Comparison of Three Fourier Transform Infrared Spectroscopy Sampling Techniques for Distinction between Lignocellulose Samples

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Lignocellulosic biomass is one of the most abundant raw materials available on earth, and the study of lignocellulose components is required for the production of second-generation biofuels. Fourier transform infrared spectroscopy (FTIR) has a demonstrated potential as a cost-effective and efficient method to distinguish between lignocellulose specimens. This study compared three FTIR modes—attenuated total reflectance (ATR), diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), and Transmission-FTIR—in their ability to distinguish samples of different lignocellulose species at varying grain sizes, as well as before and after enzyme treatment. The reproducibility among replicates and the separation between different sample groups was assessed using an adjusted "separation/scatter" metric calculated from the scores of principal component analysis (PCA). Attenuated total reflectance was most frequently the best method due to its least amount of variance among sample replicates. However, Transmission-FTIR was better than ATR for certain particle sizes or enzyme treatments. Diffuse reflectance infrared Fourier transform spectroscopy was repeatedly inferior to ATR and Transmission-FTIR, especially in terms of variability. This work provided insight into the best mode of FTIR for characterizing lignocellulose powders. Future work should test the robustness of these results with a wider range of wood species, particle sizes, enzymes concentrations, and reaction conditions.

Keywords: FTIR; ATR; DRIFTS; Transmission-FTIR; Lignocellulose; Spruce; Birch; Cellulase; Laccase

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INTRODUCTION

The analysis of lignocellulose constituents is a vital step in the industrial synthesis of second-generation biofuels (Hames *et al.* 2003). The importance of the present Fourier transform infrared (FTIR) study of lignocellulose lies within the step-wise production of biofuels; initial lignocellulosic constituents must be characterized to evaluate the potential biofuel yield, *e.g.*, by using regression analysis (Adapa *et al.* 2011). Additionally, the chemical alteration of lignocellulose after pre-treatment is important to understand. For example, lignocellulose exposure to acidic compounds and enzymes (Faix and Bottcher 1992; Stuart *et al.* 1995), and to pulverization and pyrolysis (Shen *et al.* 2010), have been studied by FTIR.

The use of FTIR for lignocellulosic analysis is extensive, as is well described in reviews elsewhere (Hames *et al.* 2003; Xu *et al.* 2013). Notably, the methods of rapid biomass analysis using FTIR and chemometrics are capable of performing complete compositional analysis, accounting for 97% to 103% of the sample mass (Hames *et al.*

2003). Efforts toward obtaining compositional information typically employ FTIR along with other analytical methods, such as wet chemical analysis (Hames *et al.* 2003; Adapa *et al.* 2011; Xu *et al.* 2013), high performance liquid chromatography (Liebmann *et al.* 2010), and Raman spectroscopy (Stewart *et al.* 1995). Additionally, thermogravimetric or pyrolysis steps are also sometimes paired with FTIR analysis of evolved decomposition products (Shen *et al.* 2010; Liu *et al.* 2011).

These reports typically only employ one or two types of FTIR analysis, *e.g.*, Transmission-FTIR (hereafter referred to simply as "Transmission"), diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), or attenuated total reflectance (ATR). When FTIR is the sole analytical technique in an article, the focus of the research tends to be the identification and comparison of peaks associated with the lignocellulosic components (Kubo and Kadla 2005; Sim *et al.* 2012). The selective use of multiple FTIR sampling techniques leads to the question of how researchers choose which mode of FTIR to use. In some cases, this may be based on the availability of ATR and DRIFTS *vs.* Transmission attachments for the FTIR instrument. However, is one mode the best for lignocellulose analysis?

To the authors' knowledge, no reported studies have systematically compared ATR, DRIFTS, and Transmission using mid-FTIR spectroscopy, evaluating their relative ability to consistently detect lignocellulose functional groups or to distinguish lignocellulose of different chemistries. This inquiry is especially important in unpredictable experimental conditions and heterogeneous mixtures. A study concerning FTIR modes could suggest a method of analysis that would best avoid spectral interferences caused by residuals from lignocellulose treatments, such as enzymes and organic mediators.

In the past, some modes of FTIR have been systematically compared in their ability to detect lignocellulose, examining spectral quality when experimental parameters were varied. Of particular relevance is a direct comparison of DRIFTS and Transmission for lignocellulose detection, by Faix and Böttcher (1992). They observed that varying lignocellulose particle sizes and KBr dilutions had a considerable impact on the quality of the spectra, such that smaller lignocellulose particle sizes and larger dilutions with KBr produced higher quality spectra, intensifying a distinct carbohydrate peak at 1100 cm^{-1} (Faix and Böttcher 1992). Following this observation, several particle sizes were also studied in this work. However, Faix and Böttcher (1992) used particle intervals all less than 100 μm, going down to 25 μm. The authors' interest was in particle sizes that would be more industrially relevant, requiring less mechanical threshing; therefore this study's particles ranged from 420 μm to 105 μm.

To the authors' knowledge, although binary combinations have been studied (*e.g.*, DRIFTS *vs.* Transmission) by Faix and Böttcher (1992), no report has systematically compared ATR, DRIFTS, and Transmission simultaneously for lignocellulose analysis. Thus, the goal of this work was to use the three modes on an identical sample set, with experimental questions related to the ability to contrast between samples from different wood species (spruce *vs.* birch) and between samples with and without enzyme digestion. Enzyme digestion is representative of a pre-treatment step to break down lignocellulose for biofuel production and may cause more subtle changes than the considerable chemical differences between wood species. Although this work was done only with mid-IR radiation (400 cm^{-1} to 4000 cm^{-1}), it could be expanded by others using the increasingly popular near-IR (Hames *et al.* 2003) spectral region.

The FTIR spectra in this work were analyzed with principal component analysis (PCA) for the unbiased description of the variance among the replicate samples. In the FTIR literature, partial least squares (PLS) analysis is frequently used to compare FTIR spectra and compositional information from wet chemical analysis (Hames *et al.* 2003; Xu *et al.* 2013). However, the goal of this present work was not to obtain chemical composition, but rather to assess the variability and contrast of the spectra obtained under different IR modes of interaction with the sample. Therefore, it was appropriate to use PCA as an unbiased method to describe the distinction between the sample groups and the spread of replicate scores. This spread of scores was quantified using a metric termed the "adjusted separation over scatter" (adj. S/S).

EXPERIMENTAL

Materials

Untreated lignocellulose sample preparation

Pre-milled samples of yellow birch (*Betula alleghaniensis* Britt.) and red spruce (*Picea rubens* Sarg.) heartwood were supplied by the University of Toronto, (Toronto ON, Canada). These were solvent extracted to remove small-molecule extractives, following ASTM standard D1105-96 (2013). Briefly, this consists of a Soxhlet extraction in a mixture of ethanol and toluene (1:0.427 v/v, respectively) for 4 h, followed by ethanol for 8 h, and water for another 4 h. The extraction was performed because extractives may inhibit lignocellulose-degrading enzymes and because structural similarity may introduce matrix effects or spectral overlap between the lignin and extractives. The extracted samples were air-dried.

The extracted birch and spruce samples were separately passed through sieves with mesh sizes of 40- (420 μm), 100- (150 μm), and 140- (105 μm) mesh. Fractions from each sieve were stored in glass containers for side-by-side comparisons of birch *vs.* spruce at each particle size. A portion of each wood also remained unsieved.

Enzymatic degradation of lignocellulose

Chemical purities and suppliers were as follows: 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS; ≥ 98% pure) and methanol (UHPLC plus grade) were supplied by Sigma Aldrich (St. Louis MO, USA); glacial acetic acid (99.9% pure) was supplied by J. T. Baker (Philipsburg NJ, USA); sodium hydroxide (48 wt.% to 52 wt.%) was supplied by Spectrum Chemical Mfg. Co. (Gardena, CA, USA); and magnesium nitrate (98% to 102% ACS grade) was supplied by Alfa Aesar (Ward Hill MA, USA).

The unsieved extracted red spruce was treated with 1:200 dilutions of either cellulase (Celluclast 1.5 L) or laccase (51003), which were both supplied by Novozymes (Bagsvaerd, Denmark). The ABTS was used as the mediator for laccase-facilitated lignin degradation, but was not used for cellulose degradation.

To provide ample material for analysis, 15.5 mL of solution was prepared with 20 mg/mL of wood loading. Aliquots of 310 mg of spruce were weighed into four 20-mL glass scintillation vials to contain: 1:200 cellulase in buffer; buffer control for cellulase; 1:200 laccase in ABTS and buffer; and ABTS/buffer control for laccase. Each vial received 11.63 mL (75% volume) of 0.1 M, 4.98 pH acetate buffer (with pH adjusted by sodium hydroxide). The laccase treatment and ABTS control received 1.55 mL of 100 mM ABTS, yielding a 10 mM final ABTS concentration. Treated 1:200 diluted samples received 77.5

μL of the corresponding laccase or cellulase protein. Distilled water was then added to each sample to reach the final target volume of 15.5 mL.

Samples were incubated in a shaker/incubator at 50 °C and 75 rpm for 35 h. The supernatants were filtered off and the solids were washed with three 20-mL portions of 1.0 M of acetic acid followed by one 20-mL distilled water rinse to remove buffer salts (Braham and Goacher 2015). Washed samples were stored in an approximately 54% relative humidity chamber prepared from a saturated magnesium nitrate solution.

Methods

FTIR analysis of lignocellulose

Infrared spectra for all samples were collected using a mid-range FTIR spectrometer (Spectrum 2; Perkin Elmer, Waltham, MA) using DRIFTS, ATR, and Transmission attachments. Spectra were obtained from 400 cm^{-1} to 4000 cm^{-1}. A scan resolution of 0.5 cm^{-1} was employed despite lower resolutions of 4 cm^{-1} being more common in literature for high-throughput analysis (Faix and Böttcher 1992). The higher resolution was chosen to more fully evaluate the abilities of the modes. Spectral contributions of atmospheric carbon dioxide and water were automatically subtracted from all collected spectra using the CO_2/H_2O correction (Spectrum, Perkin Elmer, version 10.4.3.339, Waltham, MA, USA). In all cases, equipment that contacted wood powder was cleaned between wood types using methanol. For all modes, ten scans were averaged per spectral replicate.

Attenuated total reflectance

The spectra obtained by ATR were collected using a single reflection diamond accessory (L1600107; Perkin Elmer, Waltham, MA, USA). The ATR crystal was cleaned with methanol, air-dried, and the background was collected with the crystal exposed to the air. Lignocellulose samples were scooped onto the crystal surface until complete coverage was achieved (*i.e.,* approximately 3 mg sample/replicate) and pressed onto the surface using a mechanical anvil.

Diffuse reflectance infrared Fourier transform spectroscopy

Spectra obtained by DRIFTS were collected using a diffuse reflectance accessory (L1600111; Perkin Elmer, Waltham, MA, USA). The instrumentation was blanked with metal-coated abrasive tabs (L1275106; Perkin Elmer, Waltham, MA, USA). Samples were placed into a macro cup (L1201654; Perkin Elmer, Waltham, MA, USA) using forceps, and the excess sample was removed while the remaining sample surface was smoothed using the edge of the forceps. In addition to the ten scans, a study was conducted averaging 200 scans to increase the signal-to-noise (S/N) ratio of the raw spectra, investigating whether increased scans would provide less DRIFTS spectral variance between samples, on par with ATR and Transmission.

Transmission spectroscopy

Transmission spectra were collected using a transmittance accessory (L1272269; Perkin Elmer, Waltham, MA, USA). Lignocellulose samples were diluted in a 1:100 mixture of sample to dry spectral-grade KBr. Pellets were made from this mixture by placing 200-mg aliquots between 13-mm tungsten carbide anvils in a 13-mm stainless steel die (International Crystal Laboratories, Garfield NJ, USA). The die was attached to a vacuum line for 1.5 min, then detached and placed in a pellet press (Model 4350 Bench

Top Laboratory Pellet Press, Carver Inc., Wabash IN, USA). The die was pressed to a pressure of $1.4x10^7$ Pa followed by an immediate release, then $3.2x10^7$ Pa was held for 0.5 min. The die was removed from the pellet press and evacuated for 1.0 min, then placed back in the press at $4.1x10^7$ Pa, held for 2.0 min. The resulting pellets were removed and immediately analyzed. The background was obtained from a pure KBr pellet.

PCA- Facilitated spectral processing

The FTIR spectra were analyzed over the full 400 cm^{-1} to 4000 cm^{-1} range (data not shown) and in the fingerprint region from 800 cm^{-1} to 1800 cm^{-1}. The results for the full range were consistent with those from the fingerprint region.

Principal component analysis was completed by the MATLAB R2015b (The MathWorks, Inc., Natick, MA, USA) using the PLS Toolbox v.8.0.2 (Eigenvector Research, Inc., Manson, WA, USA). The pre-processing steps were normalization and mean-centering. The scores and loadings plots completed by the MATLAB were exported to comma-separated values files and plotted in overlay using Origin b9.2.272 (OriginLab Corporation, Northampton, MA, USA) to aid in systematic comparison.

The PCA scores values for the principal component (PC) that best separated the sample groups were quantitatively analyzed in Microsoft Excel *via* the adjusted separation/scatter (adj. S/S) values. This process is illustrated in Fig. 1. The adj. S/S values were calculated by averaging the replicate scores values of both sample groups (*e.g.*, of birch and spruce), calculating the standard deviation of the replicate scores values within each sample group, adding the absolute values of the average scores values ("separation"), propagating the error of the addition using standard deviation values ("scatter"), dividing the separation value by the scatter value, and finally adjusting the S/S value by multiplying it by the total percent variance that was described by the PC. This final adjustment step is necessary because the variance described by the PC that shows chemical contrast will be lower if there is more experimental noise described by other PCs.

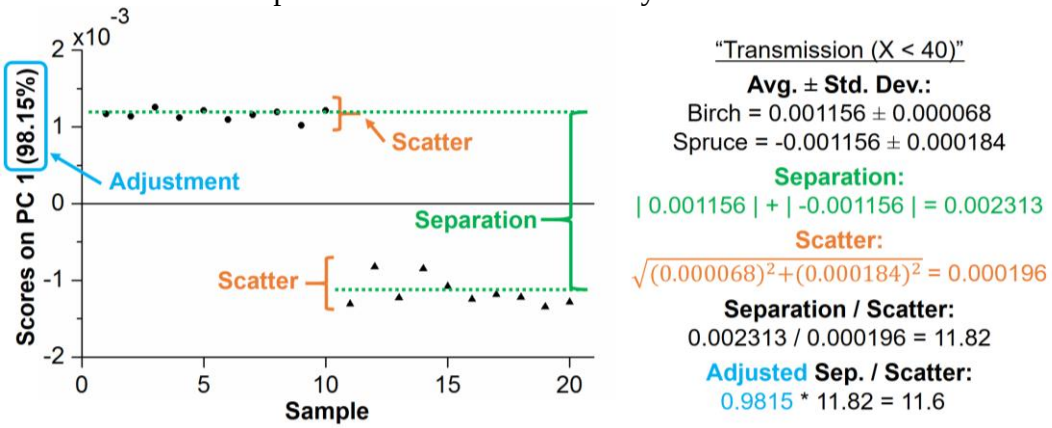

Fig. 1. Concept and example calculations for adjusted separation/scatter

RESULTS AND DISCUSSION

Ranking FTIR Modes: Example of Birch *vs.* Spruce

The PCA scores and loadings plots for the comparison of birch *vs.* spruce (Fig. 2) were organized by the FTIR mode, *e.g.*, with all four particle size ranges overlaid for ATR (Figs. 2a through 2b), for DRIFTS (Figs. 2c through 2d), and for Transmission (Figs. 2e

through 2f). The PCA data may be assessed qualitatively by a visual inspection of Fig. 2, or quantitatively using the adj. S/S metric (summarized in Table 1 for all of the data).

The difference between wood species is expected to cause clear spectral differences between the replicates for birch (shown by the circles on scores plots in Fig. 2) and those for spruce (shown by the triangles), based on differences in their total polysaccharides *vs.* lignin content, and in their lignin types. For example, spruce, as a softwood, is expected to contain predominantly G-lignin, while hardwood birch is expected to contain both G-lignin and S-lignin (Campbell and Sederoff 1996). Indeed, these samples were separated by the first principal component (PC 1) for all sieve sizes and all three FTIR modes (Fig. 2, Table 1).

Qualitatively, figures such as Fig. 2 were assessed by a visual separation between the scores between the two sample groups and the degree of deviation a single sample group exhibits; these were represented quantitatively as separation and scatter values, respectively. Separation is observed visually by the vertical position (score) of the points on the scores plots, in which each point represents a full spectrum. Points furthest from the origin correlate to a higher degree of separation, and indeed the distance on the scores plots between the average birch samples and the average spruce samples were quantified as the "separation" in the adj. S/S metric.

The most desirable result is to have a high degree of separation between the birch and spruce spectra, and to have a low variability within the replicates of each type. Low variability means that replicates within a sample group (*e.g.*, replicates of birch) have the same score, and therefore they appear as a line from left to right on the scores plot, in which the x-axis is an arbitrary sample index. Therefore, qualitatively, some of the best results appeared for ATR at particle sizes greater than 140-mesh (Fig. 2a) in which the replicates were in a tight line with little variation in the scores, and in which there was a large relative separation between birch and spruce. In contrast, DRIFTS X < 40 suffers from poor separation (Fig. 2c), and DRIFTS overall had more scatter among replicates than ATR.

These visual observations were supported by the adj. S/S values in Table 1, in which the best mode has the highest adj. S/S value. From Table 1, it was apparent that the greatest overall separation and reproducibility of scores (high adj. S/S values) were presented almost exclusively in the ATR data, with the "X < 140" samples being an exception. For each particle size interval above 140-mesh, the adj. S/S ranked ATR as best, then Transmission, then DRIFTS. For the "X < 140" sample, an inspection of Fig. 2a showed that the ATR adj. S/S dropped considerably due to higher scatter, despite similar average scores (separation). This result indicated that Transmission was the best at this smallest particle size. The greatest differentiation of all particles sizes and FTIR modes was for ATR at the "140 < X < 100" particle size, providing good separation and the least amount of deviation (adj. S/S 62.0). Particle size dependence within each FTIR mode will be discussed further below.

The inspection of scores plots alone does not provide enough information about the specifics of sample differentiation. Loadings plots (Figs. 2b, 2d, and 2f) detail what exact functional groups are responsible for the separation witnessed in the scores plots. The peaks responsible for the separation of the samples (the loadings) should be inspected for the chemistry responsible and for the S/N level of the loadings.

Table 1. Summary of Quantitative Variance Analysis

Comparison	FTIR Mode	Adj. S/S	PC #	Percent Variance
Birch *vs.* Spruce: unsieved, X < 40 (Fig. 2)	ATR *	28.4	PC 1	89.29%
	DRIFTS	3.5	PC 1	91.25%
	Transmission	11.6	PC 1	98.15%
Birch *vs.* Spruce: 100 < X < 40 (Fig. 2)	ATR *	18.3	PC 1	88.00%
	DRIFTS	4.2	PC 1	98.22%
	Transmission	6.0	PC 1	88.80%
Birch *vs.* Spruce: 140 < X < 100 (Fig. 2)	ATR *	62.0	PC 1	94.92%
	DRIFTS	5.3	PC 1	99.26%
	Transmission	7.6	PC 1	98.04%
Birch *vs.* Spruce: X < 140 (Fig. 2)	ATR	4.1	PC 1	87.35%
	DRIFTS	3.5	PC 1	97.87%
	Transmission *	8.6	PC 1	94.13%
Buffer Control *vs.* ABTS Control (Fig. 3) (Note: A lower S/S was desired here)	ATR *	0.98	PC 1	56.48%
	DRIFTS	2.2	PC 1	97.42%
	Transmission	3.1	PC 1	91.73%
Buffer Control *vs.* 1:200 Cellulase Spruce (Fig. 4)	ATR *	2.5	PC 1	85.36%
	DRIFTS	0.40	PC 2	26.83%
	Transmission	0.64	PC 1	73.33%
ABTS Control *vs.* 1:200 Laccase Spruce (Fig. 5)	ATR	0.27	PC 3	15.72%
	DRIFTS	0.39	PC 2	7.68%
	Transmission *	1.3	PC 1	81.14%

Note: Adjusted separation/scatter ratios (adj. S/S) are presented with the corresponding PC number and the percent variance described by that PC; the best mode for a given comparison is marked with an asterisk*

For example, within Fig. 2, the separation of birch and spruce was due to a variety of peaks representative of cellulose (C), hemicellulose (HC), and lignin (L). This was expected due to many differences in the hemicellulose and lignin composition between wood species (Campbell and Sederoff 1996; Poletto *et al.* 2012). However, the known higher amount of G-lignin (GL) in spruce is most evident in the ATR loadings (Fig. 2b).

Loadings should also have smooth and distinct peaks, with minimal noise. This was the case for ATR (Fig. 2b) and Transmission (Fig. 2f), but not for DRIFTS (Fig. 2d). The S/N of exemplary raw FTIR spectra for the 40-mesh spruce were calculated as approximately 1700 for ATR, approximately 78 for DRIFTS, and approximately 350 for Transmission. Therefore, a supplementary study was performed, in which the DRIFTS spectra were collected for 200 scans instead of ten scans (data not shown). The additional DRIFTS scans resulted in raw spectral S/N of approximately 300, nearly the level of Transmission data, and the loadings also appeared smoother. However, the separation between birch and spruce was poorer with 200 scans, appearing on PC 2 of the PCA models, and resulting in adj. S/S values approximately an order of magnitude lower than they had been with just ten scans. The authors hypothesize that the greatly increased analysis time for 200 scans may have caused instrumental drift to be a greater factor.

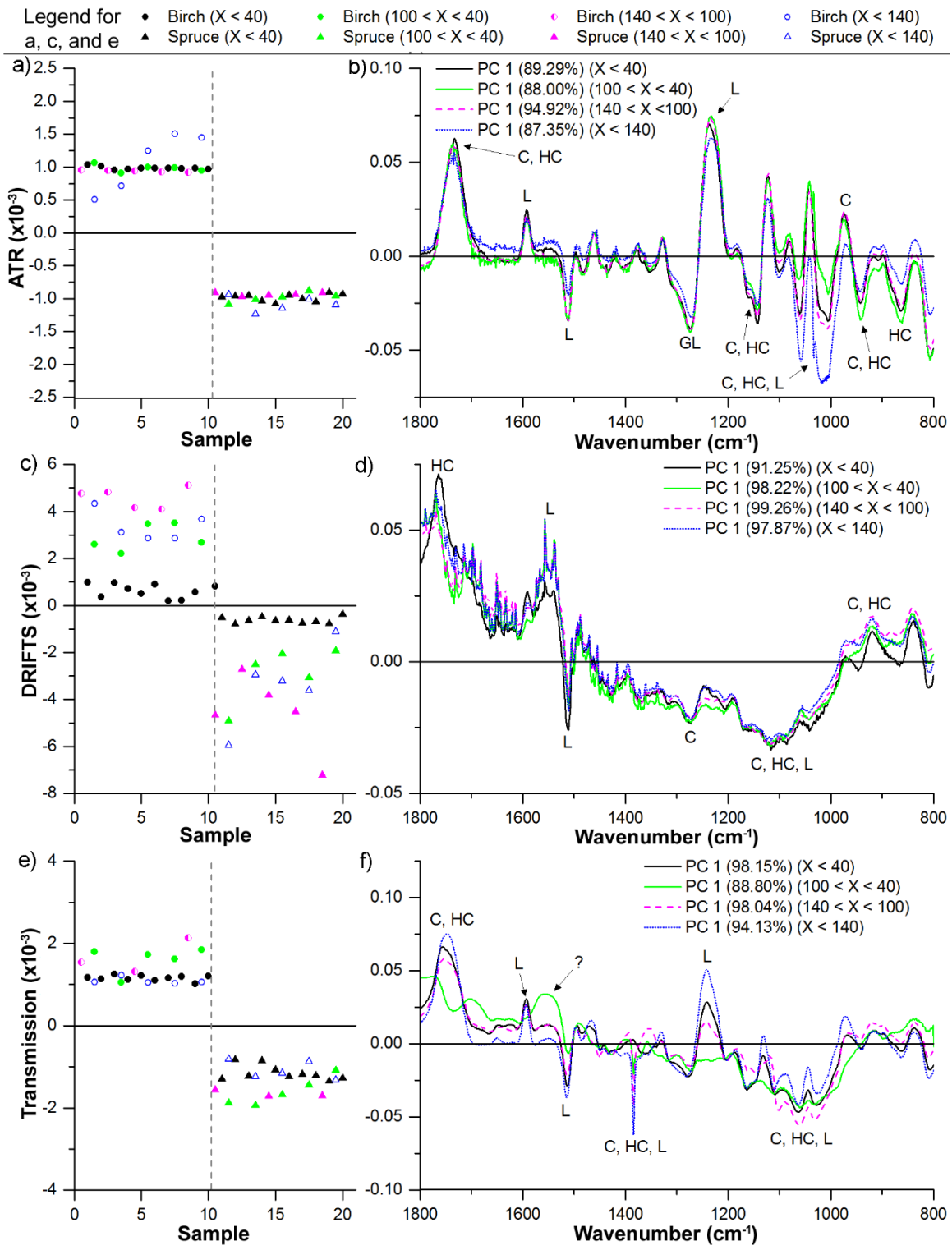

Fig. 2. Plots overlaying the scores (a, c, e) and loadings (b, d, f) for the PCA comparisons between spruce and birch of varying sieve sizes using FTIR modes of ATR (a, b), DRIFTS (c, d), and Transmission (e, f); loadings are annotated with peaks for cellulose (C), hemicellulose (HC), and lignin (L), including G-lignin (GL) (Schwanninger *et al.* 2004; Kubo and Kadla 2005; Sills and Gossett 2012); dashed grey lines between sample groups on scores are lines to guide the eyes.

Therefore, for the separation of birch and spruce, DRIFTS never produced the best adj. S/S, although it was noted that the absolute separation for DRIFTS (Fig. 2c) was greater than that for ATR and Transmission. The largest factor in the adj. S/S was the variability among replicates, which is why ATR was the best mode except for particles under 140-mesh, for which ATR had more variability and Transmission became optimal. The evaluation of the FTIR modes for certain conditions was explored further in the extended discussion of particle size below, and then in the discussion of enzyme control comparisons, cellulase treatment, and laccase treatment.

Birch *vs.* Spruce Particle Size

The birch and spruce were analyzed as a mixture of sizes (all particles "X" < 40) and also as three separate size intervals, "100 < X < 40", "140 < X < 100", and "X < 140" (Fig. 2). Faix and Böttcher (1992) reported better results for DRIFTS and Transmission at smaller particle sizes but did not study the dependence of ATR results on particle size.

Within ATR analyses, decreasing the particle size positively affected the reproducibility of ATR data, until the aforementioned 140-mesh drop in adj. S/S. In this study, the best ATR results were for the particles between 100-mesh and 140-mesh. It was not clear why a drop in adj. S/S was observed below 140-mesh. The sampling depth should be similar because the particles in ATR are pressed against the crystal, with the evanescent wave penetrating the surface. Decreasing the particle size would in theory increase sampling statistics, as more particles can be analyzed on the crystal area, alluding to a continued hypothetical improvement with smaller particles, which was not observed.

Decreasing the particle sizes did not have much of an effect on the ATR loadings plots (Fig. 2b) other than an increased carbohydrate peak intensity, perhaps due to more exposed inner cell walls from the milling and grinding of the sample. It is important to again note that ATR was the only mode to prominently distinguish the guaiacyl-lignin peak at approximately 1270 cm^{-1} (Sills and Gossett 2012), a peak necessary for a confident differentiation between hardwoods and softwoods.

Within the DRIFTS results, birch and spruce were well separated on average, but with a large amount of variability between replicates, especially between sieved spruce replicates (Fig. 2c). Additionally, as mentioned above, the DRIFTS loadings plots had high noise, and the ability to distinguish specific functional groups was greatly diminished (Fig. 2d). Nonspecific carbohydrate peaks were present in the DRIFTS loadings, with the most prominent peak being a general lignin peak at approximately 1500 cm^{-1}. Although both birch and spruce were sieved to the same sizes, the spruce particles had a larger aspect ratio, with the short dimension fitting through the sieve mesh and the longer dimension possibly contributing to greater variability.

The variation of adj. S/S for DRIFTS (Table 1) was not as large between particle sizes as it was for the other modes - it was consistently low (3.5 to 5.3). As with ATR, the greatest DRIFTS adj. S/S value was seen in the "140 < X < 100" particle sizes. The literature observation (Faix and Böttcher 1992) that smaller particle sizes increase the spectral resolution (and as a result, separation quality) using DRIFTS was not completely supported by this data, because the "X < 140" had a poorer adj. S/S. Because the IR radiation scatters through and off particles in DRIFTS, it is intuitive that the smaller particles would allow for greater homogeneity between sample portions and less variability. However, for these samples, the smallest material had more variation. Therefore, for both ATR and DRIFTS, the highest adj. S/S values were present in the "140 < X < 100" samples. This result suggests that a particle size interval of 140 < X < 100 is

possibly the most beneficial to reflectance spectral quality. Alternatively, it is possible that this aberration in the trend for the lowest sample size is more of a characteristic of the samples used than of the IR techniques, and the effect of size deserves further study.

Within the Transmission scores (Fig. 2e), the quality of the separation between birch and spruce increased with decreasing particle size, all the way to the lowest particle size studied, "X < 140", in which Transmission gave better results than ATR or DRIFTS. The better results for smaller particles with Transmission support Faix and Böttcher's (1992) earlier report. The smaller particles likely allow for a more homogenous wood distribution within the KBr pellets. However, the adj. S/S for Transmission "X < 140" was 8.6, which was less than that for Transmission for unsieved wood "X < 40" at 11.6, and remains much less than that for ATR at any particle size above 140-mesh (18.3 to 62.0).

The quality of the Transmission loadings (shown in Fig. 2f) generally improved with a decrease in particle size, with sharper and smoother peaks at lower particle sizes. However, like DRIFTS, a weakness in the Transmission loadings was the inability to detect the G-lignin peak. However, only Transmission FTIR distinguished the samples *via* a sharp carbohydrate peak at approximately 1370 cm^{-1} (Sills and Gossett 2012). In Fig. 2f, the "100 < X < 40" Transmission samples had unusual loadings, which may have been due to an inadvertent contamination, but most functional groups agreed with the other particle sizes.

Enzyme Treatments

The expectations with the enzyme treatments were that there would be no differences between the buffer control and the ABTS control (Fig. 3), that there would be a considerable loss of polysaccharide functional groups due to the cellulase treatment *vs.* the buffer control (Fig. 4), and that there would be considerable loss of lignin functional groups for the laccase treatment *vs.* the ABTS control (Fig. 5). For each figure, the ATR, DRIFTS, and Transmission PCA results were overlaid for comparison of the three modes within that scenario. With the enzyme treatments, it was postulated that because enzymes first act on the surface of particles, the differing information depths might affect the results, going from the more surface-sensitive ATR through DRIFTS, to the bulk Transmission.

Although no difference was anticipated for the comparison between the buffer control (used for the cellulase study) and the ABTS control (used for the laccase study), all three FTIR modes did distinguish these samples on PC 1 (Fig. 3a). The scatter among replicates was large, therefore the adj. S/S values were lower than for the comparison of birch and spruce (Table 1). The largest adj. S/S was observed with Transmission (3.1), then DRIFTS (2.2), and finally ATR had the lowest adj. S/S (0.98), due in part to the lower percent of variance described on PC 1 for ATR (56% *vs.* > 90%). Because a difference between these samples was not expected, ATR was designated as the best mode as it demonstrated the least difference. However, the possibility that unexpected chemistry has occurred due to the ABTS cannot be eliminated. The loadings (Fig. 3b) were noisy for DRIFTS, and it was difficult to assign functional groups to the broad features. The transmission loadings were more distinct than the ATR loadings. In ATR and Transmission, the ABTS control had more signal for the approximately 1650 cm^{-1} lignin peak assigned to the C=O stretch (Kubo and Kadla 2005). This functional group is not present in ABTS but the wavenumber may overlap with the conjugated C=C stretch (Liu *et al.* 2015; Silverstein *et al.* 2015), which may indicate that the ABTS concentration of 10 mM had been enough to leave residual ABTS (an aromatic molecule) on the sample after washing. Alternatively, ABTS is a slightly acidic molecule and therefore could have

degraded some polysaccharides (Kang *et al.* 2012), leaving more lignin in the residual solid.

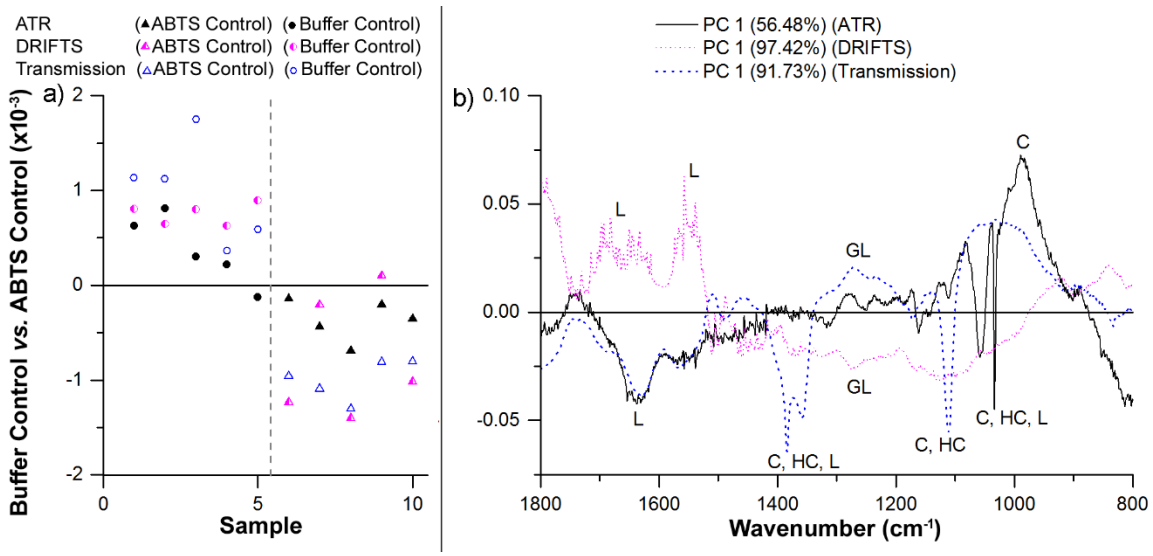

Fig. 3. Overlay of scores (a) and loadings (b) for the PCA comparisons between two controls: spruce in acetate buffer (cellulase control) and spruce in ABTS (laccase control) using three FTIR modes (ATR, DRIFTS, and Transmission); no difference was expected between these control sample groups. Loadings annotations are as described in Fig. 2.

The detection of cellulase activity (Fig. 4a) was remarkably poor with DRIFTS, with the difference between the cellulase-treated sample and buffer control present on PC 2 with 27% variance described by this PC (adj. S/S 0.4, Table 1). Transmission and ATR detected the difference on PC 1, but the Transmission data had high scatter and an adj. S/S of 0.6. The best detection of the cellulase activity was for ATR, with an adj. S/S of 2.5. Note that this was a lower adj. S/S than was observed for the more obvious contrast between the birch and spruce species, and that the scores in Fig. 4a for ATR still illustrated considerable scatter. However, of the three FTIR modes, ATR was best able to detect the action of cellulase, possibly due to the surface specificity of ATR. Furthermore, the loadings for ATR (Fig. 4b) clearly indicated the expected result that the solid residue after cellulase treatment was enriched in lignin, including G-lignin.

For the laccase treatment (Fig. 5), the greatest adj. S/S value (Table 1) was obtained with Transmission (1.26), then DRIFTS (0.39) and ATR (0.27). This separation occurred on PC 1 with an 81% variance described for Transmission, leading to its higher adj. S/S. Meanwhile, ATR did not detect the difference until PC 3. However, the laccase treatment was expected to produce a residue enriched in polysaccharides and depleted in lignin (*i.e.*, the ABTS control should appear to have more lignin). Inspection of the loadings (Fig. 5b) did not show a clear change in lignin for Transmission. The DRIFTS and ATR loadings indicated that the ABTS control was richer in hemicellulose (HC) at approximately 1750 cm^{-1} (free ester) and that the laccase treatment was enriched in the lignin-related peaks at approximately 1550 cm^{-1} (aromatic ring vibration, C-O stretch) and 1675 cm^{-1} (unconjugated C=O stretch). This enrichment of lignin for laccase-treatment was opposite of the expectation, but may be explained by the non-specific nature of these functional groups. The ATR loadings alone indicated that the ABTS control had more signal for the specific G-lignin peak around 1250 cm^{-1}, meaning that G-lignin was lost due to the laccase

treatment. Therefore, it is difficult to assign a "best" method for the laccase treatment. Based on adj. S/S alone, it was Transmission, but based on the spectral loadings, ATR was most informative.

The exploration of the appropriate particle sizes for DRIFTS should continue, as this work did not use the smaller particle sizes reported by Faix and Böttcher (1992). Furthermore, Faix and Böttcher diluted their wood particles with KBr for DRIFTS, which was not done in this work, and could be explored further. Additionally, further studies with varied substrates such as dilute acid or dilute alkali pre-treated lignocellulose would be worthwhile.

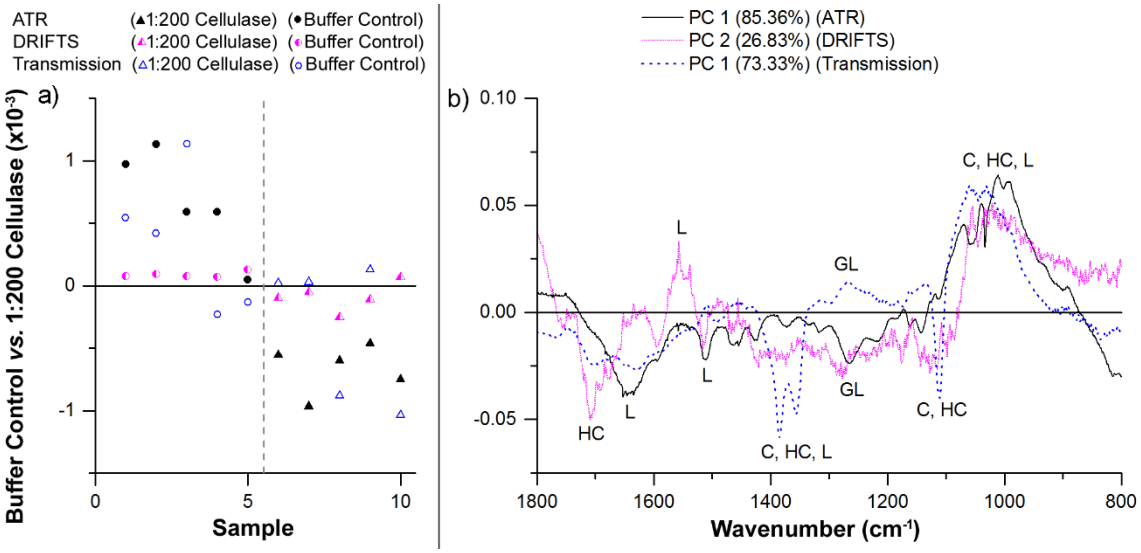

Fig. 4. Overlay of scores (a) and loadings (b) for the PCA comparisons between spruce in acetate buffer (control) and spruce treated with 1:200 cellulase using three FTIR modes (ATR, DRIFTS, and Transmission); loadings annotations are as described in Fig. 2

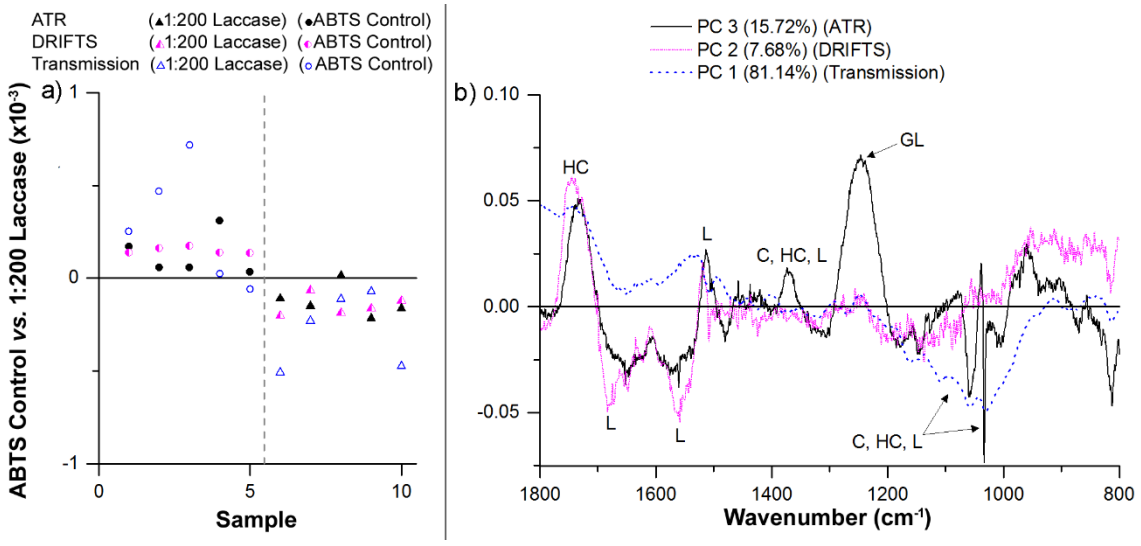

Fig. 5. Overlay of scores (a) and loadings (b) for the PCA comparisons between spruce in ABTS (control), and spruce treated with 1:200 laccase using three FTIR modes (ATR, DRIFTS, and Transmission); loadings annotations are as described in Fig. 2

CONCLUSIONS

1. Under most circumstances, attenuated total reflection (ATR) was the best mode for Fourier transform infrared (FTIR) analysis of lignocellulose powders. In five out of seven comparisons, ATR gave the best adjusted separation over scatter (adj. S/S), and ATR was the most likely method to distinguish samples based on specific G-lignin peaks, with smooth, sharp loadings. Furthermore, ATR was considerably easier to perform than either diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) or Transmission modes of FTIR, as pressing an ATR replicate against the crystal took little time.
2. Transmission was best under the other two of seven circumstances (birch *vs.* spruce, X < 140, and the laccase study). The transmission loadings were good but were less easily interpreted than the ATR loadings, due to fewer specific peaks. The occasional gains for Transmission may not be worth the extra time required to grind the powders and prepare the pellets for Transmission. The increased handling of the Transmission samples also increases the risk for contamination.
3. DRIFTS was not an ideal FTIR mode for the powdered lignocellulose in this study. The variability was high and the spectral quality was poor, often leading to uninterpretable loadings. The DRIFTS had among the highest magnitude average scores for the birch *vs.* spruce separation, showing some promise for the method. However, this high average separation was ruined by the high variability.

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