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Cellulose I crystallinity determination using FT–Raman spectroscopy: univariate and multivariate methods

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Abstract Two new methods based on FT–Raman spectroscopy, one simple, based on band intensity ratio, and the other using a partial least squares (PLS) regression model, are proposed to determine cellulose I crystallinity. In the simple method, crystallinity in cellulose I samples was determined based on univariate regression that was first developed using the Raman band intensity ratio of the 380 and 1,096 cm^{-1} bands. For calibration purposes, 80.5% crystalline and 120-min milled (0% crystalline) Whatman CC31 and six cellulose mixtures produced with crystallinities in the range 10.9–64% were used. When intensity ratios were plotted against crystallinities of the calibration set samples, the plot showed a linear correlation (coefficient of determination $R^2 = 0.992$). Average standard error calculated from replicate Raman acquisitions indicated that the cellulose Raman crystallinity model was reliable. Crystallinities of the cellulose mixtures samples were also calculated from X-ray diffractograms using the amorphous contribution subtraction (Segal) method and it was found that the Raman model was better. Additionally, using both Raman and X-ray techniques, sample crystallinities were determined from partially crystalline cellulose

samples that were generated by grinding Whatman CC31 in a vibratory mill. The two techniques showed significant differences. In the second approach, successful Raman PLS regression models for crystallinity, covering the 0–80.5% range, were generated from the ten calibration set Raman spectra. Both univariate-Raman and WAXS determined crystallinities were used as references. The calibration models had strong relationships between determined and predicted crystallinity values ($R^2 = 0.998$ and 0.984, for univariate-Raman and WAXS referenced models, respectively). Compared to WAXS, univariate-Raman referenced model was found to be better (root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP) values of 6.1 and 7.9% vs. 1.8 and 3.3%, respectively). It was concluded that either of the two Raman methods could be used for cellulose I crystallinity determination in cellulose samples.

Keywords Cellulose · Crystallinity · Raman spectroscopy · FT–Raman · Univariate · Multivariate · PLS · X-ray

Introduction

Traditionally, cellulose has been regarded as a semicrystalline material (composed of crystalline and amorphous phases). However, NMR evidence obtained in 1990s suggested that another phase, the

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paracrystalline phase, existed as well (Larsson et al. 1997). Nevertheless, in the present investigation, considering that no separately identifiable features of the paracrystalline phase have been detected in either Raman or X-ray technique, cellulose is considered as semicrystalline.

Cellulose crystallinity is defined as the mass fraction of crystalline domains in cellulose materials. This mass fraction can vary significantly in diverse materials. Crystallinity has an important effect on the physical, mechanical, and chemical properties of cellulose. For example, with increasing crystallinity, tensile strength, dimensional stability, and density increase, while properties such as chemical reactivity and swelling decrease. Some of the frequently used techniques for estimating cellulose crystallinity are wide-angle X-ray scattering (WAXS) (Segal et al. 1959; Jayme and Knolle 1964; Leppänen et al. 2009), solid state ^{13}C cross-polarization/magic-angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy (Horii et al. 1987; Newman and Hemmingson 1990; Newman 1999), and Fourier transform–infrared (FT-IR) spectroscopy (Richter et al. 1991; Hulleman et al. 1994). Although WAXS is the most extensively used technique, the X-ray method involves isolation of amorphous background from the diffraction pattern, which in the case of cellulose crystallites is not always easy for reasons that have to do with cellulose crystallites being small and, at lower crystallinities, the diffraction pattern being poorly defined (weak broad features). Texture of cellulose materials causes overestimation of the contribution from crystalline fractions. Moreover, in samples where hemicellulose and pectins are present, their contributions cause extra scattering and lead to an inaccurate measurement of the cellulose crystallinity (Andersson et al. 2003). In NMR analysis, the signals assigned to C-4 in the cellulose spectrum are used to calculate the crystallinity. However, if in addition to cellulose the sample includes hemicellulose and lignin (e.g., in wood) the ^{13}C solid state NMR spectra contain overlapping peaks, and in such cases the crystallinity calculation is based upon first separating the total NMR signal into two components, one belonging to cellulose and the other to the other non-cellulose contributors (Newman and Hemmingson 1990; Newman 1999). However, it was found that the accuracy of the NMR method depends not only upon how well the spectra can be separated

into the contributions of cellulose and non-cellulose components but also upon the lateral dimensions of the crystallites (Newman 1999). Additionally, NMR crystallinity determination involves use of curve-fitting methods that have shortcomings (Maddams 1980; Meier 2005). The deconvolution limitations are due to the fact that there are several possibilities to separate the overlapping spectral bands. In FT-IR, crystallinity calculation is also not reliable, because it is based on baseline correction and spectral deconvolution. There are additional problems as well—the quality of spectra depends on refractive index and particle size of the sample. Moreover, in diffuse reflectance FT-IR spectroscopy (DRIFT), specular reflectance causes changes in band shapes and their relative intensities (Faix and Böttcher, 1992).

Raman spectroscopy has proven to be a useful technique in the cellulose and lignocellulose field with numerous applications (Atalla and Isogai 1998; Agarwal 2008). It has become an important analytical technique for nondestructive, qualitative, and quantitative analysis of cellulose-containing materials. In particular, the FT-Raman technique has an added advantage due to its ability to successfully analyze materials that are fluorescent in conventional Raman. Moreover, in the macro mode of sampling with 1,064-nm excitation, any anisotropy associated with cellulose fiber materials is minimized because the scattering arises from the bulk of the sample and is not limited to the surface.

Crystallinity measurements using Raman on semicrystalline polymers including cellulose (Stuart 1995; Paradkar et al. 2003; Schenzel et al. 2005) have been performed. The principle behind Raman methodology is the same as in FT-IR and NMR and has to do with using the spectral features whose intensity, bandwidth, and/or position are affected by crystallinity. To develop FT-Raman-based cellulose I crystallinity quantitation method, Schenzel et al. (2005) used the weak bands at 1,462 and 1,481 cm^{-1} (CH_2 bending modes) in conjunction with spectral deconvolution. However, considering that the intensities of the selected bands are quite low and that the process of deconvolution is not free of the band fitting problems (Maddams 1980; Meier 2005), a better approach is desired. The objective of the present study was to develop another FT-Raman-based method so that cellulose I crystallinity in cellulose materials can be estimated more accurately.

Experimental

Whatman CC31 powder was from Whatman International Ltd., (Maidstone, UK). Lower crystallinity and amorphous cellulose samples were generated by grinding, for various durations, Whatman CC31 in a vibratory mill using steel balls. The milling was conducted in a cold room (5 °C) for a prescribed time. The milling times were 2.5, 5, 10, 15, 30, 45, 60, 90, and 120 min. For calibration purposes, cellulose mixtures with crystallinities in the range 10.9–64% were produced using different mass fractions of 80.5% crystalline cellulose I Whatman CC31 and completely amorphous cellulose (120 min ball-milled Whatman CC31). This group of six samples (mixtures 1–6, Table 1) along with the control and 120 min milled Whatman CC31 were classified as calibration set for univariate analysis. Other cellulose samples were obtained as follows: Avicel PH-101 (FMC Corporation, American Viscose Division, Newark, Delaware), and CF-11, Whatman CC41, and Whatman 541 paper (Whatman, UK). Black spruce, southern pine, and aspen woods were available in our laboratory and have been previously obtained in the context of other projects. Wood samples were Wiley milled to 40 mesh and extracted with acetone:water (9:1).

Alkaline H₂O₂ bleaching

The extracted 40 mesh wood samples were bleached (lignin was retained but brightened) using 5% H₂O₂ on wood (oven dry), in 0.2% MgSO₄ and 4% Na₂SiO₃ (pH 11–12). The wood consistency during bleaching was about 10% (O.D.). The bleaching was

carried out in a ziplock bag at 70 °C in a water bath. The duration of the alkaline bleaching was 90 min, with the bag being removed once at 45 min, kneaded, and returned to the bath. At the end of the treatment, the wood sample was washed with water and acidified with dilute H₂SO₄ until slightly acidic.

DP distribution in cellulose samples

The degree of polymerization (DP) distribution of microcrystalline cellulose is determined by a method in which nearly all (>97%) the microcrystalline Whatman CC31 cellulose was dissolved in 2.5 N alkali. Then aliquots of the solution are partially neutralized, which precipitates out higher molecular weight cellulose from the solution. Chemical oxidation was used to quantify the cellulose dissolved at each alkali concentration. A previously developed correlation (at Forest Products Laboratory, Madison) related the DP of soluble cellulose at different alkali concentrations.

Microcrystalline cellulose (2.5 g) was suspended in 250 mL water at 0 °C for 15 min. Then 250 mL of 5 N NaOH was added and the solution stirred at 0 °C for 60 min. Aliquots (50 mL) were partially neutralized with H₂SO₄ solutions (10 mL) at the appropriate concentrations to alkali concentrations of 1.4, 1.3, 1.2, 1.0, 0.8, and 0 N and stirred for 30 min at 0 °C. The precipitated cellulose was separated by centrifugation. The cellulose that remained dissolved in each aliquot was quantified by digesting 5 mL of supernatant with 10 mL of 0.5 N K₂Cr₂O₇ and 30 mL of concentrated H₂SO₄, then titrated with 0.1 N Fe(NH₄)₂(SO₄)₂ with ferroin indicator (see Tappi Method T203 (TAPPI Press 1991a) for similar analytic procedures). A

Table 1 % Crystallinities of calibration set samples—theoretical, WAXS, and univariate-Raman

Sample	Mass ratio control: 120 min, grams	Theoretical ^a	Segal WAXS ^b	Univariate Raman
Control	0.5000:0.0000	80.5	80.5	78.0
Mixture 1	0.4030:0.1042	64.0	56.7	62.7
Mixture 2	0.3232:0.1800	51.4	53.4	52.8
Mixture 3	0.2733:0.2188	44.8	47.0	44.8
Mixture 4	0.2053:0.2963	33.0	42.6	36.2
Mixture 5	0.1299:0.3722	20.9	26.7	23.1
Mixture 6	0.0675:0.4331	10.9	17.4	11.3
120-min milled	0.0000:0.5000	0.0	−1.0	−4.4

^a Except for control and 120-min milled which were WAXS crystallinities

^b Calculated according to Segal et al. (1959)

suspended aliquot at 2.5 N alkali and a centrifuged aliquot at 2.5 N alkali were also analyzed. A previously developed correlation at the Forest Products Laboratory with Emocel[®] 50 M was used to determine the degree of polymerization at the various levels of alkali by further isolating the cellulose fractions with acid precipitation, centrifugation, washing, and drying. TAPPI method T230 (TAPPI Press 1991b) was used to measure the viscosity of the isolated fractions dissolved in cupriethylenediamine in order to determine the DP of the cellulose fractions.

FT-Raman

Cellulose and wood samples were analyzed with a Bruker RFS 100 spectrometer (Bruker Instruments Inc., Billerica, Massachusetts). This Raman system is equipped with a 1,064-nm 1,000-mW continuous-wave (CW) diode pumped Nd:YAG laser. Approximately 0.25 g of each sample was pressed into a pellet with the help of a hydraulic press. To make a pellet, 276×10^6 dyn/cm² compressive pressure was applied. The laser power used for sample excitation was 600 mW, and 1,024 scans were accumulated. Bruker's OPUS software program was used to find peak positions and process the spectral data. From the Raman spectra, amorphous contributions in the frequency region 250–700 cm⁻¹ were removed by first normalizing (making band intensity equal) the spectra on 897 cm⁻¹ band and then subtracting the corresponding spectrum of 120-min milled sample. This was necessary because Raman spectroscopy is a semi-quantitative technique. 897 cm⁻¹ band in the spectrum was chosen because its peak height was minimally impacted by the process of ball milling. For plotting purposes, data were converted to ASCII format and then exported to Excel (Microsoft Corp., Redmond, Washington).

X-ray

Wide-angle X-ray diffraction profiles were recorded on a Bruker X-ray diffractometer with a Hi-Star 2-D area detector at the Materials Research Science and Engineering Center, University of Wisconsin, Madison. Diffractograms were obtained on the same sample pellets that were analyzed in FT-Raman. For calculating the crystallinity index, the ratio of the crystallinity part of the 002 peak to the total absolute

peak height was used. The peak present at about 22.5° (2θ) corresponds to the [002] crystal planes. The crystalline portion of the total contribution at 22.5° was calculated by Segal method (Segal et al. 1959) and involved subtracting out the amorphous contribution at 21° (2θ). The latter was the peak position in the diffractogram of 120-min milled amorphous sample.

Univariate analysis

Using OPUS, peak height was calculated by a baseline method that involved choosing a minimum intensity wavenumber near the peak (e.g., 358 and 944 cm⁻¹ for 380 and 1,096 cm⁻¹ bands, respectively) and drawing a horizontal line (from that wavenumber) under the peak. Figure 1 illustrates how peak intensity for the 380 cm⁻¹ band in mixture 4 sample was measured. In the remaining spectra, for bands 380 and 1,096 cm⁻¹, the same values of band minima (358 and 944 cm⁻¹) were used for drawing the baselines. This was done for various sets of peaks (380 and 1,096 cm⁻¹ peaks being one of the sets) that were explored in the univariate analysis. The intensity data were exported to Excel, where Raman band intensity ratios were calculated and regression models were developed.

Multivariate analysis

For multivariate data analysis, The Unscrambler 9.7 software program (CAMO Technologies, Woodbridge, New Jersey) was used. As reference values,

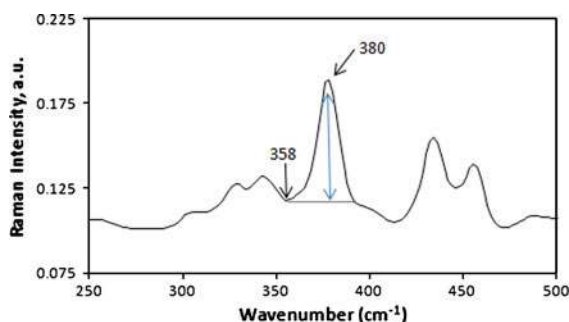


Fig. 1 Measurement of the 380 cm⁻¹ peak height in the amorphous-contribution subtracted Raman spectrum of mixture 4 sample. A minimum at 358 cm⁻¹ was chosen and a horizontal line was drawn under the peak. The peak height was measured from this base line

determined crystallinities from both univariate-Raman and WAXS were used. This was done to compare the two differently-referenced models. Calibration models were developed, using specific regions in the 250–1,550-cm⁻¹ range Raman data (Excel Raman files), with untreated and preprocessed spectra (vector normalized, 1st and 2nd derivatives). In the vector normalization the average *y*-value of the Raman spectrum was calculated first. This average value is then subtracted from the spectrum so that the middle of the spectrum is brought down to *y* = 0. The sum of the squares of all *y* values is then calculated and the spectrum is divided by the square root of this sum. The vector norm (length) of the resultant spectrum is 1. Principal component analysis (PCA) was used to reduce the original dimensions of the spectral data and represent the original data using new principal component (PC) scores that captured the maximum variation in the data. Using the first few PC variables, PCA scatter plots were generated. The “leave one out” full-cross validation technique was used to develop the partial least-squares (PLS) calibration model. The performance of the PLS models was assessed using several common statistical measures—the coefficient of determination, *R*² (a measure of the strength of the fit to the data), root mean square error of calibration (RMSEC), and root mean square error of prediction (RMSEP). Of the 20 cellulose samples, 10 were used for the PLS calibration models and the remaining 10 were used for prediction.

Results and discussion

The Raman spectrum of cellulose has been studied, and the spectral peaks have been assigned by Wiley and Atalla (1987a, b). The polarization sensitivity of most bands in oriented samples is known and has been discussed in the literature (Wiley and Atalla 1987b). The reader is referred to the literature for information on these topics.

Ball milling

Ball milling has long been known to affect the crystallinity of cellulose (Forziati et al. 1950). Raman spectra of variously ball-milled Whatman CC31 samples showed changes in most of the 250–3,700 cm⁻¹

spectral region, with the most prominent changes occurring in the fingerprint region. To visualize spectral changes occurring upon loss of crystallinity, Raman spectra, in the region 250–1,650 cm⁻¹, are shown in Fig. 2. Although a large number of bands were affected, the bands that experienced the major change are annotated. The intensity and band shape of two of these spectral features present at 380 and 1,096 cm⁻¹ were significantly affected by crystallinity modification. For example, the spectral features of 15-min ball-milled sample are significantly different from that of the control (compare Fig. 2a and e) and showed additional changes upon further ball milling (Fig. 2f or i). Nevertheless, spectra of 60-, 90-, and 120-min ball-milled Whatman CC31 (Fig. 2h, i, and j, respectively) were very similar, indicating that milling durations greater than 60 min produced very small changes in spectral features. This is supported by the crystallinity data in Table 2 (columns 2 and 3), where the values were equal to or less than 1%. In WAXS, negative values are due to the shifting of the peak position in the 120-min milled sample. On the contrary, in univariate-Raman, they arise due to the model error. In both the cases, the negative values were taken as 0% crystallinity.

WAXS diffractograms of the control and milled samples are shown in Fig. 3. Clearly, compared with the control, the 15-min ball-milled Whatman CC31 sample showed significant decline in the peak heights (compare Fig. 3a and e). The decline was accompanied by peak broadening. Further ball-milling leads to

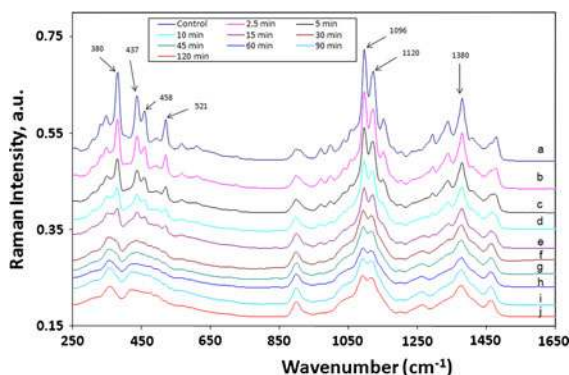


Fig. 2 Effect of ball-milling on Whatman CC31 Raman spectra; (a) control, (b) to (j) are spectra of samples that were ball-milled for 2.5-, 5-, 10-, 15-, 30-, 45-, 60-, 90-, and 120-min, respectively. Spectra were offset on the intensity scale for display purposes

Table 2 % Crystallinities of Whatman CC31 and other cellulose samples—univariate-Raman, WAXS, and multivariate-Raman (PLS)

Sample	Univariate Raman	Segal WAXS	Univariate Raman PLS	Segal WAXS PLS
Control	78.0	80.5	NA*	NA*
Mixture 1	62.7	56.7	62.1	62.4
Mixture 2	52.8	53.4	NA*	NA*
Mixture 3	44.8	47.0	40.5	42.4
Mixture 4	36.2	42.6	NA*	NA*
Mixture 5	23.1	26.7	NA*	NA*
Mixture 6	11.3	17.4	NA*	NA*
2.5-min	61.1	68.8	NA*	NA*
5-min	46.1	64.3	43.1	46.8
10-min	33.4	50.0	28.2	33.3
15-min	30.7	36.1	25.0	27.1
30-min	8.7	14.2	4.6	20.1
45-min	6.5	12.1	NA*	NA*
60-min	−0.9	0.0	NA*	NA*
90-min	−2.6	1.0	3.6	5.5
120-min	−4.4	−1.0	2.3	7.2
Whatman CC41	77.9	74.6	83.2	82.4
Whatman 541	56.2	50	NA*	NA*
CF-11	72.0	74.4	73.2	71.2
Avicel PH-101	59.5	60.9	NA*	NA*

*NA not applicable because the sample was used in the PLS calibration set

additional changes in the diffractograms, which are difficult to interpret (Fig. 3g and i). The WAXS crystallinity data, reported in Table 2 (column 3),

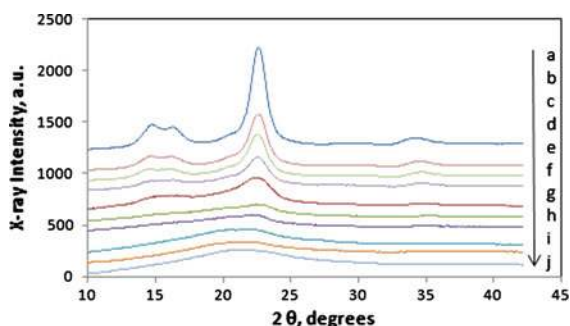


Fig. 3 WAXS diffractograms of control and ball-milled Whatman CC31: (a) control, (b) to (j) are diffractograms of samples that were ball-milled for 2.5-, 5-, 10-, 15-, 30-, 45-, 60-, 90-, and 120-min, respectively. Spectra were offset on the intensity scale for display purposes

indicated that the 60-min milled cellulose sample was completely amorphous (0% crystallinity). This was supported by the similarity of the WAXS profiles of the 60-, 90-, and 120-min milled celluloses (Fig. 3h–j).

Amorphous nature of 120 min milled cellulose

Further information was reviewed to ensure that the 120-min sample was indeed completely amorphous. When the X-ray diffractogram of the 120 min milled sample (Fig. 3j) was compared with the literature reported diffractograms of the amorphous celluloses (both mechanically and chemically produced; Forziati et al. 1950, Fig. 6; Segal et al. 1959, Fig. 5; Schroeder et al. 1986, Fig. 3; Hulleman et al. 1994, Fig. 1; Stubičar et al. 1998) it was concluded that the 120-min milled sample was completely amorphous. This conclusion was further supported by the great similarity between the band profiles of 357 and 380 cm^{-1} of 60–120 min milled samples (Fig. 4) and complete disappearance of the Raman peak at 380 cm^{-1} in the 120 min milled samples (Fig. 4). Compared to the spectrum of mixture 6 (10.9% crystalline, Table 1) 30-min milled cellulose spectrum showed a weaker 380 cm^{-1} contribution indicating that the latter's crystallinity was less than 10.9%. This shoulder was further weakened in the spectra of 45- and 60- min milled samples and in the case of the latter, became indistinguishable from the decaying profile of the band at 357 cm^{-1} . In this context, it is also important to note that the work reported by Schroeder et al. (1986) had found great similarity between the low frequency Raman spectra of chemically and mechanically produced amorphous

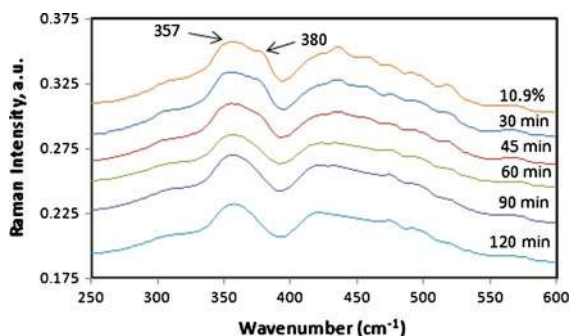


Fig. 4 Low frequency spectra comparing the band profiles of 357 and 380 cm^{-1} bands; spectrum 10.9% represents mixture 6 and spectra labeled 30–120 min correspond to 30–120 min milled Whatman CC31

celluloses and that the spectra reported there were similar to the ones in Fig. 4. Lastly, as was the case in WAXS (Fig. 3), extreme similarity between the 357 cm^{-1} -band-profiles of 60–120 min milled samples (Fig. 4) suggested that milling beyond 60-min did not additionally decrystallize cellulose and therefore, complete decrystallization was probably achieved by 60-min. This conclusion is supported by the WAXS crystallinity data in Table 2 (column 3).

Univariate analysis

To determine if, using Raman spectroscopy, a value for the crystalline fraction can be obtained in Whatman CC31 samples that were ball-milled for various durations, a two-phase (crystalline and amorphous) model was used to calculate crystallinity over a wide range of compositions (over 10%). Therefore, cellulose crystallinity, X_c , can be defined and calculated as

$$X_c = M_c/M_t = M_c/(M_c + M_a)$$

where M_c , M_a , and M_t are the crystalline, amorphous, and total mass fractions of cellulose, respectively. Since $M_c = X_{\text{control}} * M_{\text{control}}$, where X_{control} and M_{control} are the % crystallinity and mass fraction of the control sample (80.5% crystallinity, Table 1). The above equation can be re-written as

$$\begin{aligned} X_c &= X_{\text{control}} * M_{\text{control}} / (M_c + M_a) \\ &= 80.5 * M_{\text{control}} / (M_c + M_a) \end{aligned}$$

The crystallinities of the six of the eight calibration set samples (theoretical crystallinities) were calculated using the above equation and were based on the WAXS data for control and 120-min milled samples. The crystallinities were also determined by WAXS (Segal) and univariate-Raman methods (Table 1). In Raman, as in WAXS, an assumption was made that the spectrum is composed of two superimposed spectra arising from the amorphous and crystalline phases. The Raman spectra of the calibration set samples (six mixtures, amorphous, and control; Table 1) were obtained, corrected (in the low wavenumber region) for the amorphous contribution, and the peak height ratios for various bands were calculated. The low-wavenumber corrected spectra are shown in Fig. 5. The band height ratios were determined for 380/1,096, 380/2,900, 437/1,096, 437/2,900, 458/1,096, 458/2,900, 521/1,096, 521/2,900,

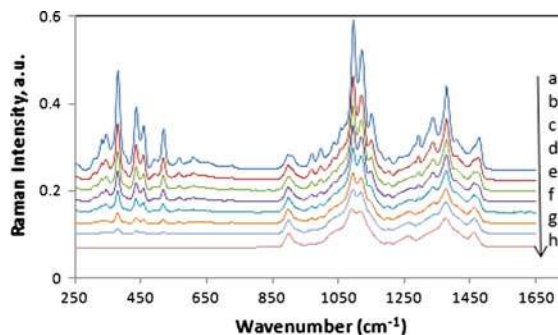


Fig. 5 Calibration set Raman spectra after subtracting amorphous spectrum in the region 250–700 cm^{-1} ; (a) control, and plots (b) to (h) are spectra of mixture 1, mixture 2, mixture 3, mixture 4, mixture 5, mixture 6, and 120-min milled cellulose, respectively. Note that in the case of 120-min spectrum the intensities below 700 cm^{-1} are all zero because of the subtraction. Spectra were offset on the intensity scale for display purposes

and 1,096/2,900. Peak height ratios were then plotted against the calibration set crystallinities. The Raman ratio plot for 380/1,096 (Fig. 6) generated excellent regression ($R^2 = 0.992$) and showed good sensitivity to crystallinity change.

Therefore, considering the excellent fit between the theoretical crystallinities and the Raman band intensity ratios, the simple univariate correlation method can be used to calculate cellulose I crystallinities in cellulose samples. The correlation equation is

$$X_{\text{Raman}} = \frac{(I_{380}/I_{1,096}) - 0.0286}{0.0065}$$

Additionally, in the case of some other peak ratios where the intensity of the $2,900\text{ cm}^{-1}$ band was used, the correlation coefficients were also excellent (not

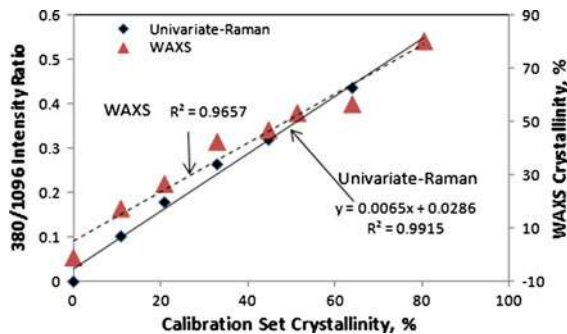


Fig. 6 Univariate analysis of the calibration set samples showing the correlation between calibration set crystallinity versus 380/1,096 Raman intensity ratio or WAXS crystallinity

reported here), but considering the fact that in FT-Raman the intensity of the $2,900\text{ cm}^{-1}$ band can be affected by the sample moisture (Agarwal and Kawai 2005), such cases were not favored. Repeat Raman scans on the seven calibration set samples produced an average standard error of $\pm 0.67\%$ and indicated that the univariate-Raman crystallinity model was reliable. The error was calculated from five replicate Raman acquisitions of each of seven different calibration samples.

Calibration set samples were also analyzed using WAXS (Table 1). The regression coefficient for the WAXS versus calibration set crystallinities was 0.966 (Fig. 6), lower than that of the Raman calibration (Fig. 6). From the Raman and WAXS crystallinity data comparison in Table 1, it is clear that the WAXS method overestimated the cellulose I crystallinity in some of the samples (e.g., compared to theoretical mixture 4, mixture 5, and mixture 6 were overestimated; Table 1). It is possible that for these samples Segal-WAXS method did not successfully separate amorphous and crystalline phase contributions.

In Fig. 7, a regression curve between the WAXS and univariate-Raman determined crystallinities for the calibration set samples showed a strong correlation ($R^2 = 0.981$). The plot indicated that univariate-Raman method can be successfully used for crystallinity estimation.

Ball milled samples

The simple method based on the univariate analysis was used for calculating the crystallinities of the 2.5-, 5-, 10-, 15-, 30-, 45-, 60-, and 90-min ball-milled Whatman CC31 samples. These Raman

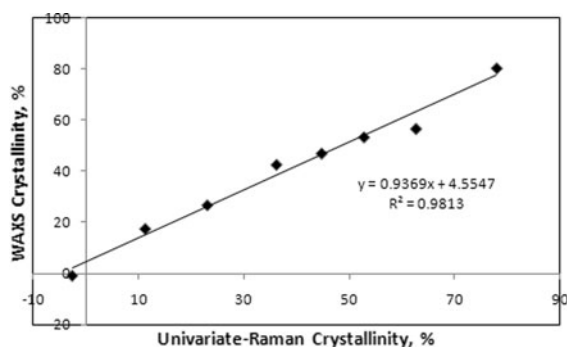


Fig. 7 Relationship between crystallinities determined by univariate-Raman and WAXS for calibration set samples

crystallinities, along with the WAXS and PLS crystallinity (see later) data, are listed in Table 2.

Once again, compared to Raman univariate Segal WAXS method generated higher cellulose I crystallinity in the ball-milled samples (Table 2, columns 2 and 3). A possible explanation is that for some samples (depending upon the crystallinity) Segal method underestimates the amorphous component in the sample diffractograms due to band profile changes (peak broadening and shifting). Higher crystallinity by WAXS methods has been reported in the literature. For example, Park et al. (2009) used three X-ray methods (peak height, peak deconvolution, and amorphous contribution subtraction) to calculate the crystallinity index of 11 cellulose samples. The three methods produced significantly different crystallinity indexes. Compared to the deconvolution approach, peak height method gave values that were significantly higher –20 to 30 points higher. Moreover, importantly enough, the differences depended upon the nature of the samples. For the 11 samples analyzed, the average crystallinity indexes for the peak height, peak deconvolution, and amorphous contribution subtraction methods were 86, 60, 68, respectively. This indicated that the index varied significantly and depended upon how the analysis was performed.

Considering that in WAXS the peak-height crystallinity calculation method gave significantly higher values compared to other X-ray and NMR methods (Park et al. 2009) the authors make the following suggestion based on the findings of the present investigation. It is proposed that, in the simple peak height method, the amorphous contribution be measured at $\sim 21^\circ$ instead of $\sim 18^\circ$. This will result in lower crystallinity values because a higher contribution of the amorphous component would be subtracted. The suggestion is supported by both the diffractogram deconvolution method which suggests a value of 21.5° (Garvey et al. 2005) and the authors' current WAXS data where for amorphous Whatman CC31 sample a value of 21° was observed (Fig. 3j). Further support comes from the literature (Teeaar et al. 1987; Hulleman et al. 1994; Stubičar et al. 1998; Isogai et al. 2009) wherein the peak position in completely amorphous cellulose is reported to be $20\text{--}21^\circ$ and not 18° (as Segal et al. 1959 reported). Although not clear why this is the case, the finding of the higher 2θ value may possibly result from the

differences in X-ray instrumentation. Nevertheless, analogous to the Segal method, the proposed approach still assumes that the amorphous contribution is same under the [002] peak as it is at 21°. Perhaps in samples that are more crystalline than completely amorphous the peak position and the profile of the amorphous component are retained. The practical effect of this suggestion would be a reduction in the overestimated values of crystallinity while still being able to use the simple peak-height method.

Testing the model assumption

To test the assumption, made earlier, that the spectrum of a semicrystalline Whatman CC31 sample is composed of two superimposed spectra arising from the amorphous and crystalline phases the Raman spectra of two samples, mixture 4 and 10-min milled (Table 2) were compared. The former sample was obtained by mixing set proportions of 80.5% crystalline and completely amorphous Whatman CC31 samples. These two samples were chosen because their univariate-Raman crystallinities are similar (~33%, Tables 1 and 2). If the assumption is valid, the spectra of mixture 4 and 10-min ball-milled samples should match. Figure 8 shows the spectra (compare Fig. 8a and b) along with the result of the spectral subtraction (Fig. 8c). The two spectra are very similar as is evident from the subtraction result (mixture 4–10-min, Fig. 8c). Therefore, the model assumption appears to be valid. The WAXS results also supported the 2 phase model although the two crystallinities differed by 7.4% (Table 2).

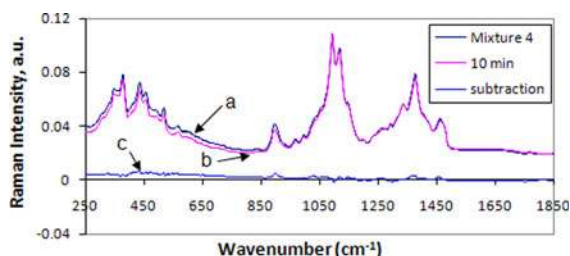


Fig. 8 Comparison of original Raman spectra of samples with similar crystallinities: (a) mixture 4, (b) 10-min milled Whatman CC31, (c) spectral subtraction result. Spectra were plotted after making the peak heights identical at 1,096 cm^{-1}

Multivariate analysis

Having shown that the univariate analysis produced a significant correlation for determining cellulose I crystallinity by Raman, it was of interest to establish whether multivariate data processing methods could be used as well.

Ten samples (control, mixture 2, mixture 4, mixture 5, mixture 6, 2.5, 45, 60-min, Whatman 541, and avicel PH-101; Table 2) were used in PLS model development and another 10 were used for prediction (mixture 1, mixture 3, 5, 10, 15, 30, 90, and 120-min, Whatman CC41, and CF-11; Table 2). Raman spectra in the region 250–1,550 cm^{-1} were used in the PLS analysis. This region was selected because the most useful changes occurred in this spectral region. Although Raman spectra were pre-processed in three different ways (vector normalized, 1st and 2nd derivatives) and analyzed using The Unscrambler, the best results were obtained for the vector normalized case. Therefore, in this section only results obtained using the vector normalized spectra are presented. The option of PLS regression with full cross validation was used for the model development. Original spectra were modeled in PLS and the results are discussed here. The models were developed with two different reference crystallinities sets—univariate-Raman and WAXS. The two PLS models are compared in Table 3.

In univariate-Raman model, first three PCs explained 100% of the crystallinity-variance. In contrast WAXS PLS model needed only two PCs. The regression coefficients for all the PCs had strong positive correlations at 380, 437, 458, 521, 1,096, 1,120, 1,330, 1,340, 1,380, and 1,475 cm^{-1} . This was the case for both the univariate-Raman and WAXS referenced models. These wavenumber positions are

Table 3 Comparison of univariate-Raman and WAXS based PLS models—results of calibration and prediction of % crystallinity

Model characteristics	Univariate Raman	Segal WAXS
RMSEC of mean (%)	1.8	6.1
RMSEP of mean (%)	3.3	7.9
R^2 -calibration	0.998	0.983
R^2 -prediction	0.995	0.973

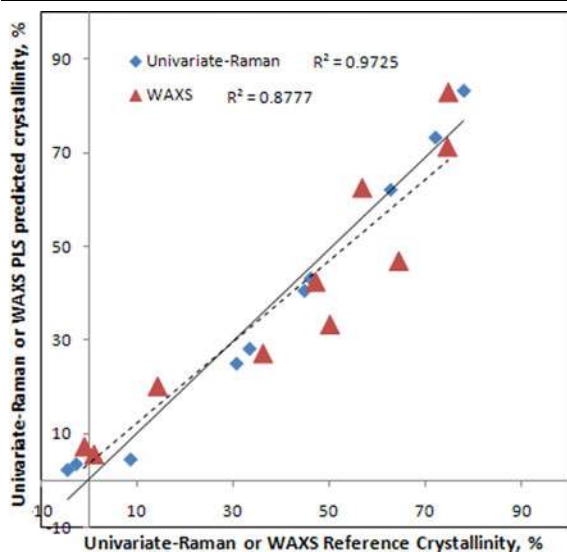


Fig. 9 Correlation between univariate-Raman determined versus PLS-Raman predicted (univariate-Raman referenced) or between WAXS determined versus PLS-Raman predicted (WAXS referenced) crystallinities

similar to where changes due to cellulose crystallinity were most prominent in the original and subtracted spectra (Figs. 2 and 5). However, the two PLS models produced significantly different RMSEC and RMSEP values (Table 3). From Table 3 it is clear that the univariate-Raman based model is superior compared to the WAXS model because for the former RMSEC and RMSEP values were about 70 and 60% lower, respectively. PLS predicted crystallinities for 10 samples are reported in Table 2 (columns 4 and 5) and the regression plots are shown in Fig. 9. Clearly, the univariate-referenced PLS model does a better job.

Raman spectra of ball-milled samples

As a function of milling duration, the Raman spectra showed many changes (Fig. 2). Sharp features present in the spectrum of high crystallinity Whatman CC31 (Fig. 2a) reduced in intensity significantly within a short duration (compare Fig. 2c with Fig. 2a). Spectra of 15- and 30-min milled samples (Fig. 2e and f) showed that the rate of phase change had slowed down and that a large part of the crystalline to amorphous change was accomplished after 30 min of ball milling. The latter observation is based on the comparison of plots in Fig. 2f and j, which indicated that the Raman features were similar and any changes

present were small. Similarity of spectra is supported by the low crystallinity of samples that were ball-milled for 30 min or longer (Table 2, WAXS and univariate-Raman). Although only the spectral modifications in the fingerprint region are shown in Fig. 2, changes were also detected in the O–H and C–H stretch regions. The cellulose fingerprint region ($250\text{--}1,500\text{ cm}^{-1}$) has contributions from CH and CH_2 bending, CH wagging, OH rocking and bending, CO and COC stretching, and anhydroglucose ring stretching, twisting, and torsion modes. The cellulose band assignments work (Wiley and Atalla 1987a) has shown that most Raman bands are coupled modes, and this situation is further complicated by the fact that the intensity, position, and width of a band are sensitive to molecular environment and intermolecular interactions. In short, although the spectra are rich in chemical information, unscrambling that information is complex. This situation is similar to the FT-IR findings where ball-milling-induced changes in the spectra of spruce wood and cellulose were shown to be associated with decline in crystallinity and/or decrease in the degree of polymerization of cellulose, and not due to other factors such as temperature change, particle size variation, and oxidation processes (Schwanninger et al. 2004).

To determine if the declining DP of cellulose had any role in the milling related observed spectral changes, the DPs of control and 120-min ball-milled Whatman CC31 samples were measured. The results, plotted in Fig. 10, indicated that there was only a

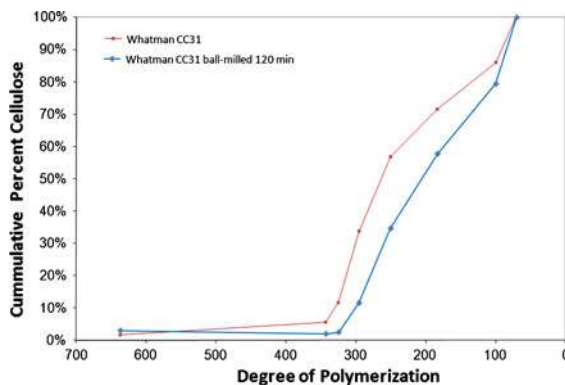


Fig. 10 Analysis of the degree of polymerization for Whatman CC31 before and after 120-min of ball-milling. The chart shows only a modest decline in the DP of the cellulose upon ball-milling

modest change in the DP. Such DP decline is not likely to cause the ball-milling-induced spectral changes that were detected. This is further supported by the study that showed that the Raman spectra of the ball-milled (low DP) and chemically regenerated (high DP) amorphous celluloses were very similar (Schroeder et al. 1986). Consequently, most of the changes in the Raman spectra are associated with decline in cellulose crystallinity and not with changes in the cellulose DP.

Other samples

Because the simple univariate method worked so well, it was used to determine crystallinities of a number of cellulose and wood samples (Table 4). To make the comparison, samples were also analyzed by the WAXS method, or, where available, the WAXS data were taken from the literature. As can be noted from Table 4, overall the Raman and X-ray crystallinities matched well. In the case of CF-11, literature-reported (Wulin et al. 2004) WAXS crystallinities were significantly different, probably because of differences in the CF-11 samples and also not knowing if the authors' used the Segal method to calculate crystallinity. The CF-11 used by Wulin et al. (2004) was a very high purity and very high crystallinity sample. Indeed, the diffractogram of CF-11 used in the present study differed from what is

reported by these authors and in particular, did not show the [021] reflection at $\sim 20.4^\circ$ (2θ). The latter was an indication of lower crystallinity sample.

In the case of wood samples, some fluorescence from lignin chromophores was encountered and it was considered prudent to brighten the samples using the alkaline H_2O_2 (lignin is bleached but not removed). Whereas the lignin-retaining bleaching resulted in small increase in the crystallinities of black spruce and southern pine (Table 4) these values were closer to the values reported in the literature (Rayirath et al. 2008 and Bertran and Dale 1985, respectively). In Table 4, literature WAXS crystallinities are assumed to be the same for untreated and alkaline-peroxide-treated woods. However, the Raman crystallinity of the untreated aspen (hardwood) was considerably lower compared to alkaline-peroxide-bleached wood (Table 4). Moreover, this value was also lower than the value reported by Bertran and Dale (1985) from WAXS measurement. This underestimation of crystallinity by Raman is probably due to the contribution of fluorescence to the intensity of the $1,096\text{ cm}^{-1}$ band making it stronger. This will make the band ratio $380/1096$ lower than would have been the case had there been no fluorescence contribution. Although the peroxide-bleached aspen and literature crystallinity data matched, the values were higher compared to softwoods. In univariate-Raman crystallinity, this can be explained by the fact that at 370 cm^{-1} lignin in

Table 4 Crystallinities of other samples—univariate-Raman and WAXS

Sample	Univariate Raman	Segal WAXS	WAXS from literature
Whatman CC41	77.9	74.6	–
Avicel-PH-101	59.5	60.9	–
CF-11	72.0	74.4	93.0 ^a
Cellulose paper Whatman 541	56.2	50.0	–
Black spruce	49.2	45.5	55.5 ^b
Black spruce, alk. H_2O_2 bleached	52.1	–	55.5 ^b
Southern pine	46.5	–	53.0 ^c
Southern pine, alk. H_2O_2 bleached	50.9	–	53.0 ^c
Aspen	44.0	–	60.3 ^d
Aspen, alk. H_2O_2 bleached	59.2	–	60.3 ^d

^a Wulin et al. (2004)

^b Rayirath et al. (2008)

^c Bertran and Dale (1985)

^d Jahan and Mun (2005)

aspen (a hardwood) has a contribution that is stronger than in softwoods. Moreover, this contribution is not removed upon alkaline peroxide bleaching. The decaying profile of the 370 cm^{-1} band enhances the peak height of the 380 cm^{-1} band. This explains why the Raman crystallinity of bleached aspen, unlike those of spruce and pine, is still somewhat higher (Table 4). Hardwood lignin and hemicelluloses are known to have contributions at or near band positions used in the calculation of Raman crystallinity (Agarwal et al. 1999; Agarwal and Ralph 1997). In any case, for hardwoods, our findings point out the necessity of further investigation so that the roles of wood fluorescence, lignin, and hemicelluloses can be better understood. On the other hand, for crystallinity determination in softwoods, unbleached samples give slightly lower crystallinity compared to what is known from the literature. Therefore, from the foregoing, it is clear that FT-Raman spectroscopy also has the potential for quantifying cellulose crystallinity in woods.

Conclusions

Quantitation of cellulose I crystallinity in various Whatman CC31 and other cellulose samples was successfully carried out by either of the two methods developed in this work. The simple method, which involved taking a ratio of the intensity of two Raman bands, was straightforward and reliable. In contrast, the second method of calculating crystallinity was a PLS regression calibration model and involved coupling of FT-Raman data with chemometrics. However, the PLS regression method was also rapid, easy to use, and reliable. The simple univariate method was applied to a set of wood samples, and in most cases, satisfactory agreement between the Raman and WAXS crystallinities was obtained.

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