of increasing pH, the increment being 2 mg/g to 4 mg/g from pH 3 to pH 8 (Fig. 1). These results confirm the literature information that Azure B binds to the acidic anionic groups (Flax and Himes, 1952; Graham, 1955). The mechanism of binding has not been elucidated before, and a tentative mechanism is proposed here. Accordingly, Azure B acts as a base that deprotonates the acidic hydroxyl group such as the phenolic OH group, which leads to an ionic bond between the protonated Azure B and the phenoxide anion. This mechanism might be affected by other adsorption conditions besides pH. To find out suitable contact time and temperature, further tests undertaken at pH 7 aimed at elucidating the adsorption kinetics and isotherm type.

3.3 Adsorption kinetics and the effect of temperature on adsorption

Kinetics of the adsorption process was studied using WS, SGB, and oat husk materials. Increasing temperature from 15 °C to 50 °C decreased the equilibrium adsorption capacities of WS slightly (Fig. 2a). Regardless of the temperature the adsorption proceeded rapidly within the first hour, and a plateau was nearly reached after four hours. At a two-fold higher adsorbate/adsorbent ratio, q_e increased only slightly when the contact time was prolonged beyond 8 h (Fig. 2b). It is possible that the adsorption process is affected by porous sections of the adsorbent where mass-transfer limits the site-specific adsorption. Substrate accessibility and unspecific adsorption also affect enzymatic hydrolysis of lignocellulosic materials (Mooney et al., 1998) in a process where the reaction time is typically 24 h or longer (Pihlajaniemi et al., 2014). To provide sufficient time for the adsorption process to reach equilibrium, further experiments were carried out with 24 h contact time at 25 °C.

3.4 Equilibrium isotherms

Equilibrium isotherms were determined for adsorption of Azure B on WS, SGB, oat husk, and the lignin and cellulose materials in order to study their surface properties. The fitted parameters of the Langmuir and Redlich-Peterson equations of the test materials are summarized in Table 3. With the native plant materials, adsorption was better modeled by the Redlich-Peterson equation compared to the Langmuir equation (Fig. 3). WS showed throughout higher equilibrium adsorption capacities (q_e) than SGB or oat husk materials. Markedly higher q_e values were obtained with the lignins. The Redlich-Peterson isotherm was not fitted for WS lignin, but the adsorption was sufficiently well modeled by the

Langmuir isotherms with both materials. GreenValue lignin showed 1.5-fold higher maximum adsorption capacity (X_m=543 mg/g) than WS lignin (Table 3). This difference is probably the result of different physicochemical structures of the two lignins, stemming from the isolation conditions. In contrast to the lignins, both cellulose materials showed low X_m values for Azure B, suggesting that only moderate encapsulation or physisorption into/onto cellulose occurred (Table 3). The difference between the measured q_e and the values predicted by the Langmuir models for WS, SGB, and oat husk materials (Fig. 3) suggests that Azure B could have been removed from solution also by secondary phenomena such as encapsulation. The Langmuir isotherm is based on monolayer adsorption, and the q_e data of each of the lignocellulosic materials fitted well to the linearized equation ($0.92<R^2<0.99$) (Table 3). The amount of acidic hydroxyl groups in the plant materials was thus determined based on the maximum adsorption capacities predicted by the Langmuir equation.

3.5 Effect of extractives and particle size on adsorption

Adsorption of Azure B on WS and extractive-free wheat straw (WS-EF) was studied to find out the effect of extractives on adsorption. Both materials were used as adsorbents under identical conditions. Based on total lignin content of the adsorbent, equilibrium adsorption capacities of WS and WS-EF for Azure B were 99.6 mg g^{-1} ± 0.5 mg g^{-1} and 104.1 mg g^{-1} ± 0.2 mg g^{-1}, respectively. Hence, the effect of extractives was considered small. Differing particle size did not affect adsorption of Azure B on WS because at identical conditions WS milled to pass 1 mm and 0.2 mm sieves gave q_e values of 42.0 mg/g and 40.4 mg/g, respectively. The difference between the values was within the experimental error.

3.6 Amount of surface-accessible acidic hydroxyls and corresponding surface area of lignin in wheat straw, sugarcane bagasse, and oat husk materials

Surface chemistry of lignocellulose defines many of its properties in heterogeneous reaction systems, for instance in enzymatic hydrolysis (Kristensen et al., 2007). The amount of surface-accessible acidic hydroxyl groups in WS, SGB, and oat husk materials was determined using maximum equilibrium adsorption capacities (Table 3). Correction for binding of Azure B on non-lignin constituents was made based on the equilibrium adsorption capacity of Emcocel 50M, microcrystalline cellulose that did not contain lignin (Table 1). Values for the correction factor *b* were 0.94, 0.91, and 0.90 for WS, SGB, oat husk materials, and 0.997 and 0.999 for WS lignin and GreenValue lignin. Surface area of lignin (SA) was calculated from Eq. 6 and specific surface area of lignin (SSA) from Eq. 7. The results show that the amount of surface-accessible acidic hydroxyls (0.21 mmol/g) and SA (84 m^2/g) of WS were 1.7-fold higher than in SGB or oat husk materials (Table 4). Much higher amount of surface-accessible acidic hydroxyls was obtained with GreenValue lignin and WS lignin that showed 1.16 mmol/g and 1.77 mmol/g of acidic hydroxyls, respectively. The amount of total acidic hydroxyls shown by the ^{31}P NMR analysis of the fully soluble lignins (Table 2) suggests that 48% and 37% of these groups were quantified by the Azure B adsorption method in GreenValue lignin and WS lignin in solid state, reflecting the proportion of surface-accessible acidic hydroxyls. These proportions in the two lignins might be due to morphological differences as the WS lignin contained more ash, but less carbohydrate than GreenValue lignin (Table 1).

The amount of surface-accessible lignin on WS surfaces was estimated using data in Table 4. Comparison of SSA of wheat straw (354 m^2/g lignin) and SSA of WS lignin (555 m^2/g lignin) or GreenValue lignin (768 m^2/g lignin) suggested that 64% to 46% of acidic lignin was surface-accessible in straw. This comparison is based on the assumption that native lignin on WS had similar morphology and chemical structure as the isolated lignins. Therefore, both native and isolated lignins would contain equal proportions of surface-accessible acidic hydroxyls. In contrast, if assuming that WS contained only phenolic hydroxyls among the acidic OH groups, the ratio of the amount of surface-accessible acidic OH groups in WS (0.89 mmol/g lignin) to the amount of phenolic groups (^{31}P NMR) of WS lignin (2.76 mmol/g lignin) or GreenValue lignin (2.79 mmol/g lignin) would suggest that 32% of native straw lignin was surface-accessible. Although isolated lignin may not fully represent native wheat straw lignin, these estimations of the proportion of surface-oriented lignin in straw provide an important advancement to the earlier knowledge. Previously, the surface coverage of lignin has not been altogether measured, and even the total surface areas obtained by Brunauer–Emmett–Teller (BET) analysis of pretreated corn stover have been said to be 10 to 15 fold underestimated (Chundawat et al., 2011). In the current study, the estimated proportion of lignin accessible for Azure B in wheat straw suggests that lignin is largely located on the porous surfaces and in the conductive tissues such as the xylem where lignin has been mentioned to be unwettable (Laschimke, 1989). This is consistent with the fact that lignin is the principal component of water conducting tissues of vascular plants, and that isolated oat husk lignin increases water resistance of lignocellulosic fibers (Sipponen et al., 2010).

3.7 Remarks concerning analysis of oxidized samples

Effect of cellulose and hemicellulose together on adsorption of Azure B was studied using holocellulose that contained 8.7% of lignin (Table 1). It was observed that despite its low lignin content, the equilibrium adsorption capacity of holocellulose was slightly higher (43.0 mg/g) than obtained with wheat straw (40.4 mg/g) in similar conditions (100 mg adsorbent in 50 mL of 0.1 g L^{-1} Azure B at pH 7). The acidic sodium chlorite treatment used in preparation of holocellulose had thus produced a very large amount of anionic groups that were new adsorption sites for Azure B. In addition, as sodium chlorite can oxidize polysaccharides such as starch (Hebeish et al., 1992) and cellulose (Kumar et al., 2013), oxidation of hemicelluloses might have also occurred. In fact, the acid-chlorite treatment decreases the degree of polymerization of cellulose, although to lesser extent in the presence of lignin (Kumar et al., 2009; Hubbell and Ragauskas, 2010). In general, selectivity of delignification reactions on lignin is limited due to the associated polysaccharides (Gierer, 1986). Unless the selective oxidation of lignin can be confirmed, prudent interpretation of results from oxidized lignocellulosic materials is recommended.

3.8 Equations for single-point determination of surface-accessible acidic hydroxyls and corresponding surface area of lignin

In order to make the high-throughput analysis possible, the presented adsorption data was used for developing a single point procedure for determining the surface-accessible acidic hydroxyls in solid materials. The adsorption conditions were selected based on the adsorption isotherms of the plant materials (Fig. 3) so that sufficient decrease in absorbance

is obtained. The varying ratio of lignin to Azure B (x, on dry weight basis) had a linear effect on q_e of the materials, giving the following correction factor formulae:

Wheat straw (R^2=1.00): $F_{WS} = 1.5038 - 0.1261x$

Sugarcane bagasse (R^2=0.95): $F_{SGB} = 1.3841 - 0.0786x$

Oat husk (R^2=0.97): $F_{Oat\ husk} = 1.4599 - 0.0889x$

The ratio of X_m to q'_e (predetermined ratio of maximum equilibrium adsorption capacity to the equilibrium adsorption capacity at the conditions of the single-point procedure) was 1.64, 1.55, and 1.52 for WS, SGB, and oat husk materials. The following equations were obtained for calculating the amount of surface-accessible acidic hydroxyls based on the q_e measured by the single-point procedure:

Wheat straw: $\text{Surface-accessible acidic OH } \left(\frac{mmol}{g}\right) = 0.91F_{\text{WS}} \frac{1.64q_e}{305.83}$

Sugarcane bagasse: $\text{Surface-accessible acidic OH } \left(\frac{mmol}{g}\right) = 0.88F_{\text{SGB}} \frac{1.55q_e}{305.83}$

Oat husk: $\text{Surface-accessible acidic OH } \left(\frac{mmol}{g}\right) = 0.87F_{\text{Oat husks}} \frac{1.52q_e}{305.83}$

The respective surface area of lignin (SA) is obtained by multiplying the surface-accessible acidic hydroxyl content with molar surface area of Azure B (397 m^2/mmol).

4 Conclusions

Surface area of lignin was measured in wheat straw, sugarcane bagasse, oat husk, and two isolated lignin materials, based on the surface-accessible acidic hydroxyl groups, as determined by cationic dye adsorption. Direct information of lignin can be obtained in its native state using this approach. In summary, this method should turn out useful and complimentary to other characterization techniques of lignin and lignocellulose in solid state. Ongoing experiments focus on applying the method for characterizing solid lignocellulosic fractions from various pretreatments. The authors encourage other investigators to apply the method for characterizing native and pretreated herbaceous and wood materials.

Supplementary material description:

Figure S1 showing the proposed mechanism of Azure B binding to the acidic hydroxyl group of lignin. Figure S2 showing the effect of lignin to Azure B ratio on equilibrium adsorption capacity in the developed single-point method, and the corresponding correction factors for WS, SGB, and oat husk materials.

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Table 1. Composition of the lignocellulosic materials (g/100 g) as analyzed by acid hydrolysis.

Material	Glc[a]	Xyl[a]	Ara[a]	Gal[a]	Ac[b]	KL[c]	ASL[d]	Total lignin[e]	Ash
Wheat straw	39.0	23.7	1.6	0.9	1.9	21.8	1.8	23.7	4.2
Sugarcane bagasse	40.8	22.4	2.1	0.8	3.0	22.0	1.8	23.8	3.0
Oat husks	31.0	31.4	3.1	1.3	2.7	20.4	2.4	22.7	4.6
WS-EF	38.4	23.3	3.0	1.2	1.8	20.0	1.4	21.4	5.1
WS lignin	nd[f]	0.8	0.2	nd	nd	79.2	3.7	83.0	4.0
GreenValue lignin	0.2	2.7	0.9	0.1	na[g]	86.4	5.2	91.6	1.4
WS holocellulose	49.7	28.2	3.7	1.2	na	4.1	4.6	8.7	na
Whatman I	96.6	nd	nd	nd	na	0.8	0.3	1.1	na
Emcocel 50M	95.7	1.4	0.4	nd	na	nd	0.2	0.2	na

[a]:Anhydrous sugars: Glc, glucose; Xyl, xylose; Ara, arabinose; Gal, galactose. [b]:Ac, acetyls; [c]:KL, Klason lignin; [d]:ASL, acid-soluble lignin. [b]: Total lignin=KL+ASL. [f]:nd, not detected; [g]:na, not analyzed. Various data shown in the table are mean values of duplicate analyses having average deviations from the mean < 5%.

Table 2. Amount of aliphatic, phenolic (S and G condensed at 5-position, G, H), and carboxylic hydroxyls (mmol/g) in the two lignin materials before and after acetylation, as determined by ^{31}P NMR.

Material	Aliphatic	Phenolic			Carboxylic	Total	
		S + R-5-G[a]	G	H		Phenolic	Acidic
WS lignin	1.67	1.19	0.93	0.17	0.83	2.29	3.13
Ac-WS lignin[b]	0	0	0	0	0.51	0	0.51
GreenValue lignin	1.92	1.26	0.82	0.48	1.18	2.55	3.73
Ac-GreenValue lignin[b]	0	0	0	0	0.61	0	0.61

The average deviation from the mean value shown in various columns was < 5% on average. [a]: guaiacyl unit condensed at 5-position of the aromatic ring. [b]: Single determinations of acetylated lignins that were not completely soluble were carried out.

Table 3. Fitted parameters of Langmuir and Redlich-Peterson equations for Azure B adsorption on different materials.

Material	Isotherm	R^2	X_m [a]	X'_m	a_L	a_r	b_r	K_r
Wheat straw	Langmuir	0.991	68.9 ± 0.3	291	0.099	-	-	-
	Redlich-Peterson	0.998	-	-	-	0.292	0.858	9.72
Sugarcane bagasse	Langmuir	0.923	45.5 ± 0.6	191	0.051	-	-	-
	Redlich-Peterson	0.999	-	-	-	0.406	0.843	7.42
Oat husks	Langmuir	0.958	41.2 ± 1.0	181	0.079	-	-	-
	Redlich-Peterson	0.998	-	-	-	0.913	0.737	10.04
WS lignin	Langmuir	0.972	356 ± 2	429	0.313	-	-	-
GreenValue lignin	Langmuir	0.899	543 ± 2	593	0.306	-	-	-
	Redlich-Peterson	0.999	-	-	-	0.166	1.048	118.6
Whatman I	Langmuir	0.973	1.65 ± 0.28	-	0.049	-	-	-
Emcocel 50M	Langmuir	0.943	5.63 ± 0.25	-	0.008	-	-	-

[a]: The value after ± indicates standard deviation from the mean value. Units of the parameters: X_m (mg g^{-1}); X'_m (mg g^{-1} lignin); a_L (L mg^{-1}); a_r (L mg^{-1})^{b_r}; b_r (); K_r (L g^{-1}).

Table 4. Amount of surface accessible acidic hydroxyls and estimated surface area of lignin in WS, SGB, oat husk, and isolated lignin materials.

Material	Amount of surface-accessible acidic hydroxyls	Surface area of lignin	
	(mmol g^{-1} material)	SA^a (m^2 g^{-1} material)	SSA^b (m^2 g^{-1} lignin)
Wheat straw	0.21	84	354
Sugarcane bagasse	0.13	53	225
Oat husks	0.12	48	211
WS lignin	1.16	460	555
GreenValue lignin	1.77	703	768

[a]: SA, surface area of lignin was calculated from Eq. 6 [b]: *SSA*, specific surface area of lignin was calculated from Eq. 7.

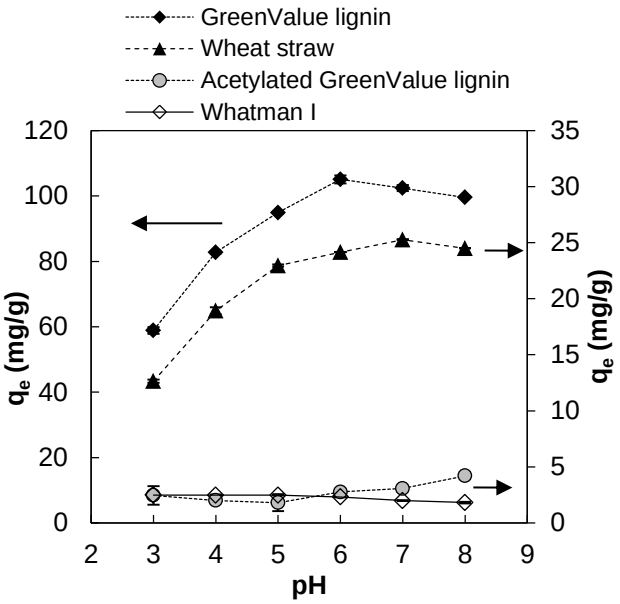

Figure 1. The effect of pH on equilibrium adsorption capacity of WS, GreenValue lignin, acetylated GreenValue lignin, and Whatman I filter paper. Experiments with 24 h contact time at 25 °C in each series were carried out using 0.1 g L^{-1} Azure B in pH buffer solution and constant amount of the adsorbent: lignin materials (50 mg lignin in 50 mL Azure B), WS and Whatman I (100 mg in 25 mL Azure B).

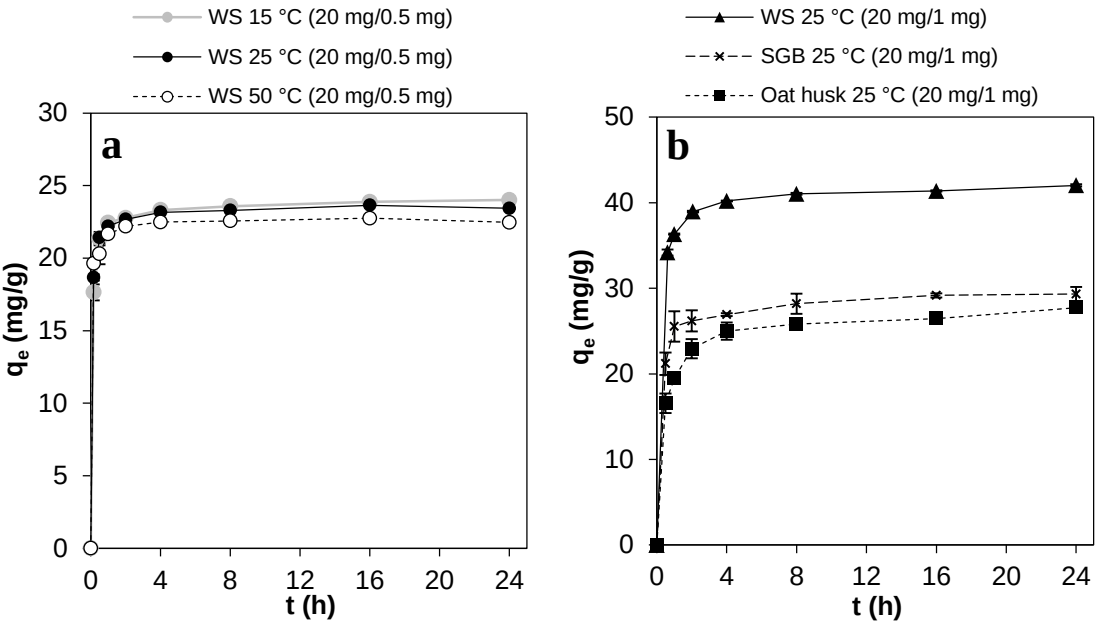

Figure 2. Time-course of adsorption of Azure B on lignocellulosic materials. (a) Adsorption on WS at 15 °C, 25 °C, and 50 °C. (b) Adsorption on wheat straw (WS), sugarcane bagasse (SGB), or oat husk materials at 25 °C. The values in brackets indicate the dry weight ratio of the adsorbent to Azure B from 0.1 g L^{-1} solution at pH 7.

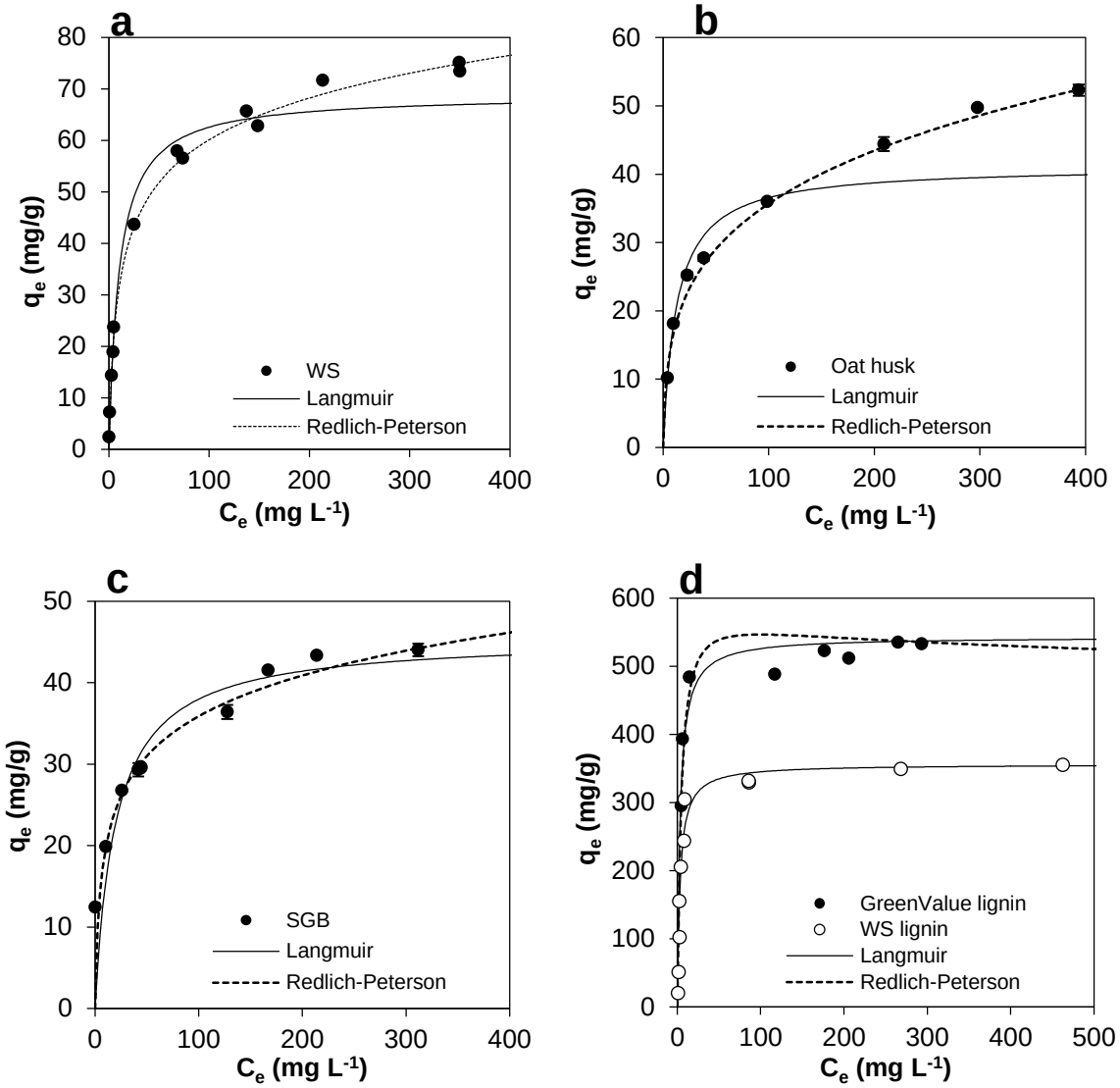

Figure 3. Equilibrium isotherms of Azure B adsorption on WS (a), oat husk (b), SGB (c), and WS lignin and GreenValue lignin (d) materials. Experimental data obtained from experiments with 24 h contact time at 25 °C is fitted to Langmuir (——) or Redlich-Peterson (----) equations.