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Evaluation of Fifteen Cultivars of Cool-Season Perennial Grasses as Biofuel Feedstocks Using Near-Infrared

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ABSTRACT

Cool-season (C3) perennial grasses have a long history of cultivation and use as animal forage. This study evaluated 15 cultivars of C3 grasses, when harvested in late June for increased biomass yield, as biofuel feedstocks using near- infrared spectroscopy (NIR) based partial least square (PLS) analysis. These grasses were grown near Iliff, CO, for three growing seasons (2009–2011). The carbohydrate composition and released carbohydrates (total glucose and xylose released from dilute acid pretreatment and enzymatic hydrolysis [EH]) were predicted for samples from the study using NIR/PLS. The results were analyzed from a biofuels perspective, where composition combined with harvest yield provided information on the carbohydrate yield available for biomass conversion processes, and released carbohydrate yield provided information on the accessibility of those carbohydrates to conversion methods. The range in harvest yields varied more among cultivars (2900 kg ha^{-1}) than did the range in carbohydrate composition (56.0 g kg^{-1}) or released carbohydrates (60.0 g kg^{-1}). When comparing carbohydrate yield to released carbohydrate yield between cultivars, an efficiency as high as 87% release of available carbohydrates was obtained for pubescent wheatgrass [*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey 'Mansaka'], with a low of 71% for hybrid wheatgrass [*Elytrigia repens* (L.) *nevski ' pseudoroegneria spicata* (PURSH) A. Love 'Newhy']. Though hybrid wheatgrass had the lowest release efficiency, its high harvest yield resulted in release of more total carbohydrates than half the other cultivars analyzed. This suggested that harvest yield, carbohydrate release, and carbohydrate composition, together-play significant roles in biofuel feedstock evaluation.

Core Ideas

- Harvest yield varies more across species than sugar content and accessibility.
- Harvest yield and sugar accessibility are both critical parameters for conversion.
- Near-infrared/partial least square models are valuable for quickly evaluating biomass for bioconversion.

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Replacing petroleum gasoline with lignocellulosic-derived fuels requires significant development of biofuel feedstocks themselves, as well as logistics for harvest and transport, and optimization of the biorefining process. Biomass development requires feedstocks that can produce sufficient yields with specific qualities to maintain feedstock supplies for continuous use in biorefineries (Anderson and Akin, 2008). Parrish and Fike (2010, p. 30) proposed a list of characteristics the ideal energy crops should possess:

"Perennial (probably), High-yielding (certainly), With minimal or modest inputs (for economic and environmental/resource reasons), Readily adopted (not requiring overly novel cultural practices), Desirable feedstock qualities (with minimum of anti-quality factors), and Well-adapted to the biorefinery's locale (since biomass cannot be transported over great distances)"

They suggest that perennial species are more likely to provide consistently high yields, as well as soil protection with lower inputs, making them both economically and environmentally desirable (Parrish and Fike, 2010). Though such herbaceous feedstocks as sorghum [*Sorghum bicolor* (L.) Moench], miscanthus [*Miscanthus* × *giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize (*sacchariflorus* × *sinensis*)] and switchgrass [*Panicum virgatum* (L.)] have already garnered much attention as biofuel feedstocks, it is unlikely that only one grass, or even three, will provide a feedstock solution to all geographic regions globally or in the United States. For biofuels to be truly viable, it is far more plausible that a diverse set of crops, either in monocultures or mixtures, will need to be vetted for varying geographic regions to provide the greatest yields and ease of conversion to cellulosic fuels.

The C4 perennial grasses, miscanthus and switchgrass, have drawn significant attention as biofuel feedstocks. The C4 grasses have some of the greatest yield potentials as well as high resource use efficiency making those attractive options for further

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Abbreviations: ADF, acid detergent fiber; AS, autosampler; ASE, automated solvent extractor; CSU, Colorado State University; DM, dry matter; EH, enzymatic hydrolysis; ET, evapotranspiration; FT-NIRS, fourier transform near- infrared spectroscopy; HSD, honest significant difference; LAP, laboratory analytical procedure; NDF, neutral detergent fiber; NIRS, near-infrared spectroscopy; NREL, National Renewable Energy Laboratory; PLS, partial least squares; RMSEC, root-mean-square-error of the calibration; RMSECV, root-mean-square-error of the cross validation; RMSEP, root-mean-square-error of the prediction; SNV, standard-normal-variate.

development (van der Weijde et al., 2013). Alternatively, C3 perennial grasses have not garnered as much attention in the biofuels sector, but many potential advantages suggest they should be. The C3 perennial grasses have historically been grown as animal forages owing to their superior digestibility and high nutritive value (Burns and Fisher, 2010). Use of these crops as biomass feedstocks can take advantage of the crop development, agronomic knowledge, and production infrastructure already available. With increased production, this dual acting feedstock could promote further utilization of marginal lands by livestock producers (Sanderson and Adler, 2008). Another potential advantage of C3 perennial grasses is the availability of both forage and bioenergy markets which could reduce adoption risk to the producer compared to a crop only targeted to bioenergy outlets. Finally, a diversity of bio-energy crops and production regions can give stability and resilience to bioenergy systems (Jordan et al., 2007).

Many C3 perennial grass crops have already been selected for high digestibility and feed value. These selection criteria correlate with reduced lignin content, which may be advantageous for use of these crops as bioenergy feedstocks. Lignin, a complex structural phenolic polymer, impedes hydrolyses of cellulose and hemi-cellulose for fermentation (Anderson and Akin, 2008). Bioenergy crops with reduced lignin have potential for greater conversion efficiencies (Sarath et al., 2008). Because of this, efforts are underway to reduce lignin in bioenergy target species like switchgrass (Pedersen et al., 2005). Another approach is to increase the polysaccharide to lignin ratio (Lorenz et al., 2009). The C3 grass species utilized for forage have already been selected for these qualities.

An analysis of animal feed quality often consists of neutral detergent fiber (NDF), acid detergent fiber (ADF), and crude protein. While these forage analysis methods have some utility in evaluating biomass for bioconversion, a more detailed knowledge of cell wall composition and recalcitrance toward bioconversion processes is generally needed (Udén et al., 2005; Wolfrum et al., 2009; Vogel and Jung, 2001). The cell wall polysaccharides cellulose and hemicellulose, as glucan and xylan are of greatest interest as the two most abundant structural carbohydrates in herbaceous feedstocks for chemical conversion to ethanol or other value added products (Per, 1993). As previously described, an understanding of lignin content can provide an estimation of recalcitrance or a plants natural ability to resist extraction of these valuable carbohydrates (Jung et al., 1992; Johnson et al., 1995; Thammasouk et al., 1997). While rumen digestibility or organic matter digestibility can provide information about biomass recalcitrance a more tailored approach to screen feedstocks for susceptibility to deconstruction is useful. One specific and readily used deconstruction process incorporates dilute acid pretreatment of the biomass to solubilize hemicellulose; followed by enzymatic hydrolyses of the cellulose. Deconstruction assays which measure released carbohydrates, defined as the total sugar yield of glucose and xylose from dilute acid pretreatment and enzymatic hydrolysis, provide information about the accessibility of these cell wall carbohydrates for biochemical conversion. An understanding of both composition and released carbohydrate yield is valuable for feedstock development and successful biofuels production; although a feedstock may be high in glucan and xylan, those carbohydrates may not be readily accessible for deconstruction as influenced by the lignin composition (Sims et al., 2010; Sharma et al., 2013; Fiorese et al., 2014; Brethauer and Studer, 2015).

In addition to more tailored wet chemical methods, the use of NIR/PLS to predict composition and biomass deconstruction outcomes is well established in the literature (Vogel et al., 2010; Lindedam et al., 2010; Huang et al., 2012; Wolfrum et al., 2013b; Xu et al., 2013; Hames et al., 2013; Sluiter and Wolfrum, 2013; Payne and Wolfrum, 2015). These high throughput methods provide a useful means by which a small number of biomass samples can be evaluated by wet chemical methods and then used to predict a much larger number of samples of a similar species or population. Well-developed NIR models for single plant species often require hundreds of samples. However, multi-species models can circumvent this issue by incorporating a smaller number of plants from a given species as well as capitalize on additional information from different yet similar plant species. Multi-species models have been developed and are in use for both woody and herbaceous feedstocks (Mika et al., 2003; Adler et al., 2009; Chataigner et al., 2010; Hodge and Woodbridge, 2010; Monono et al., 2012; Dale et al., 2013).

The South Platte Irrigation Research and Demonstration Project in Iliff, CO, was developed by the Department of Crop and Soil Sciences, Colorado State University (CSU) to investigate water conserving crop systems on an irrigated study site intended for animal forage. The 3.5 ha (8 acre) study area was planted with 15 perennial cool-season grass cultivars (Table 1), representing 12 species, to evaluate the effect of different irrigation strategies and harvest times on the grass cultivars. The 15 C3 perennial grasses were chosen based on their productivity under limited irrigation typical to eastern Colorado. These grasses reach their peak maturity in early summer, and as the plants begin to senesce, late-season harvest dates could have lower biomass yields (Niu et al., 2005). As yield is an important factor for both animal forage and biofuels feedstock production, an early spring harvest corresponding to specific production phases of the selected C3 grasses was necessary. An early June harvest would correspond to the boot to early heading stage of the plants maturity, depending on the individual species. This strategy would seek to harvest the grasses at high nutrition (crude protein) and digestibility (low NDF and ADF) as it pertains to animal feed. In contrast, a late June harvest, corresponded to the mid- to late heading stage of the plant, would seek to maximize dry matter yield and structural carbohydrate composition (cellulose and hemicellulose) as well as a likely and important reduction in crude protein.

We examined the 15 cool-season perennial grass cultivars for three growing seasons using the late June harvest date, which was the nontraditional harvest date for animal feed and sought to maximize biomass yield over animal feed quality. The primary objective of this work was to compare the results of carbohydrate yield based on sample composition to released carbohydrate yield based on a laboratory-scale deconstruction assay. This compares the herbage production of each grass cultivar for composition as it related to structural glucan and xylan within the plant, to the herbage production of the grass as it reacted to dilute acid pretreatment and EH, as an average of 3 yr. We chose to average the data over 3 yr because we were more interested in illustrating the differences in cultivars between these two important biofuels measurements than a focus on individual yearly performance. This study demonstrates the need for developing biomass feedstocks with sizeable carbohydrate content but tempered with a consideration

Table 1. Species and cultivar of cool-season, perennial grasses cultivated for the South Platte Irrigation Research and Demonstration Project near Iliff, CO.

Species	Cultivar	Scientific name	Acronym
Crested wheatgrass	Hycrest	*Agropyron cristatum × desorturum*	CWG
Hybrid wheatgrass	Newhy	*Elytrigia repens* (L.) *nevski × pseudoroegneria spicata* (PURSH) A. Love	HWG
Intermediate wheatgrass	Beefmaker	*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey	IWG
Pubescent wheatgrass	Manska	*Thinopyrum intermedium*	PWG
Slender wheatgrass	San Luis	*Elymustrachycaulus* ssp. *trachycalaus*	SWG
Tall wheatgrass	Jose	*Thinopyrum ponticum*	TWG
Western wheatgrass	Rosana	*Pascopyrum smithii*	WWG
Experimental meadow brome	Nebraska (BR-0317-28)	*Bromus biebersteinii* Roem. & Schult	EMB
Smooth brome	Newell	*Bromus inermis* Leyss.	SB
Hybrid brome	Bigfoot	*Bromus inermis × B. biebersteinii*	HB
Meadow brome	Cache	*Bromus biebersteinii* Roem. & Schult	MB1
Meadow brome	Montana	*Bromus biebersteinii* Roem. & Schult	MB2
Russian wildrye	Bozoisky-Select	*Psathyrostachys junceus*	RWR
Tall fescue	Fawn (endophyte-Free)	*Schedonorus arundinaceus* (Schreb.) Dumort	TFF
Tall fescue	Jessup MaxQ	*Schedonorus arundinaceus* (Schreb.) Dumort	TFQ

of recalcitrance or accessibility of those carbohydrates to a specific deconstruction process. Both of these measurements must then be paired with the contribution of harvest yield as measured in kilograms per hectare.

A second objective of this work was to show the practical application of developing robust multi-species NIR/PLS multivariate calibrations for screening of large sample sets for cell wall composition and extractable sugar yields. Instead of developing several single species models we were able to perform chemical analysis on a smaller select set of samples, add them to a well-developed multi-species herbaceous feedstock model and quickly obtain predicted information on hundreds of samples. Because chemical analyses can be expensive and time consuming, breeding programs and field studies will require these tools to quickly evaluate the large numbers of samples they often generate to determine the best plants for further study and optimization as well as best field practices.

MATERIALS AND METHODS

Field Experiment

A field experiment was established in 2008 at the South Platte Irrigation Research and Demonstration Project near Iliff, CO (40°45′ N, 103° 3′ W, elevation 1165 m). Soils at the site were classified as Loveland clay loam (fine-loamy over sandy or sandy-skeletal, mixed, superactive, calcareous, mesic Fluvaquentic Endoaquoll) and Nunn clay loam, wet (fine, smectitic, mesic Aridic Argiustoll). Soil analysis prior to establishing the grasses indicated levels of P(31 mg kg^{-1} by $NaHCO_3$ extraction) and exchangeable K (902 mg kg^{-1}) were sufficient for high yields without fertilization. The experimental design was a split-plot within a randomized complete block with three replications. Main plots consisted of 15 cultivars of cool-season forage grasses (Table 1) that were seeded in the spring of 2008 using a no-till drill (Model 3P605NT, Great Plains Mfg., Inc., Salina, KS) with 17 cm row spacing. Each main plot was split in half with one subplot harvested about 1 June and the other 3 wk later. Main plots measured 4.6 by 18.3 m, and subplots 2.3 by 18.3 m. Plots were irrigated using a linear-drive sprinkler system, and the irrigation schedule was determined based on local calculations of evapotranspiration (ET) for irrigated forage grasses (CoAgMet, 2011). Irrigation totaled 260, 400, and 80 mm in 2009, 2010, and 2011, respectively. Nitrogen (as urea) was applied uniformly across all plots during the spring of each growing season at a rate of 90 kg N ha^{-1}. The grasses were allowed to establish during the 2008 growing season with data collection beginning in 2009.

For this study, only samples from the second harvest date (third week of June) were analyzed. At this point, all of the grasses were in the late reproductive to early seed development stages of growth (i.e., peak biomass production) except for tall wheatgrass which matures later and was only in the early heading growth stage. Plots were harvested in June of 2009, 2010, and 2011 using a Lacerator Green Chopper (Gruetts Inc., Potter, WI) with an attached weigh bin to collect forage from a 1.5 by 16.5 m area of each plot. Grasses were harvested at a 10 cm cutting height. A subsample of approximately 600 g was collected from each plot as harvested materials entered the weigh bin using a net to capture a random sample along the entire plot length.

Sample Preparation and Study Sample Selection

Subsamples were dried at 55°C for a minimum of 72 h in a forced-air oven to determine dry matter (DM) content. The yield of each plot was then determined by multiplying the DM content of each subsample by the wet plot weight and converting to a kg ha^{-1} basis. Samples were then milled to a 2 mm particle size using a shear mill (Wiley Model 4, Arthur H. Thomas Co., Philadelphia, PA) and then homogenized using a cyclone mill (Cyclotec Model 1093, Foss Corp., Eden Prairie, MN). Prepared samples were then placed in sealed plastic containers and stored at room temperature until analyzed.

For the study reported in this manuscript, focus was placed on 135 samples consisting of the 15 grass cultivars, over three harvest years, and for three individual sample replicates for each harvest year. Irrigation and harvest date were kept constant using full season irrigation and a late June harvest. The composition (glucan, xylan, and lignin) and carbohydrate release data for these samples were generated using the NIRS-based mixed herbaceous feedstock models described below. None of the 135 samples was directly analyzed by wet chemical means.

Calibration Samples and Model Development

Samples ($n = 58$) were selected for use as calibration or validation samples to develop robust NIRS-based PLS mixed herbaceous feedstock models for composition and carbohydrate release from pretreatment and EH. These samples were different from the 135 samples previously described and measurements from the 58 samples were not used as part of the subsequent evaluation of individual cultivars, but only used to develop the models. These 58 samples represented the three harvest years, early and late June harvests as previously described, full season irrigation, and included the following species: tall wheatgrass referred to as TWG [*Thinopyrum ponticum* (Podp.)Z.–W. Liu & R.–C. Wang 'Jose'], meadow brome referred to as MB1 [*Bromus biebersteinii* Roem. & Schult 'Cache'], tall fescue endophyte free referred to as TFF [*Schedonorus arundinaceus* (Schreb.) Dumort (endophyte free)], russian wildrye referred to as RWR [*Psathyrostachys junceus* (Fisch.) Nevski 'Bozoisky-Select'], pubescent wheatgrass referred to as PWG, crested wheatgrass referred to as CWG [*Agropyron cristatum* × *desorturum* (Fisch. ex Link) J.A. Schultes 'Hycrest'], western wheatgrass referred to as WWG [*Pascopyrum smithii* (Rydb.) Á. Löve 'Rosana'], and smooth brome referred to as SB [*Bromus inermis* Leyss. 'Newell']. Field replicates were combined to generate a single composite sample for analysis. These composite samples were scanned by FT-NIRS and subjected to compositional analysis and carbohydrate release assay as dried at 55°C. Though the objective of this work was to analyze samples from a late June harvest, samples from both the early and late June harvest dates were included to increase the robustness of the resulting models.

A mixed herbaceous feedstock model for the prediction of structural glucan and xylan, as well as lignin and ash was developed. Additional details as well as calibration and validation statistics for this predictive model are reported in Supplemental File 1. The mixed herbaceous feedstock model used to predict carbohydrate release was identical to that of Payne and Wolfrum (2015). Further details on the development of this model including validation and calibration statistics are reported in that publication. In summary, partial least square multivariate calibrations were developed using Unscrambler X 10.3 (UnscramblerX, 2013) software. A PLS-1 model was developed to predict the sum of glucose and xylose released following pretreatment and enzymatic hydrolysis. A PLS-2 model was developed to predict composition which included glucan, xylan, lignin, and ash. A PLS-1 model relates a single independent variable such as glucose/xylose release to the dependent variable, in this case the spectra. A PLS-2 model relates more than one independent variable, such as the constituents for composition, to the dependent variable.

Composition and Carbohydrate Release

The 58 samples selected for compositional analysis were analyzed using the NREL suite of Laboratory Analytical Procedures (LAP) (National Renewable Energy Laboratory, 2014). The history and uncertainties associated with these methods have also been published (Sluiter et al., 2010; Templeton et al., 2010). Cell wall or structural glucan and xylan as well as lignin were determined using these methods along with the free sugars sucrose and glucose. Measured constituents were corrected to a dry weight basis and were reported as g kg^{-1}.

As previously mentioned soluble free sugars were measured using the NREL LAPs. The 58 samples were also analyzed for starch composition using the Megazymes Total Starch assay (AOAC Method 996.11, AACC Method 76.13, and ICC Standard method no. 168) (Megazyme International, 2016). These measurements were meant to assess the contribution these sugars might have to a carbohydrate release assay. The average starch composition with standard deviation was 8.4 ± 0.10 g kg^{-1} and the glucose composition (from free glucose and sucrose) was13.3 ± 5.9 g kg^{-1}. These contributions were considered negligible and were excluded from the sum of carbohydrates for composition. Samples analyzed for composition were also subjected to a high throughput carbohydrate release assay developed by Wolfrum et al. (2013a). This assay included a dilute sulfuric acid pretreatment followed by enzymatic hydrolysis. The acid pretreatment was performed using an ASE 350 (Dionex, Sunnyvale, CA) at a constant temperature of 130°C for 7 min using 3.0 g of sample and 30 mL of a 0.01 g kg^{-1} sulfuric acid solution. The enzymatic hydrolysis assay was similar to the NREL LAP for enzymatic hydrolysis (National Renewable Energy Laboratory, 2014). Carbohydrates released through this sequential assay were calculated as a sum of glucose and xylose released following pretreatment and enzymatic hydrolysis. This was defined as the mass of carbohydrates released, sum of glucose and xylose, per unit of dry biomass. A more detailed description of this calculation and its assumptions is provided by Wolfrum et al. (2013a).Yields were defined as the product of carbohydrates released (g kg^{-1}) and harvest yield (kg ha^{-1}).

Near-Infrared Spectroscopy

Both the 58 sample set for model development and the 135 samples that were predicted with the model were scanned at a 2 mm particle size and less than 5% moisture using a Thermo Antaris II FT-NIR spectrometer. Samples were scanned in duplicate using the 40 place autosampler carousel which used disposable 2 dram borosilicate vials for sample presentation. Duplicate scans were averaged for prediction. Each sample scan was an average of 128 scans using the wavenumber range of 3300 to 12000 cm^{-1} with a resolution of 8 cm^{-1} (3.857 cm^{-1} data spacing).

All spectra were mathematically preprocessed prior to use. Spectra were first transformed using the standard-normal-variate (SNV) to correct for light scatter and variations in particle size. Following SNV, the Savitzky–Golay first derivative, second order polynomial, with 21 point smoothing was applied to the spectral data to correct for baseline variations (Savitzky and Golay, 1964). The spectral range was reduced to 4000 to 8000 cm^{-1}. This was done to eliminate spectral regions associated with increased noise that did not contribute significantly to composition and total carbohydrate release model development.

Data Analysis

Analysis of variance using Tukey Honest Significant Difference (TukeyHSD) was performed using the open source statistical software package R (R Development Core Team, 2013) and the *agricolae* package to compare harvest yield, carbohydrate composition, carbohydrate yield, released carbohydrate yield, lignin composition, and the difference between carbohydrate yield and release yield for all 15 cultivars. The experimental design was a randomized complete block design with three replications. Main effects included in the analysis of variance were cultivar and year, where cultivar and year were considered fixed effects and field replication considered a random effect. Mean differences were considered significant at $\alpha = 0.05$.

Other statistical comparisons were calculated in the Minitab 16.2.4 statistical software package (Minitab, 2010) which included *t* tests and correlation coefficients. Statistical differences between carbohydrate yield and released carbohydrate yield for a single species were determined by a paired *t* test. The Pearson product moment correlation coefficient was used to determine correlations between lignin and carbohydrate yield and released carbohydrate yield. Mean differences and correlations were considered significant at $\alpha = 0.05$.

To further compare trends in sample populations, TWG, PWG, CWG, WWG, intermediate wheatgrass or IWG [*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey 'Beefmaker'], HWG, and slender wheatgrass or SWG [*Elymustrachycaulus* (Link) Gould ex Shinners ssp. Trachycalaus 'San Luis'] were assigned to the wheatgrass class of cool-season grasses. Smooth brome, MB1, meadow brome or MB2 [*Bromus biebersteinii* Roem. & Schult 'Montana'], hybrid brome or HB [*Bromus inermis ′ B. biebersteinii* Leyss. and Roem. & Schult 'Bigfoot'], and experimental meadow brome or EMB [*Bromus biebersteinii* Roem. & Schult 'Nebraska' (BR-0317-28)] were assigned to the brome class of grasses; TFF and tall fescue Jessup MaxQ or TFQ [*Festuca arundinacea* Schreb. 'Jessup MaxQ'] were assigned to the fescue class, and the single sample RWR, assigned to the wildrye class of grasses.

RESULTS

Harvested biomass yields were measured for the 15 grass cultivars for each of three growing seasons. Year ($F_2 = 36.4$, $P < 0.001$) and cultivar ($F_{14} = 11.9$, $P < 0.001$) were statistically significant for harvest yield, however the cultivar × year interaction was not significant ($F_{28} = 0.93$, $P = 0.57$). This was likely due to differences in weather among years, especially precipitation. Annual precipitation was 281, 380, and 492 mm in 2009, 2010, and 2011, respectively. While 2009 and 2010 were both drier years than the long-term average (450 mm), 2011 was wetter than average with an especially wet spring. The result of individual plots over 3 yr were averaged for each of the 15 grass cultivars and are provided in Fig. 1. Certain classes of grass were more productive than others and there was a statistically significant difference among cultivars, as previously stated with an HSD value of 1161 kg ha^{-1}. The wheatgrasses were the most productive while the fescues were the least productive. Tall wheatgrass, SB, IWG, HWG, CWG, PWG, and SWG were the highest yielding cultivars in that order and were not significantly different from each other with a range of 5946 to 5011 kg ha^{-1}. The lowest yielding cultivars were the two fescues (TFQ 3732 kg ha^{-1} and TFF 3050 kg ha^{-1}) which were not significantly different from one another, but significantly different from the highest yielding group of cultivars. Most species of wheatgrass fell into the highest yielding species range except WWG at 4418 kg ha^{-1}. The brome cultivars were all similarly mid- to low yielding with a range of 4626 to 4386 kg ha^{-1}, with the exclusion of SB at 5777 kg ha^{-1} which was statistically significantly higher.

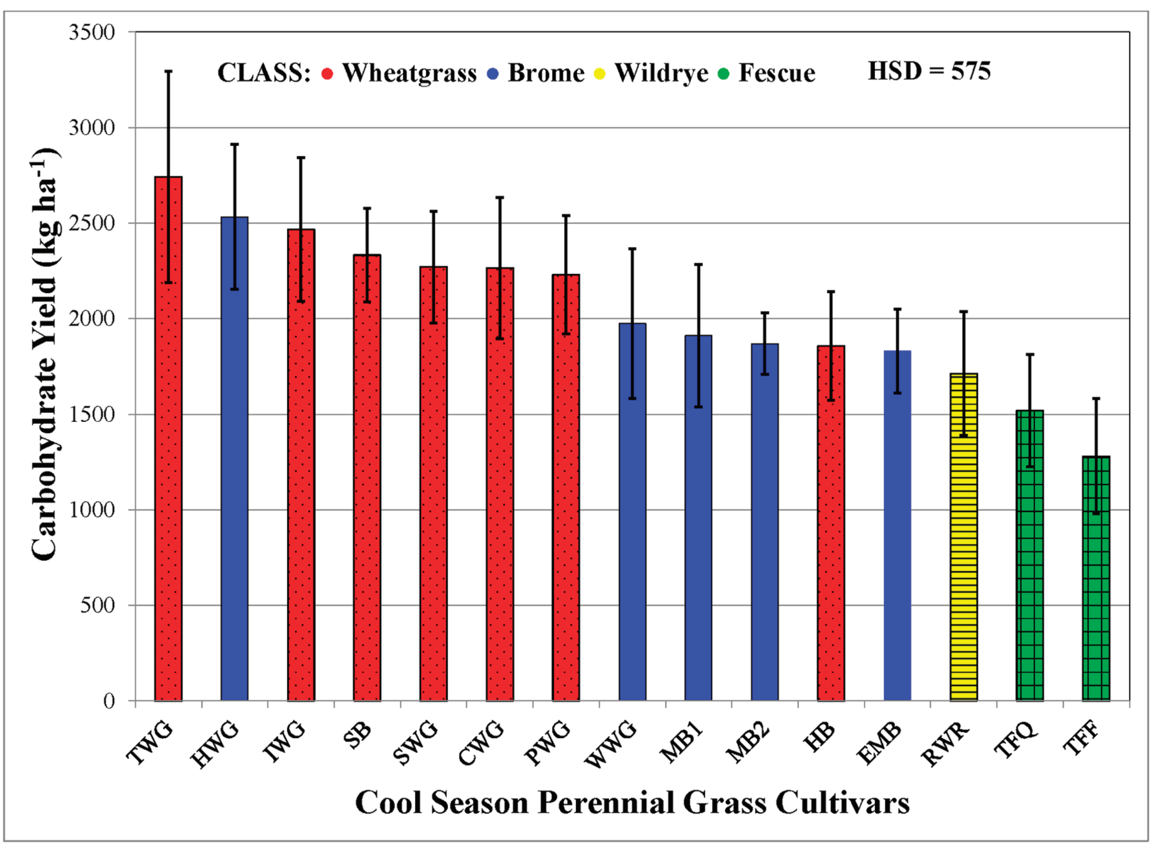

Fig. 1. Harvest yield for 15 cultivars of cool-season grasses averaged over 3 yr. The graph lists the individual grass cultivars by acronym provided in Table 1 and colors them based on the four represented classes. The yield of each cultivar was an average of nine plots, across 3 yr (2009, 2010, and 2011). All plots were harvested in late June of each year. Tukey's honest significant difference among cultivars was reported ($\alpha = 0.05$). Error bars correspond to a 95% confidence interval.

Figure 2 illustrates the mean carbohydrate composition of the 15 grass cultivars as the sum of glucan and xylan reported on a gram per kilogram dry weight basis. They were reported without anhydro correction to indicate their structural polymeric cell wall origin and were different than free or soluble carbohydrates, which were considered negligible in this study. The range in carbohydrate composition between the highest and lowest cultivars was 56 g kg^{-1}, with TWG possessing the highest content at 458 g kg^{-1} and HB possessing the lowest at 402 g kg^{-1}. There were statistically significant differences among cultivars ($F_{14} = 10.7, P < 0.001$) and year ($F_2 = 6.5, P = 0.002$) with an HSD of 30 g kg^{-1}. There was also a significant cultivar × year interaction ($F_{28} = 2.1, P = 0.004$). Similar to the ranking of harvested biomass yields, the grasses largely separated by class with the wheatgrasses possessing the highest average carbohydrate content. Species TWG, HWG, SWG, WWG, and IWG, as a group were significantly higher in carbohydrate composition than most other species, and TWG, HWG, and SWG were higher than all other brome, fescue, and wildrye cultivars.

The product of carbohydrate composition and harvest yield, referred to here as carbohydrate yield, is illustrated in Fig. 3. Carbohydrate yield was the sum of glucan and xylan multiplied by the harvest yield which was measured in kg ha^{-1} and averaged across the three growing seasons. There were statistically significant differences among cultivars ($F_{14} = 11.7, P < 0.001$) and year ($F_2 = 30.4, P < 0.001$) with an HSD of 575 kg ha^{-1}. The cultivar × year interaction was not significant ($F_{28} = 1.0, P = 0.49$). Again, the wheatgrasses demonstrated some of the greatest carbohydrate yields. The highest carbohydrate yielding cultivars were TWG, HWG, IWG, SB, SWG, CWG, and PWG with a range of 2742 to 2231 kg ha^{-1} of carbohydrates respectively, and were not statistically significantly different from one another. Experimental meadow brome, RWR, TFQ, and TFF filled out the bottom of the list and were not statistically different from one another at 1831, 1713, 1520, and 1280 kg ha^{-1} of carbohydrates.

Figure 4 illustrates the combined result of average carbohydrate release and harvest yield, where the sum of glucose and xylose released from pretreatment and EH was multiplied by the harvest yield and averaged across the three growing seasons. Referred to here as released carbohydrate yield, results were reported by grass cultivar and class. There were statistically significant differences among cultivars ($F_{14} = 17.7, P < 0.001$) with an HSD of 485 kg ha^{-1} carbohydrates released. Year was also significant ($F_2 = 35.4, P < 0.001$), however the cultivar × year interaction was not significant ($F_{28} = 1.0, P = 0.43$). Tall wheatgrass, IWG, SB, and PWG were the highest yielding cultivars for release with a range of 2626 to 2247 kg ha^{-1} and were not significantly different from one another. Russian wildrye, TFQ, and TFF demonstrated the smallest amounts of released carbohydrates with a range of 1520 to 1098 kg ha^{-1} and were not significantly different from one another. There was some re-ordering of rank for individual species between carbohydrate yields and released yields, but only two individual species demonstrated any substantial difference in ranking. Hybrid wheatgrass had the second highest carbohydrate yield at 2533 kg ha^{-1} but, only the fifth highest carbohydrate release yield at 2107 kg ha^{-1}, while PWG had the seventh highest carbohydrate yield at 2231 kg ha^{-1} with the fourth highest release of those carbohydrates at 2247 kg ha^{-1}.

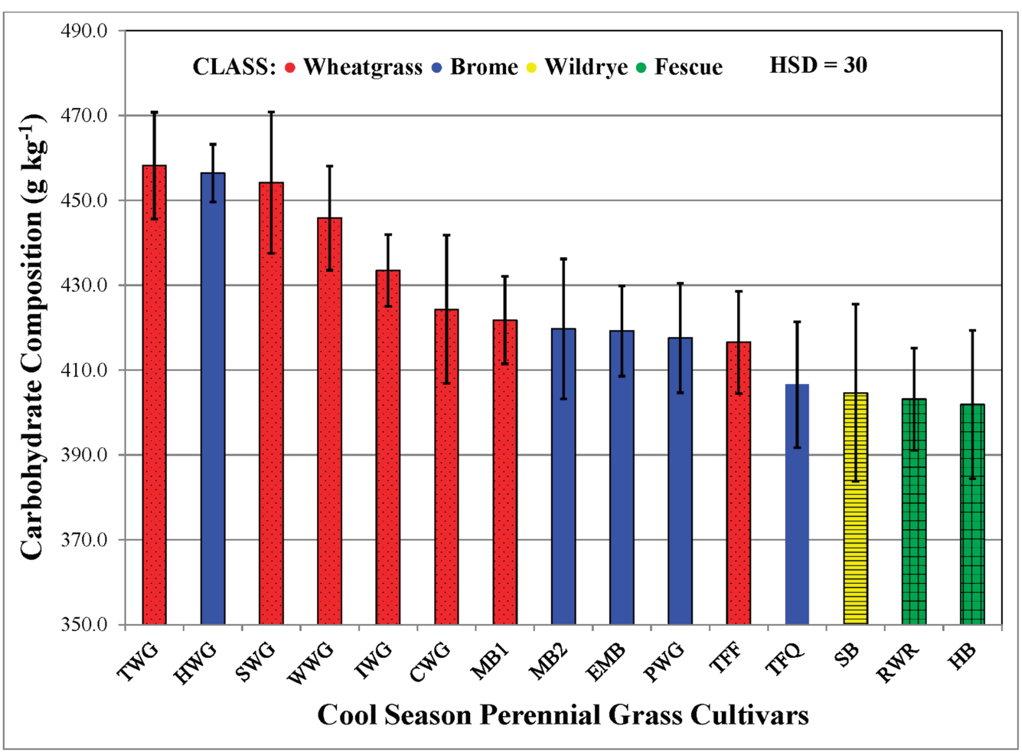

Fig. 2. Mean carbohydrate (glucan and xylan) composition of 15 cultivars of cool-season grasses. The graph lists the individual grass cultivars by the acronyms provided in Table 1 and colors them based on the four represented classes. The carbohydrate composition of each cultivar was an average of the sum of glucan and xylan predicted for nine plots, across 3 yr (2009, 2010, and 2011) for each cultivar. Carbohydrate composition was reported on a g kg^{-1} dry weight basis and "glucan" and "xylan" refer to the structural (cellulose and hemicellulose) carbohydrates quantified. Tukey's honest significant difference among cultivars was reported ($\alpha = 0.05$). Error bars correspond to a 95% confidence interval.

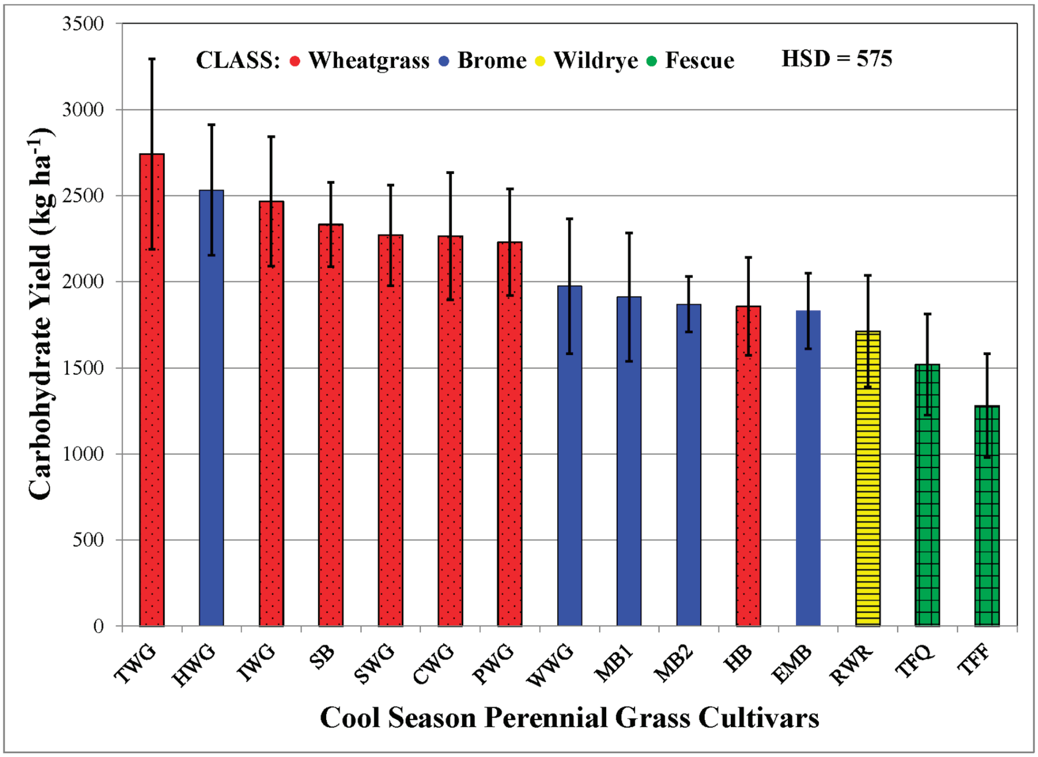

Fig. 3. Carbohydrate (glucan and xylan) Yield (kg ha^{-1}) for 15 cultivars of cool-season grasses. The graph lists the individual grass cultivars by the acronyms provided in Table 1 and colors them based on the four represented classes. The carbohydrate yield of each cultivar was the product of carbohydrate composition (sum of glucan and xylan) and harvest yield. For each cultivar, this was an average across nine plots and 3 yr (2009, 2010, and 2011). Tukey's honest significant difference among cultivars was reported ($\alpha = 0.05$). Error bars correspond to a 95% confidence interval.

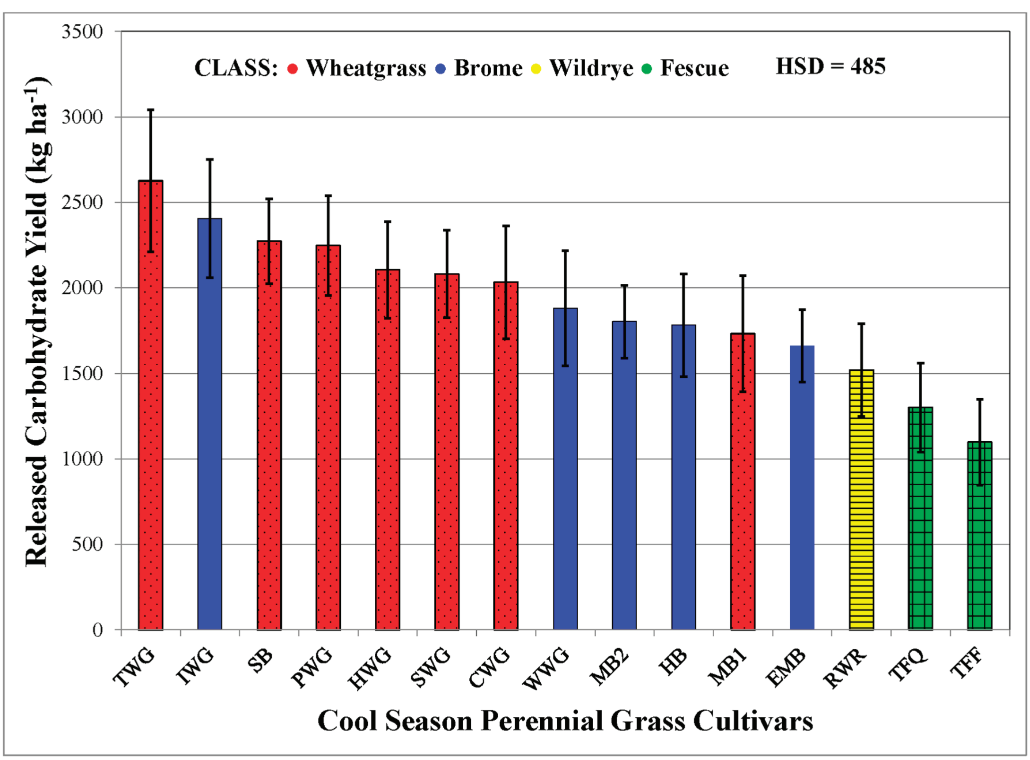

Fig. 4. Released carbohydrate (glucose and xylose) Yield (kg ha^{-1}) for 15 cultivars of cool-season grasses. The graph lists the individual grass cultivars by the acronyms provided in Table 1 and colors them based on the four represented classes. The released carbohydrate yield of each cultivar was the product of carbohydrate release (sum of glucose and xylose), from pretreatment and enzymatic hydrolysis, and harvest yield. For each cultivar, this was an average across nine plots and 3 yr (2009, 2010, and 2011). Tukey's honest significant difference among cultivars was reported ($\alpha = 0.05$). Error bars correspond to a 95% confidence interval.

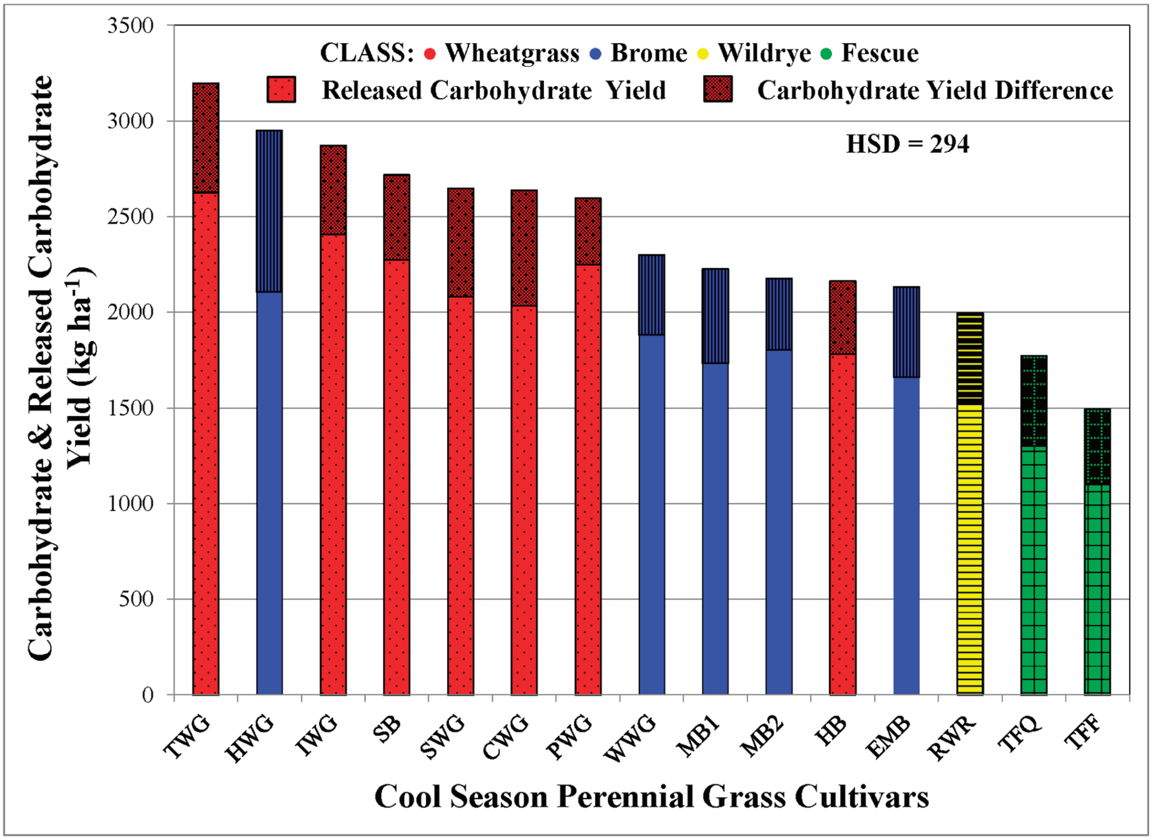

Fig. 5. Comparison of carbohydrate yield and released carbohydrate yield (kg ha^{-1}) for 15 cool-season grasses. The graph lists the individual grass cultivars by the acronyms provided in Table 1. Previously, carbohydrate yield was reported as the product of predicted glucan and xylan composition, and yield. For the purpose of comparison to release, glucan and xylan have been converted to glucose and xylose and their sum multiplied by harvest yield. Released carbohydrate yield of each cultivar was the product of predicted glucose and xylose release from pretreatment and enzymatic hydrolysis and harvest yield. For each cultivar, this was an average across nine plots and 3 yr (2009, 2010, and 2011). Tukey's honest significant difference for the difference between the carbohydrate yield and release yield among cultivars was reported (α = 0.05).

Figure 5 directly compares carbohydrate yield to released carbohydrate yield for each cultivar. Carbohydrate composition (g kg^{-1}) and carbohydrate yield (kg ha^{-1}) were previously reported on an anhyro basis (glucan and xylan) in Fig. 2 and 3. However, carbohydrate yield was converted to glucose and xylose for Fig. 5. This was necessary to support a direct comparison to carbohydrate release yields which were reported on a hydrated basis. All cultivars demonstrated statistically significant differences at the 95.0% confidence level between their carbohydrate yield and their released carbohydrate yield after deconstruction assay. There were statistically significant differences among cultivars (F_{14} = 4.3, P < 0.001) for the magnitude of this difference with an HSD of 294 kg ha^{-1}. Year was also significant (F_2 = 29.2, P < 0.001), however, the cultivar × year interaction was not significant (F_{28} = 1.3, P = 0.20). The average difference and standard deviation between carbohydrate yield and release across cultivars was 488 ± 124 kg ha^{-1}.

As illustrated in Fig. 5, HWG resulted in the largest difference between composition and release at 844 kg ha^{-1} which equated to a 71% release of available carbohydrate content. The HWG was also significantly higher than most other cultivars as a group for this difference, excluding CWG, TWG, and SWG. These four species were not significantly different from one another with a range of 844 to 565 kg ha^{-1} difference in carbohydrate yield to released carbohydrate yield. The PWG had the smallest difference at 348 kg ha^{-1}, equating to an 87% release of its carbohydrate composition. It was important to compare these differences as a percentage of the total carbohydrate yield or the percentage of carbohydrates released and not just as an absolute difference between two measured values. Based on the HSD value of absolute difference, PWG was only significantly different and lower than one other species, HWG. The HWG was the species with the greatest difference at 844 kg ha^{-1}. While HWG had the greatest absolute differences, HWG, TFQ, and TFF at 71, 73, and 74% had the lowest percentage of carbohydrates released based on total carbohydrate herbage production, with TFQ (471 kg ha^{-1}) and TFF (394 kg ha^{-1}) having smaller absolute differences by comparison. There were significant differences among cultivars (F_{14} = 7.4, P < 0.001) for the ratio of released carbohydrate yield to the total carbohydrate yield with an HSD of 8%. Year was also significant (F_2 = 38.5, P < 0.001), however the cultivar × year interaction was not significant (F_{28} = 1.4, P = 0.12). The PWG had the highest released carbohydrate yield efficiency at 87%. However, based on HSD, it was not significantly different than SB, IWG, TWG, WWG, MB2, HB, and SWG, with a released carbohydrate yield efficiency range of 84 to 79%, respectively.

To further explain trends in the data, correlations were investigated between different variables. There was a moderate negative correlation between lignin composition and carbohydrate release (r = −0.398, P < 0.001). There was a moderate positive correlation between lignin composition and the difference between carbohydrate composition and carbohydrate release ($r = 0.420$, $P < 0.001$). There was a strong positive correlation between lignin yield and released carbohydrate yield ($r = 0.905$, $P < 0.001$).

DISCUSSION

The primary objective of this work was to compare the carbohydrate composition of a set of cool-season grasses to their practically accessible carbohydrates using a specific method of biomass deconstruction. This was accomplished by analyzing a relatively small number of cool-season grass samples, 58, over 3 yr, harvested in June, using primary methods. These primary methods included laboratory analytical procedures for composition and a dilute acid pretreatment and EH assay for deconstruction or carbohydrate release. This data along with NIR spectra of the 58 perennial C3 grass samples was integrated into a large data set of herbaceous feedstocks to develop NIR-based PLS models to predict composition and carbohydrate release. These models were then used to predict the composition and release of a larger population, 135 samples, of cool-season grasses using only the late June harvest. The performance of these models, including validation and calibration statistics and descriptive statistics of the sample population, is outlined in greater detail in Supplemental File 1 for composition and Payne and Wolfrum (2015) for carbohydrate release. These predicted values were then combined with measured dry matter harvest yields to evaluate the potential of these grasses as biofuels feedstocks and to highlight the utility of this process for feedstock evaluation.

There was much greater variability in harvest yield across cultivars than in carbohydrate (glucan and xylan) composition or accessibility of those carbohydrates to conversion. Differences in yield between cultivars were as much as 3000 kg ha^{-1} between the highest and lowest producers, TWG (5946 kg ha^{-1}) and TFF (3050 kg ha^{-1}), and as little as 11 kg ha^{-1} between CWG (5337 kg ha^{-1}) and PWG (5326 kg ha^{-1}). For TWG and TFF, this equated to a difference in yield of about 50%. The average cultivar yield and standard deviation was 4800 ± 800 kg ha^{-1}. In terms of average carbohydrate composition of the individual cultivars, there was less than a 60 g kg^{-1} difference between the highest (TWG) and lowest (TFF) cultivars with the average carbohydrate composition and standard deviation being 426 ± 20 g kg^{-1}.

Carbohydrate yield provides information about what is theoretically possible to derive from these particular grasses. Carbohydrate yield is not necessarily what can be practically converted with any particular bioconversion process. For an indication of how accessible these carbohydrates were to conversion to monomeric carbohydrates, samples were subjected to a carbohydrate release assay. Carbohydrate release revealed similarly narrow results with an average carbohydrate release and standard deviation of 390 ± 30 g kg^{-1}. The wheatgrass class of grasses had the greatest carbohydrate yields, but this was largely a result of each individual species substantial harvest yield. Therefore, yield had a significant influence on interpretation of the results when calculated as carbohydrate yield and released carbohydrate yield. This was consistent with data presented by Monono et al. (2013) for individual and mixed herbaceous species grown in North Dakota using irrigated and non-irrigated plots. They concluded that dry matter yields were the main driver behind differences in ethanol yield potentials. Of particular interest, the C3 species tall wheatgrass, intermediate wheatgrass and wildrye were assessed among others including the C4 switchgrass species. For their work switchgrass outperformed other species for biomass yields in irrigated plots, while C3 grasses outperformed switchgrass on non-irrigated plots. This continues to lend merit to the need for well-studied suitable species for specific geographic locations were water resources are low.

The greatest difference, when comparing carbohydrate yield to released carbohydrate yield within a single cultivar, was exhibited by HWG at a difference of 844 kg ha^{-1} of carbohydrates. In this case only 71% of the carbohydrates in this particular cultivar were released by dilute acid pretreatment and EH. Russian Wildrye, TFF, and TFQ had similarly low and not statistically significantly different percentages of released carbohydrate yields at 74, 73, and 72%, respectively. Though HWG had the least accessible carbohydrates according to this study, its high harvest yield was enough to result in release of more total carbohydrates than half the other cultivars analyzed. In this case, yield overcame recalcitrance and highlights the importance of the yield contribution. Neglecting the yield contribution, selecting this species for deconstruction could mean an almost 29% loss in carbohydrates toward conversion to ethanol or other valuable products.

In contrast, PWG was one of the more efficient species with an 87% release of sugars and the smallest difference between carbohydrate yield and released carbohydrate yield (348 kg ha^{-1}), according to this study. The PWG was also one of the higher yielding species. With respect to HSD (8%) for release efficiency, PWG though ranked the most efficient was not significantly different than SB, IWG, TWG, WWG, MB2, HB, and SWG, in that order with SWG at 79%. Of these similarly efficient cultivars, TWG, IWG, SB, PWG, and SWG were the highest preforming for carbohydrate release yield (in that order) and not significantly different from one another.

With respect to ranking, TWG had the highest harvest (5946 kg ha^{-1}) and carbohydrate yields (2742 kg ha^{-1}) with one of the highest percentages of available carbohydrates released (84%). With respect to plant maturity at time of harvest, one advantage of using a late harvest to evaluate these 15 C3 grasses was that it minimized the effects of cultivar maturity. While all of the cultivars evaluated were in heading/reproductive stages, TWG was in the early heading stage and the rest were in late reproductive to early seed development stages. Nevertheless, TWG's carbohydrate composition was not different than most of the other species in the wheatgrass class (excluding CWG and PWG), suggesting that maturity differences were not as important as species differences. To explain the difference in sugar release efficiencies across cultivars, cell wall composition, in particular, lignin content is one obvious component to examine. There was a moderate negative correlation between lignin composition and carbohydrate release. This would stand to reason as lignin content increases, carbohydrates are generally more difficult to remove. Similarly, there was a moderate positive correlation between lignin composition and the difference between potential and accessible carbohydrate composition. Therefore, as lignin composition increased, so did the difference between the carbohydrates content of the plant and the accessibility of those carbohydrates. However, a strong positive correlation between lignin yield and released carbohydrate yield existed. A strong positive correlation was likely the result of greater yielding species having greater amounts of both lignin and carbohydrates for release. These results were similar to those reported by Lorenz et al. (2009) and Lorenzana et al. (2010) for corn stover composition and glucose release or convertibility using similar methods of composition and total carbohydrate yield analysis.

In terms of lignin, the average lignin composition of the 15 C3 grasses was lower in comparison to the general range in lignin reported by Ragauskas et al. (2014) for other prominent biofuel feedstocks. With respect to species, RWR, CWG, and HWG had some of the highest average lignin content (131, 129, and 129 g kg^{-1}) with some of the lowest percentages of carbohydrates released (77, 77, and 72%) as might be expected. Similarly, IWG and PWG had some of the lowest lignin values at 113 and 116 g kg^{-1}, with some of the highest release efficiencies at 84 and 87%, respectively. However, SB had the highest average lignin content at 132 g kg^{-1}, and one of the highest carbohydrate release yields at 84%. Similarly, HB had high average lignin content at 125 g kg^{-1} and high sugar release efficiency at 82%. Conversely, TFQ had one of the lowest average lignin values at 116 g kg^{-1} with one of the lowest release efficiencies at 73%. While some of these findings may seem contrary, the lignin content was similar among all cultivars with an average value and standard deviation of 123 ± 6.0 g kg^{-1} with an HSD of 6.8 g kg^{-1}. Therefore, it was difficult to suggest that differences in lignin content alone were useful for predicting or explaining recalcitrance in this case. It is more likely that a combination of characteristics that differ across individual species and cultivars would provide more complete information about recalcitrance and better explain the difference in released carbohydrate yield results presented here. These could include not only lignin content, but the specific ratio of phenylpropanoids comprising the lignin, the way it specifically cross-links with hemicellulose, as well as the crystallinity of the cellulose and the presence of non-lignified cell wall phenolic acid esters known to be prevalent in grasses and how these specific molecular characteristics are influenced by plant maturity at harvest (Jung et al., 1992; Mansfield et al., 1999; Zhang and Lynd, 2004; Anderson and Akin, 2008; Vogel et al., 2010).

It can be useful to focus on composition and harvest yield potential rather than deconstruction efficiency, as biochemical conversion methods can vary, specifically deconstruction methods (physical, chemical, thermal, biological, and combinations thereof). While this specific set of cool-season grasses generally suggest lower glucan and xylan compositions than some of the top preforming biofuel feedstocks as reported by Ragauskas et al. (2014), again these cool-season grasses generally had lower lignin levels than most of those same reported grasses at peak biomass. The C3 grasses have also been effectively adapted for the variable climate experienced in the semiarid eastern plans of northern Colorado and by extension to other regions with similar soil and climates. The potential of these grasses as biofuels could further be optimized by tailoring harvest times to the specific maturity of each species. Not only could this contribute to better harvest yields in some species but lower lignin levels and a reduction in protein content. Cool-season grasses typically grown as animal forage have higher protein concentrations than are typically desirable for biochemical conversion. Gillette (2011), who evaluated the 2009 harvest in her Master's thesis, reported that crude protein levels were an average of 175 g kg^{-1} for the early June harvest of 2009 which was optimal for animal forage production. Samples analyzed in this study suggested an average protein composition of less than 100 g kg^{-1} for the late June harvest across three growing seasons. Excessive protein can be problematic for certain deconstruction routes for a number of reasons: reduced cellulose digestibility, Mallard reactions, catalyst poisoning, NOx emissions (Murray et al., 2008). However, research is progressing into feasible ways to recover this protein as a valuable coproduct (Dale et al., 2010; Chiesa and Gnansounou, 2011; Leberknight et al., 2011; Wernick and Liao, 2013). Dilution of the protein by blending with a feedstock lower in protein composition is another option. Despite these issues, Gillette observed dramatic decreases in protein concentrations in most species after seed head emergence, excluding RWR, and she suggested further delays in harvest could reduce protein content even further. Therefore, if the protein content could not be valorized or diluted by other means, there is room for further reductions based on harvest timing.

CONCLUSION

Cool-season perennial grasses are an interesting biofuels feedstock given their long history of use as livestock forages. This study demonstrated considerable harvest yield differences among C3 grass cultivars for a nontraditional late June harvest across three growing seasons. The harvest yield significantly influenced the yields associated with carbohydrate composition and the release of those carbohydrates. The wheatgrass class of grasses exhibited the greatest harvest yields and subsequently the greatest carbohydrate yields which were a result of each individual cultivars substantial harvest yield. Specifically, TWG was ranked with the highest harvest and carbohydrate yield, and demonstrated the greatest released carbohydrate yield as well. However, PWG, SB, and IWG were all higher yielding and statistically similar to TWG in their conversion efficiencies for released carbohydrate yields. The RWR, TFF, and TFG were the lowest yielding cultivars and their conversion efficiencies for released carbohydrate yields were also low. The HWG had the worst conversion efficiency between carbohydrate yield and released carbohydrate yield. However, as one of the higher yielding species it released more carbohydrates than half of the other species despite is low conversion efficiency.

Care should be taken in extrapolating the results of this study to the growth of perennial cool-season grasses in other environments and under different management practices. However, these results are valuable in demonstrating the importance of biomass yields on carbohydrate composition, and the importance of total carbohydrate yield estimates to final outcomes. The question then becomes which problem is the easiest to solve: increasing yield in the field, increasing carbohydrate accessibility through genetic modifications, or an alternative deconstruction process? The best solutions will likely involve improvements to multiple areas simultaneously. This study also demonstrates NIR/PLS as a valuable method of analysis in this evaluation process. With predictive tools for composition and total carbohydrate yield, decisions can be made more quickly toward more productive and cost effective solutions at various steps in the biofuels production pathway.

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SUPPLEMENTAL MATERIAL

Supplemental material was provided to describe the mixed herbaceous feedstock PLS-2 model used for prediction of feedstock composition. This material included a table (Table S1) of descriptive statistics for composition of the samples set used for the calibration model and the sample set used for validation of the model. Table S2 includes summary statistics evaluating the PLS-2 calibration model while Table S3 provides summary statistics for the validation set predicted on the PLS-2 model. Figures S1 and S2 depict predicted versus measured results for both the calibration and validation sample sets. The calibration model included six different feedstocks: corn stover (34), sorghum (35), miscanthus (34), switchgrass (14), rice straw (14), and a variety of cool-season grasses (52) as described in this manuscript. Validation of the calibration model included 18 samples from the six different feedstock types that were not used to build the calibration model.

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