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High-Throughput Method for Determining the Sugar Content in Biomass with Pyrolysis Molecular Beam Mass Spectrometry

Robert W. Sykes[1,2] · **Erica L. Gjersing**[1,2] · **Crissa L. Doeppke**[1,2] · **Mark F. Davis**[1,2]

Abstract There is an important need to assess biomass recalcitrance in large populations of both natural and transgenic plants to identify promising candidates for lignocellulosic biofuel production. In order to properly test and optimize parameters for biofuel production, the starting sugar content must be known to calculate percent sugar yield and conversion efficiencies. Pyrolysis molecular beam mass spectrometry (py-MBMS) has been used as a high-throughput method for determination of lignin content and structure, and this report demonstrates its applicability for determining glucose, xylose, arabinose, galactose, and mannose content in biomass. Biomass from conifers, hardwoods, and herbaceous species were used to create a 44 sample partial least squares (PLS) regression models of py-MBMS spectra-based sugar estimates on high-performance liquid chromatography (HPLC) sugar content data. The total sugar py-MBMS regression model had a R^2 of 0.91 with a 0.17 mg/mg root mean square error of validation indicating accurate estimation of total sugar content for a range of biomass types. Models were validated using eight independent biomass samples from multiple species, with predictions falling within errors of the HPLC data. With a data collection time of 1.5 min per sample, py-MBMS serves as a rapid high-throughput method for quantifying sugar content in biomass.

Keywords Glucose · Xylose · Recalcitrance · Prediction · Herbaceous · Conifer · Hardwood · Bioenergy

✉ Robert W. Sykes
robert.sykes@nrel.gov

[1] National Bioenergy Center, National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA

[2] BioEnergy Science Center, 15013 Denver West Parkway, Golden, CO 80401, USA

Introduction

With the introduction of the Renewable Fuel Standard, requiring the use of cellulosic biofuels, a surge in research has focused on determining the optimum conditions for converting biomass to fuels. One key factor of these studies is the carbohydrate content of plants, specifically on the two most abundant sugars of glucose and xylose. Sugars have a variety of possible routes for fuels synthesis including microbial conversion to ethanol or other transportation fuels and chemicals [1], pyrolysis to high energy content oils and gasses, or gasification to alcohols or alkanes [2]. All of these routes are directly affected by the chemical composition of the biomass starting material and the method used to release the sugars from the biomass. A simple and effective method to determine the sugar content of starting biomass material is often needed to optimize the process of converting biomass to fuels.

A variety of methods have been developed to quantify sugar content in biomass. The standard method for determining the composition of biomass uses a procedure that is both labor- and time-intensive, requiring gram quantities of biomass and taking close to 2 weeks for the full analysis [3–8]. With improvements in genetic modification and transformation of plants, the time and quantity requirements of traditional methods of sugar determination are no longer sufficient for the number of plants that need characterization. High-throughput methods for screening biomass are needed, and the review paper by Lupoi et al. [9] describes the benefits and drawbacks of many of these methods. An NMR method for determining sugar content in biomass hydrolysates was recently published [10], but the method still requires the time-consuming hydrolysis process. Techniques that use whole biomass are of greater interest for high-throughput applications because of the reduced amount of sample preparation and manipulation. Near-IR techniques can be combined with chemometrics to predict biomass composition in a nondestructive manner [11, 12]. However, near-IR regression coefficients can be hard to interpret due to the molecular overtones of the fundamental

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bands residing in the mid-IR region causing weak and poorly resolved peaks [13]. The near-IR technique is also very sensitive to moisture content necessitating meticulous sample preparation and storage to ensure accurate results [14].

Pyrolysis molecular beam mass spectrometry (py-MBMS) provides an information-rich chemical fingerprint of each sample and has been used to study the cell wall composition of herbaceous biomass [15, 16], the decomposition of carbohydrates [17], and the production and analysis of bio-oils [18]. More recently, py-MBMS has been used for high-throughput screening for lignin content and S/G ratio of biomass [19–21]. Although the pyrolysis is destructive, only a small amount (~4 mg) of sample is needed for analysis, and the technique has proven to be highly valuable and reliable in the years since it was introduced. While py-MBMS data has primarily been used to estimate lignin properties such as content and composition, the mass spectrum contains additional information about the plant that can be utilized through chemometrics. During pyrolysis, the biomass is fragmented into individual molecular fragments with heat in the absence of oxygen. These molecular fragments retain the information of its parent structures and are used to quantify the composition of the starting material. This paper demonstrates a method of using partial least squares (PLS) modeling to correlate py-MBMS spectra with sugar contents, determined from traditional wet chemical analysis, in order to predict sugar contents from the py-MBMS spectra.

Materials and Methods

Biomass Samples

Biomass samples used in this study were identical to the Gjersing et al. [10] study, with the exception of several samples that were fully consumed in the previous study. Briefly, switchgrass, wheatstraw, poplar, pine, and eucalyptus samples were selected from historical datasets analyzed at the National Renewable Energy Laboratory that cover the natural range of sugar content in plants. The biomass material was milled to pass through a 20 mesh screen using a mini Wiley mill. For starch removal and ethanol extraction, approximately 200 mg of biomass material was wrapped in a standard mesh tea bag. Starch was removed from the samples using commercial amylases described in Decker et al. [22]. Samples were ethanol extracted using a soxhlet for approximately 24 h to remove natural oils and phenolic compounds.

Hydrolysis

A scaled-down version of the NREL Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin from Biomass" [3] was used for preparing hydrolysates. Autoclave pressure tubes (2–5 mL) were loaded with 7.5 mg (±0.5 mg) milled and extracted biomass. The samples were then mixed with 75 μL of 72 % H_2SO_4 and placed in a 30 °C water bath for 1 h. After the samples were removed from the water bath, 2.1 mL of water was added to each tube and the tubes were autoclaved at 121 °C for 1 h. The samples were allowed to cool to room temperature, and the liquid hydrolysate fraction was then decanted into 15-mL conical tubes and neutralized with $CaCO_3$ to a pH of 7. The neutralized samples were spun at 5000 rpm for 10 min; the liquid hydrolysate fraction was filtered at 2 μm to ensure all solids were removed from the solution.

HPLC

High-performance liquid chromatography (HPLC) was performed as specified in the NREL Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin from Biomass" [3]. An Agilent Infinity 1220 Series HPLC system with a Bio-Rad HPX-87P column was used. The injection volume was 10–50 μL for each sample, with a mobile phase of HPLC-grade water at a flow rate of 0.6 mL/min. The column temperature was 80–85 °C with a run time of 35 min.

Pyrolysis Molecular Beam Mass Spectrometry

Plant cell walls containing cellulose, hemicellulose, and lignin are pyrolyzed, and the information-rich vapors are analyzed using a molecular beam mass spectrometer (Fig. 1). A commercially available molecular beam mass spectrometer designed specifically for biomass analysis and an autosampler was used for pyrolysis vapor analysis [23, 24]. Approximately 4 mg of air-dried 20 mesh biomass was introduced into a quartz pyrolysis reactor at 500 °C with 0.9 L/min helium carrier gas flow via 80-μL deactivated stainless steel Eco-Cups provided with an autosampler [19]. Mass spectral data from m/z 30–450 were acquired over a 90-s acquisition period on a Merlin Automation data system version 3.0 using low-energy (17 eV) electron impact ionization. Low-energy electron impact reduces fragmentation due to ionization in the mass spectra [23]. Two technical replicate spectra of each sample were collected.

Data Analysis

Multivariate analysis including principle component analysis (PCA) and partial least squares (PLS) modeling was performed on mean normalized spectral data (m/z 30–450) using The Unscrambler v. 9.7 (CAMO A/S, Trondheim, Norway). The number of factors used in PLS models was chosen based on recommended values from the statistical software, as well as interpretation of the

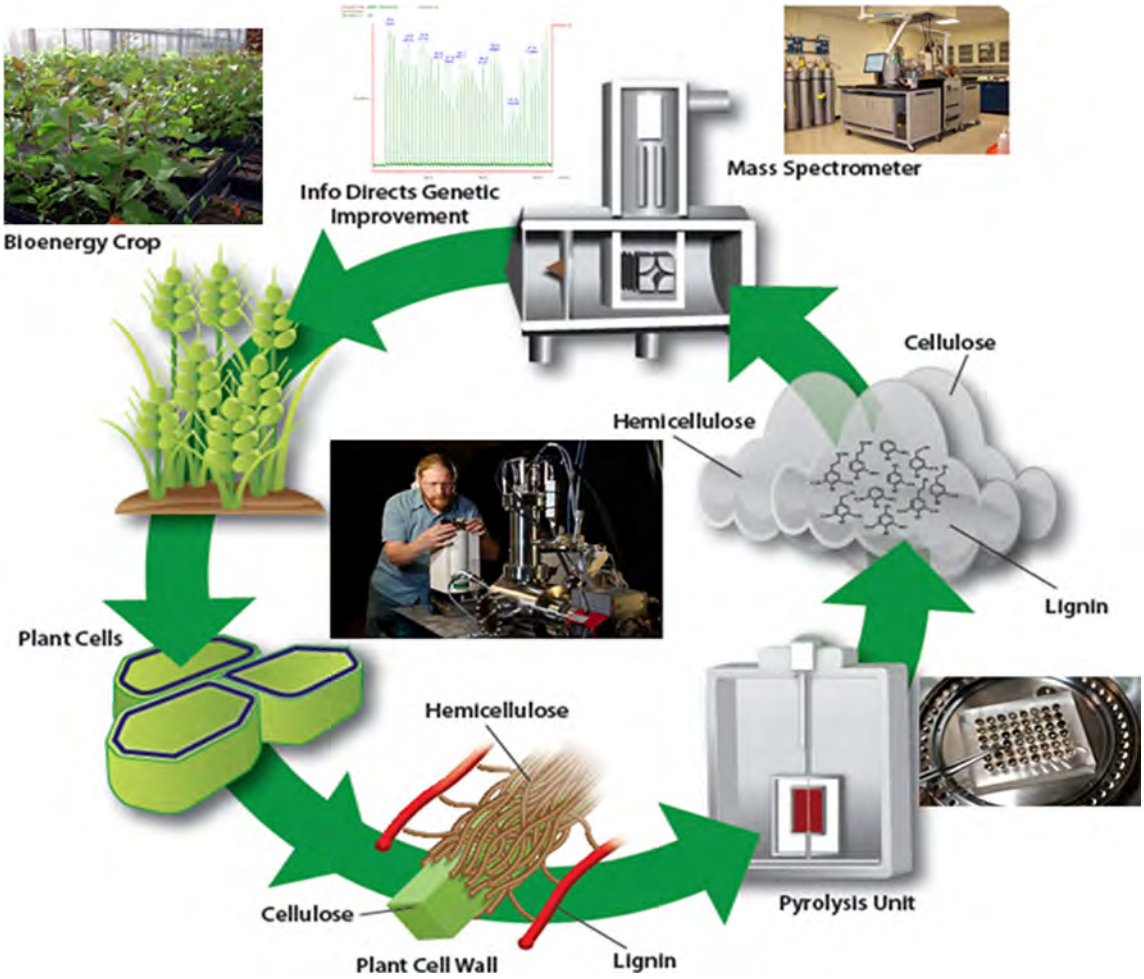

Fig. 1 Experimental depiction of a pyrolysis molecular beam mass spectrometry study. Figure created by Anthony Castellano. Photos provided by Dennis Schroeder and Robert Sykes

regression coefficients of the models. The number of factors selected for the models never exceeded the value recommended by the software but was lowered if the interpretation of the regression coefficients was simplified and the integrity of the model was not compromised. The full cross validation option was in The Unscrambler used for all PLS models to ensure accurate error calculation. Full cross validation, also known as leave-one-out (LOO) cross validation, involves leaving one sample out of the dataset and calculating a submodel with the remaining samples. This process is repeated exhaustively for all samples, creating a series of submodels. Once the series of submodels are created, all prediction residuals are combined to compute the validation residual variance and the root mean square error of prediction. The Unscrambler statistical software package uses these calculations to determine the appropriate number of factors used for PLS models to prevent overfitting, the description of random error or noise. The predictions of the various sugar contents were also produced using The Unscrambler statistical software.

Results and Discussion

Principal Component Analysis

Pyrolysis MBMS spectra were analyzed using PCA to cluster the samples together based on similar mass fragment peak intensities (Fig. 2). PCA is a multivariate statistical technique that excels at visually mapping differences between samples in large datasets. The samples in this study separate into three distinct groups (Fig. 2): conifers, hardwoods, and herbaceous samples along the *x*-axis or principal component 1 (PC1). The unique chemistry of these three groups results largely from differences in lignin monomer structure. Conifer lignin content consists of guaiacyl (G) lignin monomers, hardwood lignin includes both syringyl (S), and G lignin monomers, and

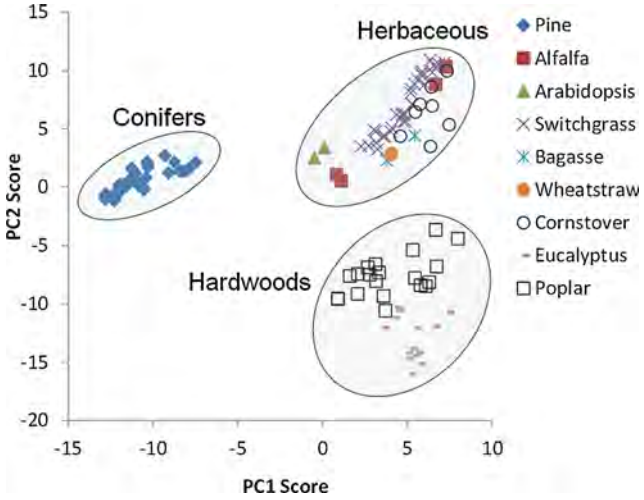

Fig. 2 Principal component analysis plot of conifer, hardwood, and herbaceous samples

herbaceous samples have S and G monomers but a higher proportion of *p*-coumaryl (H) lignin monomers than conifers and hardwoods [25]. Samples with positive PC1 scores (hardwoods and herbaceous) in Fig. 2 have higher S lignin content than samples with negative PC1 scores (conifers).

PLS Regression Models

PLS models were constructed using HPLC sugar content data and MBMS spectra from 44 mixed biomass samples, from switchgrass, wheatstraw, poplar, pine, and eucalyptus samples. Models were created for the two major sugars, glucose and xylose, and the minor sugars, arabinose, galactose, and mannose (Figs. 3 and 4). In addition, a PLS model was constructed for total sugar content (Fig. 5). The major sugar models, glucose and xylose, have R^2 values of 0.83 and 0.97, respectively, indicating good correlation between traditional HPLC measured sugar contents and py-MBMS spectra.

The results presented here are in agreement with the nuclear magnetic resonance (NMR) method for sugar determination reported in Gjersing et al. [10] with R^2 values for glucose (0.82) and xylose (0.93). Xu et al. [26] also reports similar values with an R^2 of 0.87 for glucan and 0.78 for xylan using near-infrared spectroscopy. The arabinose model with R^2 of 0.85 has a good correlation with HPLC. Both the py-MBMS and NMR galactose models display a poor correlation, with R^2 of 0.60 compared to R^2 of 0.66 by NMR [10], most likely due to the fact that the detection limit of the HPLC method of 0.05 mg/mL causing higher error at these low galactose

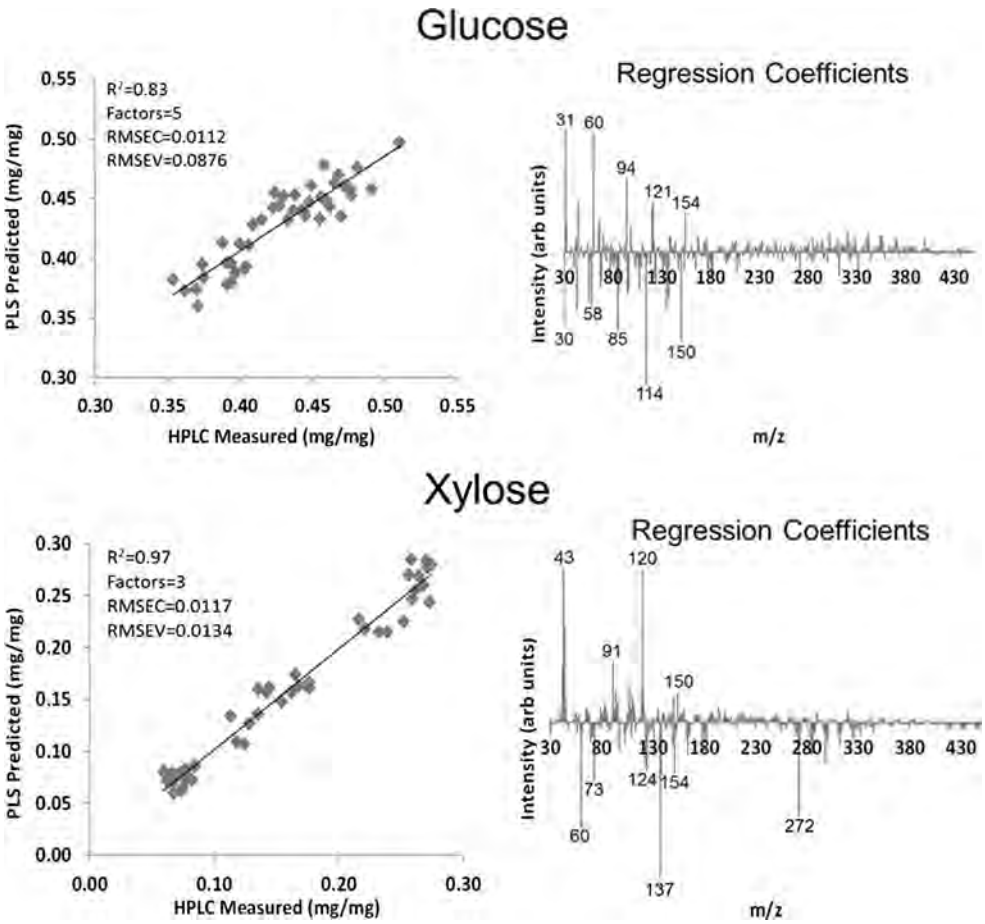

Fig. 3 PLS regression models for the two major sugars in biomass, glucose, and xylose, and their associated regression coefficients. R^2 of validation, number of factors used to create the model, root mean square error of calibration (*RMSEC*), and root mean square error of validation (*RMSEV*) are shown

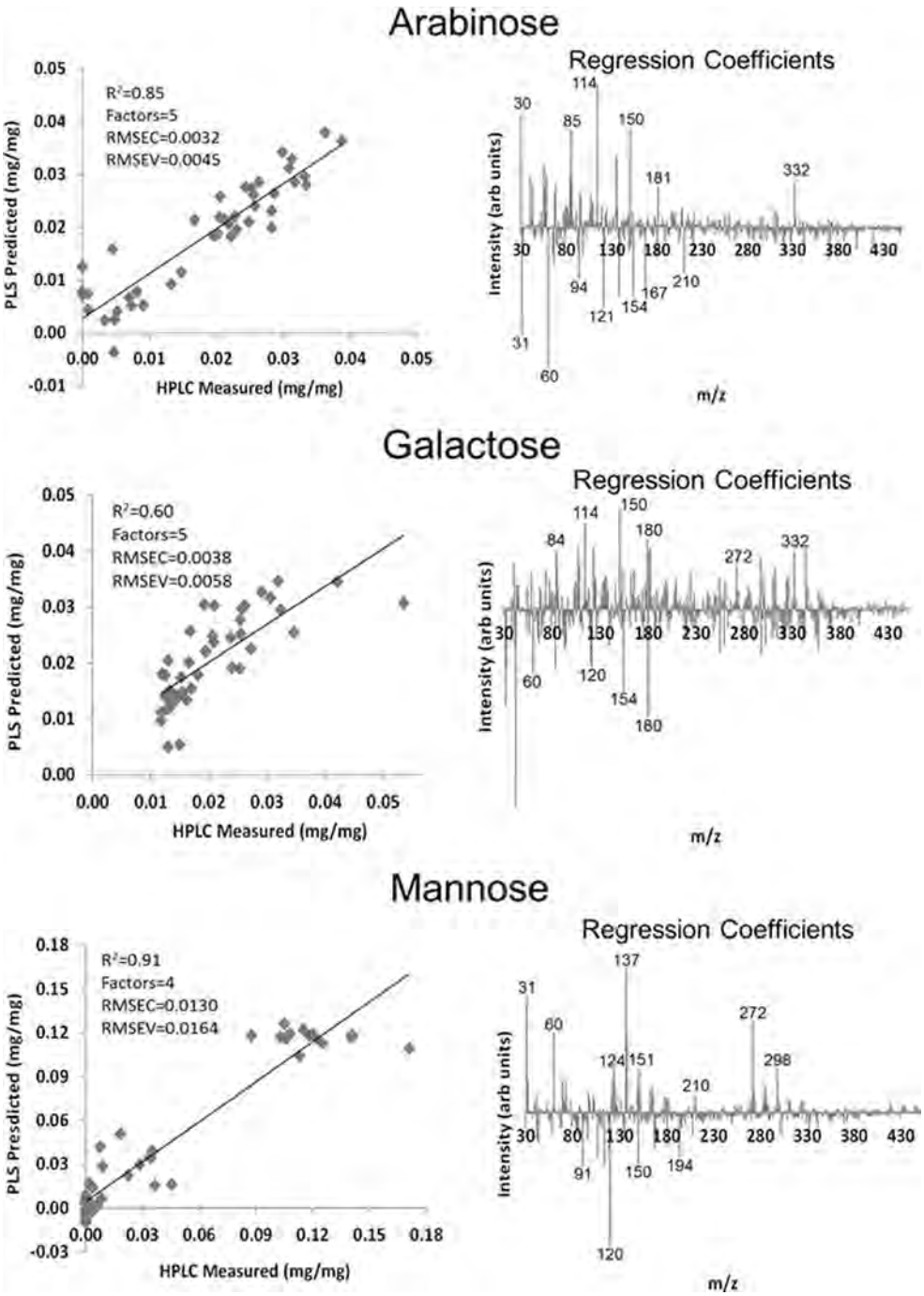

Fig. 4 PLS regression models for the minor sugars in biomass, arabinose, galactose, and mannose, and their associated regression coefficients. R^2 of validation, number of factors used to create the model, root mean square error of calibration (*RMSEC*), and root mean square error of validation (*RMSEV*) are shown

contents. While the mannose model results in a good R^2 value of 0.91, the model is skewed by the conifer samples that typically have high mannose content compared to all other samples, essentially making this a two-point regression model that has limited validity (Fig. 4). Combining the major and minor sugars together for a total sugar model results in a model with an R^2 of 0.91 (Fig. 5).

Additional models were also created for each biomass category (hardwood, conifer, and herbaceous) for comparison to models containing samples from all categories. The individual biomass category models generally resulted in lower R^2 values than models using all the samples available and, therefore, are not presented here. For example, using hardwood samples to predict glucose and total sugars resulted in R^2 values of 0.76 and 0.79, respectively, compared to 0.83 and 0.91 when all samples were used in the model. Errors for calibration and validation associated with the individual biomass category models were also approximately 25 % higher. Combining the hardwood, conifer, and herbaceous samples together into one dataset extended the overall range of the sugar content in

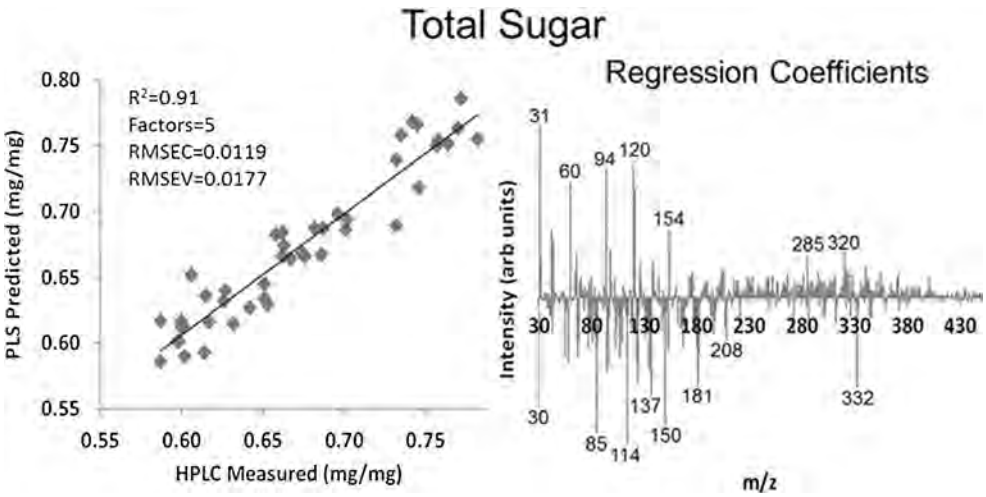

Fig. 5 PLS regression model for total sugars in biomass and associated regression coefficients. R^2 of validation, number of factors used to create the model, root mean square error of calibration (*RMSEC*), and root mean square error of validation (*RMSEV*) are shown

the models and helped elucidate which compounds were driving the predictions of the various sugar component contents.

One advantage the py-MBMS technique has over other high-throughput techniques is the ability to directly attribute spectral peaks to compounds associated with parent chemical structures. The regression coefficients associated with the PLS models for glucose and xylose are shown in Fig. 3. The regression coefficients consist of molecular fragments associated with lignin, cellulose, and hemicellulose shown in Table 1. In the glucose PLS model, a prominent C6 sugar peak (m/z 60) appears in the positive regression coefficients, while C5 sugar peaks (m/z 85 and m/z 114) are in the negative regression coefficients indicating an inverse relationship between cellulose and hemicellulose (Table 1) [23]. Analyzing the regression coefficients for the xylose PLS model shows that the model is driven primarily by lignin content, with S and G lignin fragments in both the positive and negative regression coefficients (Fig. 3). Peak 120 (vinylphenol), which is a major low molecular weight lignin peak from grasses, is also prominent in the positive

Table 1 Peak and precursor assignments in mass spectra of lignified samples

m/z	Assignment	Precursor Type
57, 73, 85, 96, 114	C5 Sugars	
57, 60, 73, 98, 126, 144	C6 Sugars	
94	Phenol	H, S, G
120	Vinylphenol	H, S, G
124	Guaiacol	G
137[a]	Ethylguaiacol, homovanillin, coniferyl alcohol	G
138	Methylguaiacol	G
150	Vinylguaiacol, coumaryl alcohol	G
152	4-Ethylguaiacol, vanillin	G
154	Syringol	S
164	Allyl-+propenylguaiacol	G
167[a]	Ethylsyringol, syringylacetone, propiosyringone	S
168	4-Methyl-2,6-dimethoxyphenol	S
178	Coniferyl aldehyde	G
180	Coniferyl alcohol, vinylsyringol, α-D-glucose	S, G
182	Syringaldehyde	S
194	4-Propenylsyringol	S
208	Sinapaldehyde	S
210	Sinapyl alcohol	S

From Evans and Milne [23]

[a] Fragment ion

regression coefficients in Fig. 3 [23]. While the vinylphenol molecule can be associated with H, S, and G lignin monomers, here, we attribute m/z 120 as a fragment of *p*-coumarate, which acylates the side chains of the phenylproanoid polymer backbone [27]. Regression coefficients for the minor sugars show similar trends, but due to the inherently small content range among the samples, the coefficients are more complicated (Fig. 4). The total sugars model in Fig. 5 has C6 sugar and S lignin molecular fragments in the positive regression coefficients and C5 sugar and G lignin molecular fragments in the negative regression coefficients. The large presence of lignin peaks in the sugar models is expected due to their intimate relationship within the cell wall.

The py-MBMS technique also allows visual inspection of the spectra in real time to see if changes in cell wall chemistry have occurred. Genetically modifying biosynthetic pathways may result in new compounds appearing in plants that are not naturally occurring. These compounds can ultimately cause additional errors associated with PLS models as they are not accounted for in the original model. Pyrolysis MBMS can quickly highlight these types of compounds by visual inspection of spectra from transgenic plants compared to controls. Peaks that are unique or present in different ratios compared to controls can be studied for identification and quantification.

Future implementation of these sugar cell wall component PLS models will require that calibration samples be included with each py-MBMS dataset to ensure accuracy and robustness. The inclusion of calibration samples in each dataset negates differences in detector response associated with spectrometer tuning and electronic drift over time. Direct comparison of py-MBMS spectra between datasets is not recommended due to the possible instrumental and experimental errors mentioned above. Multiple standards totaling 10 % of

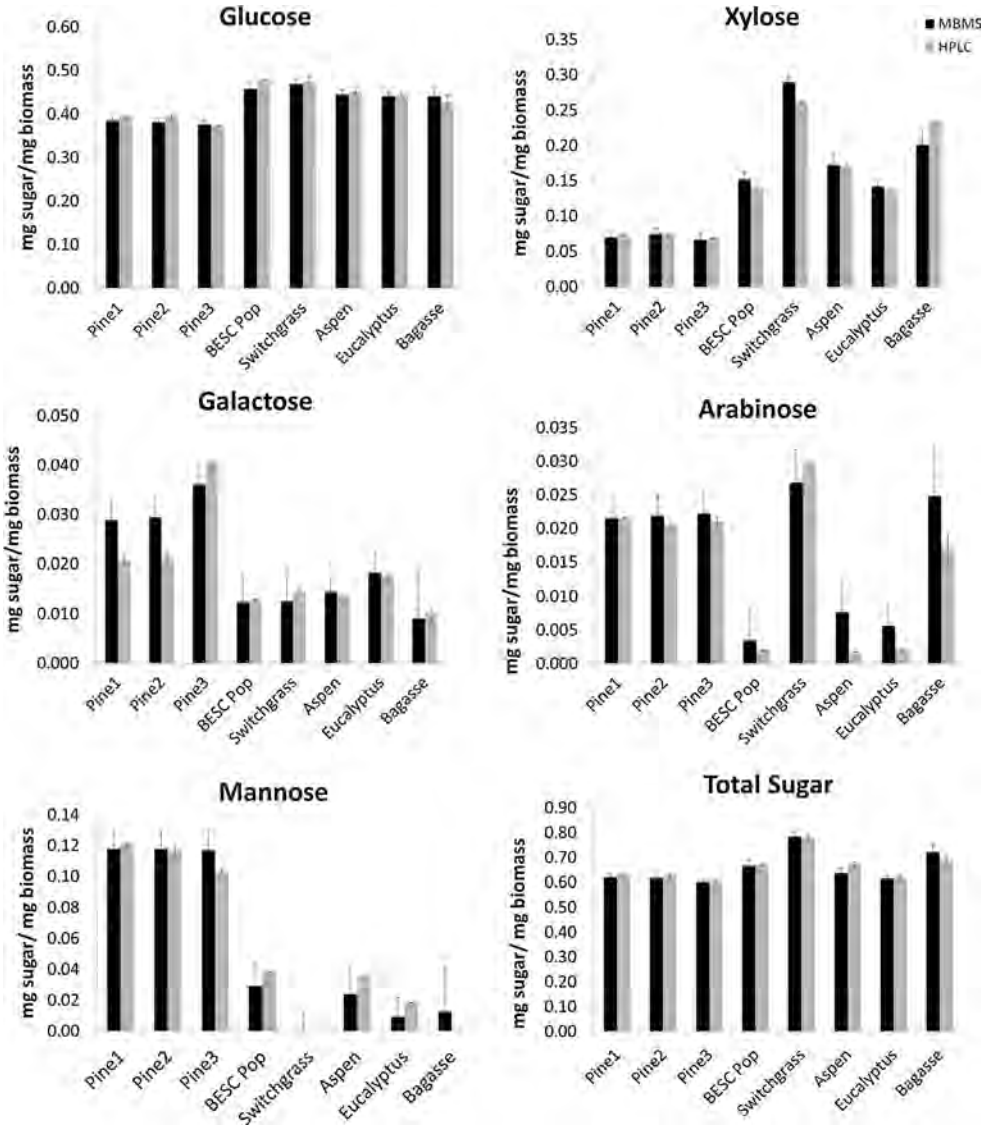

Fig. 6 PLS sugar content predictions using py-MBMS spectra for glucose, xylose, mannose, galactose, arabinose, and total sugar content. *Black bars* represent sugar content predictions using py-MBMS, and *gray bars* represent HPLC measured sugar contents

the samples are included with each experiment to provide a measurement of instrument drift.

PLS Model Validation

The PLS regression models were validated by using eight randomly selected biomass samples: three conifer (pine), three hardwood (eucalyptus and *Populus*), and two herbaceous (bagasse and switchgrass). The major and minor sugar contents were predicted using PLS regression and compared to the HPLC measured values (Fig. 6). Results from the HPLC data and the py-MBMS model predictions agree within error, where the py-MBMS PLS error bars indicate the root mean square error of prediction (RMSEP) and the HPLC error bars are the standard deviation of replicate samples (Fig. 6). Error bars on the minor sugars are higher due to relatively small amount of each sugar and limited range in the biomass. These sugar content predictions verify the ability of py-MBMS as a substitute for HPLC sugar measurement in a high-throughput capacity. The overall sugar content range and the variety of species present in the dataset make the PLS models very robust. This is especially useful with increased use of transgenic samples, where models must predict chemical composition that pushes the boundaries of the normal range of natural variation.

Conclusions

Pyrolysis molecular beam mass spectrometry is shown to be a potential high-throughput method for determining glucose, xylose, arabinose, galactose, and mannose content in biomass. PLS regression models of py-MBMS spectra have strong correlations with total sugar content models with an R^2 of 0.91 with a 0.17 mg/mg root mean square error of validation. Models were validated using eight independent biomass samples from multiple species, with the sugar content predictions falling within errors of the HPLC data. The short (1.5 min) sample analysis time for quantifying sugar content in biomass is a significant improvement of traditional HPLC methods and equivalent to other techniques such as FT-NIR and NIR. In addition, the py-MBMS technique can provide superior molecular structural information associated with quantification of other cell wall components such as lignin and S/G ratio. Development of these techniques allows researchers to fully analyze larger experiments that were not feasible previously due to time and money constraints. High-throughput techniques contribute to continued improvement and discovery of lignocellulosic feedstocks used for biofuel production.

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Conflict of Interest The authors declare that they have no competing interests.

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