Composition Analysis of Canola and Intermediate Wheatgrass Biomass and the Effects of Extraction

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Knowing the composition of biomass is critical for determining accurate yields of renewable chemicals and fuels; however, nonstructural components can affect the results of standard composition procedures, leading to inaccurate reactant amounts. To remove these nonstructural components, solvent extractions can be done, but the impact on composition values has not been well-reported. For this study, compositional analysis was performed on as-received canola (*Brassica napus*) and intermediate wheatgrass (*Thinopyrum intermedium*), as well as ethanol, water, and water/ethanol extracted biomasses. Water/ethanol extraction of the intermediate wheatgrass resulted in significantly lower xylose and both acid soluble and insoluble lignin amounts when compared to the as-received analysis. Since sugar was removed during the extractions, it is recommended to use the as-received composition values for glucuronoarabinoxylans; however, the extractives may interfere with the lignin analysis and therefore, the extracted lignin values are likely more reflective of the composition.

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INTRODUCTION

In recent years, there has been a significant amount of research on upgrading lignocellulosic materials (herein referred to as "biomass") to chemicals and fuels due to theoretical carbon neutrality (Romo *et al.* 2018; Jeswani *et al.* 2020; Job *et al.* 2022). In order to improve the economics of biomass processing, all major fractions of the biomass will need to be utilized. Alternative crops, such as oilseed and perennial forages, allow for harvesting part of the plants, and then upgrading the lignocellulosic portion to add further value to the process. For example, the oilseed could be harvested, and then the remaining biomass upgraded to bioderived chemicals. However, to accurately determine the yield of products that result from biomass conversion, an accurate composition profile of the sugar and lignin content is necessary.

Biomass consists of three major fractions, cellulose, hemicellulose, and lignin, that can be readily upgraded into chemicals and fuels. Cellulose is comprised of linked glucose monomers and makes up 25 to 40% of the dry weight of the feedstock, while hemicellulose is primarily glucuronoarabinoxylans (GAXs) and can make up 10 to 30% dry weight of the feedstock. The third major component of biomass, lignin, is a phenolic polymer and comprises between 17 to 24% of the structure of grasses (Saake and Lehnen 2007; Buchanan *et al.* 2015) and ranges from 6.1 wt% for corncob and up to 35% for softwood

pine (Cai *et al.* 2017). Due to these wide ranges of composition and differences that can occur due to precipitation amount, temperature (Zhang *et al.* 2019), and even time of year the biomass was harvested (Sawatdeenarunat *et al.* 2015), assumptions about the percentages of sugars and lignin in biomass cannot be made. To accurately calculate product yields resulting from biomass upgrading, the precise biomass composition must be known.

Although there are several methods for quantifying biomass composition (Cai *et al.* 2017), a common method of quantifying the major fractions is performing acid hydrolysis on the biomass sample to break down the polymeric sugars into monomers and then measuring the concentrations of the different sugars using chromatography and using spectrophotometry to determine the lignin amount (Sluiter *et al.* 2012). However, biomass also contains nonstructural components, such as nonstructural sugars, ash, protein, and salts, and these can interfere with sample composition analysis (Sluiter *et al.* 2005). These nonstructural components can be removed using appropriate solvents so that extractive-free biomass remains for further testing (Sluiter *et al.* 2011). If the extractives are not removed, the hydrophobic extractives may inhibit hydrolysis, and artificially high lignin values may occur due to unhydrolyzed carbohydrates condensing with the lignin (Sluiter *et al.* 2005). Some solvents reported in literature for extraction include acetone (Singh *et al.* 2021; Smit and Huijgen 2017), supercritical CO_2 in an ethanol-water cosolvent (Lv *et al.* 2013), water, and ethanol (Toribio-Cuaya *et al.* 2014; Hickey *et al.* 2021; Cai *et al.* 2017). Acetone and supercritical CO_2 pretreatment are typically used to fractionate biomass to increase the effectiveness of later processing. Both water and ethanol are used in the standard National Renewable Energy Laboratory (NREL) NREL/TP-510-42619 procedure (Sluiter *et al.* 2005) and the TAPPI T 204 cm-97 (2007) method that present specific procedures for removing solvent-soluble materials from biomass via a Soxhlet apparatus, which allows the solvent to reflux over the biomass and the extracted materials to be collected in a flask below the biomass. Water extraction is recommended to remove inorganic material and nonstructural sugars, while ethanol extraction is recommended to remove waxes, chlorophyll, and other minor components (Sluiter *et al.* 2005).

Previous work has shown that both ethanol and water extractions on switchgrass, corn stover, and fescue affected the biomass composition results. Individually, both water and ethanol extractions reduced the Klason lignin values and affected the apparent glucan content in most cases (Thammasouk *et al.* 1997). Composition analysis of extracted biomass is generally seen as giving a more accurate estimate of cellulose in the feedstock by removing noncellulosic, glucose-containing compounds. However, it is important to note that for biomass upgrading reactions the total amount of C6 sugars, including the nonstructural sugars, are critical and extraction would reduce the actual glucose value due to sucrose removal, leading to inflated product yields. The lower Klason lignin values were hypothesized to be due to the removal of solvent-soluble compounds that would otherwise become insoluble in the acid conditions used in Kalson lignin determinations, which leads to overestimating the initial amount of lignin prior to upgrading (Thammasouk *et al.* 1997). Additional research on non-herbaceous biomass determined that ethanol extraction was important to determine the Klason lignin content of biomass (Hickey *et al.* 2021). Both research groups demonstrated that a solvent extraction influenced the compositional analysis of a feedstock, but it is unknown what solvents are best suited for a certain feedstock, as relative proportions of solvent-soluble components will vary between species. For example, corn stover was found to have much higher water-soluble extractives than ethanol-soluble extractives. Additionally, Thammasouk *et al.* (1997) did not evaluate the

effect of water extraction followed by ethanol extraction, which is recommended for herbaceous biomass in the NREL/TP-510-48087 Summative Mass Closure laboratory analytical procedure (Sluiter and Sluiter 2011).

In Montana, wheat-based annual cropping systems are increasingly diversified with industrial and food grade *Brassica napus* (canola) oilseed crops and perennial forages such as *Thinopyrum intermedium* (intermediate wheatgrass). This paper presents the composition results of these two herbaceous biomass species, canola and intermediate wheatgrass, that have undergone ethanol, water, and water/ethanol extractions to determine the effect of extraction on composition.

EXPERIMENTAL

Materials and Methods

The 'Manska' intermediate wheatgrass and 'Hyola 357 Magnum' canola-quality *B. napus* were provided by the USDA-ARS in Sidney, MT. The canola was harvested at two sites: the Froid and Rasmussen (referred to as Ras) farms while the intermediate wheatgrass was harvested from the Froid farm. The Ras farm is located 8 km (5 mi) north of Sidney, MT (47°46´ N, 104°14´ W, 688 m above mean sea level), and the Froid farm is located 11 km (6.8 mi) north of Culbertson, MT (48°14´ N, 104°29´ W, 655 m above mean sea level). Both sites are mapped as Williams loam (fine-loamy, mixed, superactive, frigid Typic Argiustolls) with 0.5% to 1% slope. The above ground biomass was collected the day before combine harvest in 2012 and had any oil seeds removed. The remaining biomass was then dried at 55°C for 7 days, ground using a 1 mm sieve catch, and stored in zipped plastic storage bags. Prior to analysis, each feedstock was sieved between No. 16 (1.18 mm) and No. 30 (0.6 mm) mesh sizes (Fig. 1) as this is the range used in biomass experiments within the lab, and the samples were stored in glass vials under ambient conditions.

Fig. 1. Biomass was sieved to a particle size of 0.6 to 1.18 mm including: a) intermediate wheatgrass grown at the Froid location, b) canola grown at the Froid location, and c) canola grown at the Ras location.

Biomass Moisture Content

A Mettler-Toledo ME204E analytical balance was used to weigh 5 g of biomass onto a metal weigh tray of known weight. The biomass sample was spread evenly on the weigh tray and placed in the Kett FD100 moisture determination balance and heated to the set point of 250 °C. The heat automatically shut off when the balance no longer recorded a change in mass and the moisture content was displayed, which took between 4 and 8 min. The biomass sample was then reweighed on the analytical balance to confirm the moisture content displayed by the FD100.

Extractions

A 4 g sample of feedstock was weighed to the nearest 0.1 mg, placed in a cellulose thimble, and then set in a Soxhlet apparatus. The extractions took place with 150 mL of either 18.2 MΩ water or 95% ethanol. Ethanol extractions were performed according to the TAPPI T 204 cm-97 (2007). The ethanol reflux was set at approximately 6 cycles/h for a total of 30 total solvent reflux cycles over a 5-hr period. Water extractions were performed similarly to the TAPPI procedure, but instead followed the total extraction time recommended by the NREL/TP-510-42619 laboratory analytical procedure (Sluiter *et al.* 2005), which was 20-24 h until the solvent was clear. Water-ethanol extractions were done by performing a water extraction, drying the biomass, and then performing an ethanol extraction. After each extraction, a small liquid sample (~0.5 mL) was taken for HPLC analysis and the remaining solvent was transferred to a pre-weighed beaker and evaporated to 25 mL over a hot plate. The beaker and its contents were dried overnight in an oven at 105 °C. The percent extractives were calculated from the dried extractives and the oven-dry mass of the initial feedstock added to the thimble.

Biomass Compositions

Using the NREL/TP-510-42618 laboratory analytical procedure (Sluiter *et al.* 2012), feedstocks were hydrolyzed in 72% H_2SO_4, diluted with DI water, and autoclaved for 1 h to convert cellulose and hemicellulose into their monomeric constituents. The liquid and solids were separated by vacuum filtration using a filtering crucible. The solids were heated to 105 °C to determine the dry residue weight and then heated to 575 °C to ash the solids so that the acid insoluble lignin could be calculated. Using the filtered liquid, the acid soluble lignin content was determined using a UV-Vis spectrophotometer to measure the absorbance at 320 nm that was then converted to a mass using an absorptivity value of 30 L/(g cm), which was according to the guidelines for corn stover in the NREL method. The monomeric forms of sugar in the liquid filtrate were quantified with high performance liquid chromatography (HPLC) and sugar recovery standards that were made according to the same laboratory analytical procedure (NREL/TAP 510-42618; Sluiter *et al.* 2012).

RESULTS AND DISCUSSION

Extraction and Composition Analysis

To evaluate the effect of extraction method, different extractions followed by biomass composition analysis were carried out using the intermediate wheatgrass (Table 1) and canola from both the Froid (Table 2) and Ras (Table 3) sites. Four protocols were followed for each biomass type: no extraction (n/a), ethanol extraction (EtOH), water extraction (H_2O), and water extraction followed by ethanol extraction (W/E). The composition data shown in Tables 1 through 3 are on a weight percent basis for as received, oven dried biomass both in structural polymers as well as nonstructural monomeric components.

Removal of extracted materials and their inclusion in the composition improved mass balance closure for each of the tested biomasses for all extractions, which was expected since the extractives are unaccounted for in the as received method. It should be noted that variability in the dryness of the extractives can lead to variability in extractive weight percentages (Milne *et al.* 1992) due to small amounts of moisture possibly remaining after oven drying causing variability in the measurements.

Table 1. Composition of Intermediate Wheatgrass as Determined by the Analysis of As Received, Ethanol, Water, and Water/Ethanol (W/E) Extracted Methods

Extraction	n/a	EtOH	H_2O	W/E
Moisture	4.4	4.4	4.2	4.4
Total glycans	59.9	55.5	59.7	55.2
Glucose	33.5 (0.6)	32.2	36.1	32.7 (1.7)
Xylose	24.2 (0.9)	21	21.1	20.2 (0.3)
Arabinose	2.3 (0.2)	2.2	2.5	2.3 (0.3)
Total Lignin	21.5	17.4	17.8	16.6
AIL	18.6 (0.8)	15.1	16.3	15.3 (0.5)
ASL	2.8 (0.1)	2.4	1.8	1.6 (0.1)
Ash	3.1 (0.2)	4	4.3	3.9
Extractives	n/a	8.3	16.8	19.5
Total mass balance	89	90	103	100

Note: For Tables 1-3, all values shown are w/w on an As Received Basis and represent the fraction of the initial oven dry weight made up by each component. Standard deviations are shown in parentheses where applicable.

Table 2. Composition of Froid Canola as Determined by the Analysis of As Received, Ethanol, Water, and Water/Ethanol (W/E) Extracted Methods

Extraction	n/a	EtOH	H_2O	W/E
Moisture	4.8	4.8	4.4	5.3
Total glycans	50.3	52.9	60.6	52.4
Glucose	29.7 (0.9)	31.2 (0.4)	36.6 (2.4)	31.6
Xylose	20.0 (0.6)	21.0 (0.1)	23.4 (2.1)	20.3
Arabinose	0.7 (0.03)	0.7 (0.01)	0.7 (0.1)	0.5
Total Lignin	23.2	22.4	26.5	22
AIL	21.3 (0.6)	20.7 (1.1)	25.0 (2.2)	20.7
ASL	1.9 (0.2)	1.8 (0.1)	1.7 (0.2)	1.4
Ash	0.9	1	2	0.1
Extractives	n/a	4.1	10.9	13.5
Total mass balance	79	85	104	93

Table 3. Composition of Ras Canola as Determined by the Analysis of As Received, Ethanol, Water, and Water/Ethanol (W/E) Extracted Methods

Extraction	n/a	EtOH	H_2O	W/E
Moisture	4.9	3.8	4.7	3.9
Total glycans	55.2	57.2	57.3	54.1
Glucose	31.3 (0.5)	32.4	33.4	31.4
Xylose	22.9 (0.2)	23.9	23.1	22
Arabinose	1.0 (0.04)	0.98	0.81	0.72
Total Lignin	21.7	23.5	23.3	23.7
AIL	19.9 (1.1)	21.8	21.9	22.5
ASL	1.8 (0.04)	1.7	1.5	1.3
Ash	1.1	0.2	0.2	5.9
Extractives	n/a	2.2	8	9.8
Total mass balance	83	87	94	97

For intermediate wheatgrass (Table 1), 8.3% of the biomass was extracted using ethanol and 16.5% with water. When a water extraction was performed followed by an ethanol extraction, 19.5% of the biomass was extracted, indicating that approximately 3

wt% of the extractives could only be removed with ethanol, but that the extractives are not mutually exclusive to the solvent as also reported in work by Hickey *et al.* (2017). As can be seen in Fig. 2 for the intermediate wheatgrass, analysis of the extraction solvent showed that the water extraction had the same amount of sugars as the W/E sample. That is, no additional sugars were quantifiable in the ethanol extraction that followed the water extraction, confirming that water removed the majority of easily accessible sugars that were seen in the ethanol only extraction. As mentioned, ethanol extractions remove the waxy materials such as sap and resin (Sluiter *et al.* 2012), which is likely the 3 wt% of biomass that was exclusively removed using ethanol.

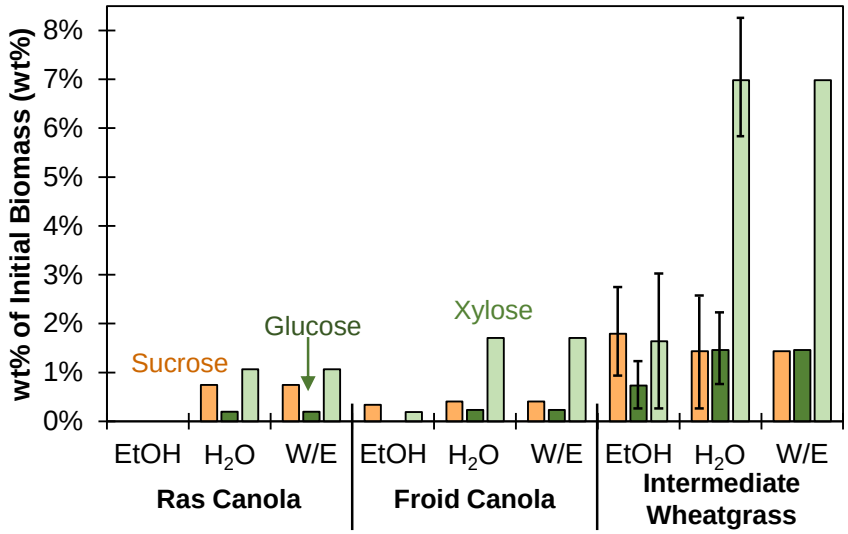

Fig. 2. Amount of sucrose (■), glucose (■) and xylose (■) in the extractives after ethanol, water, or water/ethanol extractions of canola and intermediate wheatgrass. Standard deviation error bars were added to the analyses with at least three values.

Both canola samples had a lower amount of extractives compared to intermediate wheatgrass; ethanol extracted 2.2 to 4.1% and water extracted 8 to 10.9% for the Ras and Froid canola, respectively. In the case of the water/ethanol extractions, 9.8 to 13.5% of extractives were removed, which were within experimental error of each other. In the case of the Froid canola, less than 1.5% of extractives were removed using either water or ethanol. However, this means that of the 4.1% removed by ethanol extraction, 1.5% of that portion was also removed by water, indicating that only 2.6% of extractives were removed with ethanol alone. Due to the small percentage removed, ethanol extraction may not be necessary for accurate compositional analysis of canola. This is confirmed when comparing the lignin values in Table 2. The total, acid insoluble, and acid soluble lignin values are all within error for the as received and ethanol extracted samples.

The extractives from the intermediate wheatgrass and canola were analyzed for composition and all samples, excluding the Ras canola extraction with ethanol, had xylose peaks present ranging from 0.2 wt% to 7 wt% of the initial biomass and most, excluding the ethanol extracted canola samples, had glucose peaks as well, ranging from 0.2 wt% to 2.9% of the initial biomass (Fig. 2). The extractions that used water had higher amounts of both xylose and glucose present, which was expected since sugar is more soluble in water compared to ethanol (Alves *et al.* 2007). These sugars were likely the nonstructural sugars since the cellulose and hemicellulose are insoluble in water and ethanol (Hickey *et al.*

2021). It is worth noting that the ethanol extraction after water extraction did not contain detectable amounts of sugars in any sample and therefore, the bars for W/E and H_2O are equivalent in Fig. 2. The canola sample had a maximum amount of 2 wt% sugars extracted whereas the intermediate wheatgrass had up to 8.4 wt% of the initial sugars removed during the extraction process. This could indicate that intermediate wheatgrass contains more nonstructural carbohydrates or that the sugars in intermediate wheatgrass are more susceptible to removable and are easier to access than for the canola sample. Nonstructural carbohydrates include important transport molecules such as sucrose and storage carbohydrates such as amylose and amylopectin. Their amounts have been shown to be highly variable, fluctuating seasonally as well as daily, as sucrose is converted to starch and back (Hartman and Trumbore 2016). Amylose and amylopectin are both insoluble in ethanol; however, a small fraction of amylose can be extracted with hot water (Green *et al.* 1975).

Impact of Extraction on Major Fractions

Although it can be useful to quantify the extractives, the carbohydrates and lignin content of biomass are typically the fractions of greater interest, and extractives can interfere with the characterization of these materials (Sluiter *et al.* 2011). In the case of the carbohydrate and lignin analysis, the composition of both the intermediate wheatgrass and canola were approximately 30% glucose, 20 to 24% xylose, less than 2% arabinose, and 17 to 27% lignin depending on extraction method. Water extractions resulted in the highest amount of glucose compared to the as-received compositions (Tables 1 to 3), while changes in content of other sugars depended on the extraction method and biomass.

For the intermediate wheatgrass, t-tests showed that xylose, AIL, ASL, and total lignin were significantly different for the non-extracted and water/ethanol extracted compositions (Fig. 3C). For all variables, the non-extracted values were higher than the water/ethanol extracted biomass. The lower lignin values are similar to previous results (Thammasouk *et al.* 1997; Hickey *et al.* 2021) where Thammasouk *et al.* (1997) determined that the extracted, lower lignin values would be more accurate since the lignin values of the non-extracted biomass likely contained condensed extractives as well, which is also mentioned in the LAP document (Sluiter *et al.* 2011). Because extractives make up 20% of the intermediate wheatgrass and ash is 3% (Table 1), that certainly could be the case. Figure 3D confirms this, as it shows that for each of the extraction methods, there is a positive correlation between the difference in total lignin percent (as calculated by the as received biomass minus the extracted biomass lignin percent) and the amount of extractives. That is, the more extractive present, the greater the difference in total lignin value. However, the choice of extraction solvent did not play a significant role as for a given biomass. All extraction results were within error of each other excluding the water extraction for the Froid canola that was high due to the variability in measurements.

The significantly lower xylose percent for the water/ethanol extracted sample can likely be explained by the amount of xylose that was extracted for the intermediate wheatgrass sample, which was 7.0 wt% of the initial biomass (Fig. 2). As mentioned previously, the sugars in the intermediate wheatgrass may be more accessible than for the canola sample or more nonstructural sugars may be present. For the non-extracted versus extracted canola (Figs. 3A and 3B), there was less of a difference than with the intermediate wheatgrass for glucose and lignin values. Both canola samples had less extractives and less ash (Table 3), indicating that the lower amounts of extractives such as nitrogen containing compounds, waxes, chlorophyll, and ash present may interfere less with the subsequent

acid hydrolysis than in the case of the intermediate wheatgrass (Godin *et al*. 2011). Figure 3E shows that for each of the extraction methods, there was less of a correlation between the difference in xylose percent (as calculated by the as received biomass minus the extracted biomass xylose percent) and the amount of extractives. The difference in values seems to be based on biomass than related to amount of extractives present, with the intermediate wheatgrass having the largest difference for each of the extractions. Interestingly, for a given biomass, all extracted compositions regardless of solvent choice resulted in similar xylose differences. As reported by Hickey *et al.* (2021), since the cellulose and hemicellulose should not be soluble in ethanol, the difference is likely the removal of free mono and oligosaccharides, which may be completely removed regardless of solvent.

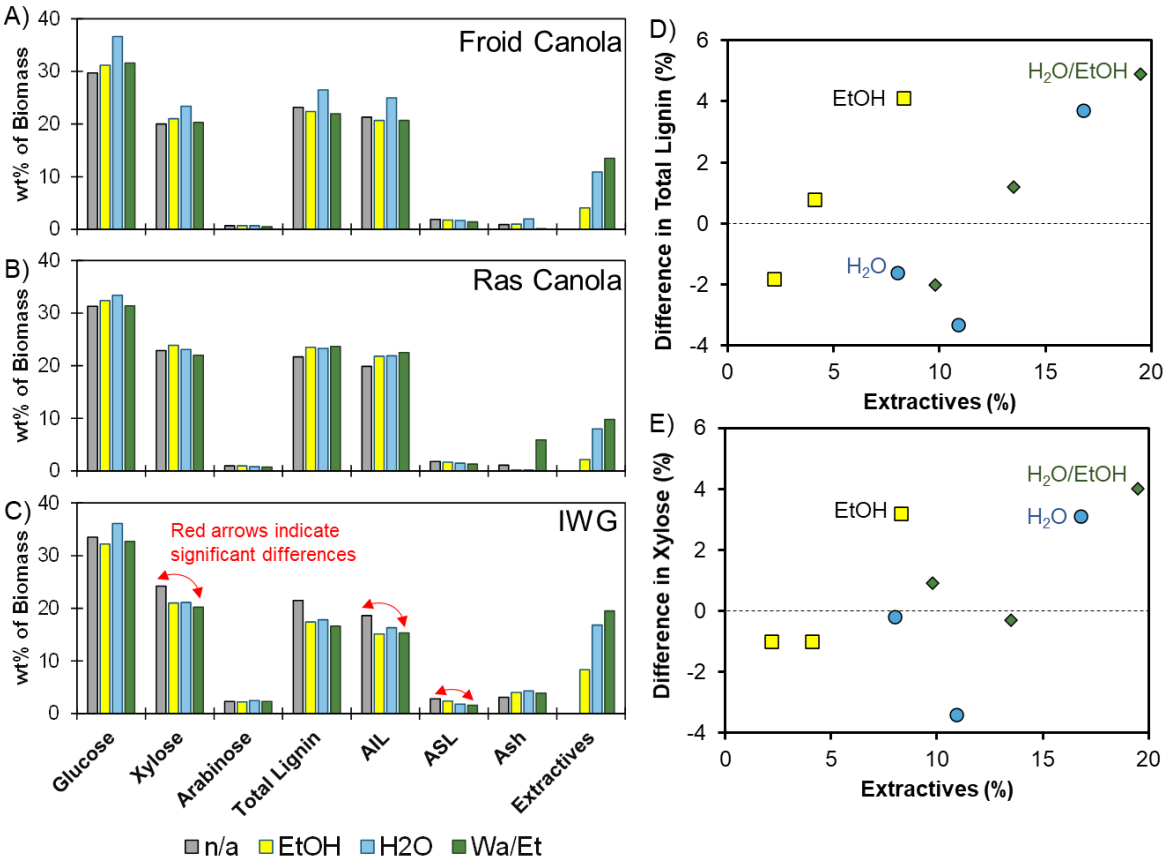

Fig. 3. Compositions of A) Froid canola, B) Ras canola, and C) intermediate wheatgrass biomass for non-extracted (■), ethanol extracted (■), water extracted (●), and water/ethanol extracted (♦) biomasses and the relationship between D) total lignin and E) xylose of the as received and extracted composition analyses. Statistical analysis was only performed on the intermediate wheatgrass due to sample size.

CONCLUSIONS

1. Intermediate wheatgrass contains more nonstructural sugars than canola, as indicated by the high amounts of sugars in the extractives after water, ethanol, and water/ethanol extractions. Therefore, in the case of biomass processing for upgrading to chemicals and fuels, the total amount of sugars should be determined by composition analysis on as received biomass with no extractions performed.
2. If the lignin composition is desired, it is suggested to perform, at minimum, an ethanol or water extraction prior to compositional analysis on intermediate wheatgrass for improved accuracy due to the amount of extractives present. For canola samples, no extraction was necessary.
3. Further studies of herbaceous biomass species should be considered to determine the effect of extractions on biomass composition. In particular, focus on the nonstructural sugars in the extraction solvent could help determine trends in which biomasses require extraction prior to composition analysis.

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