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Improved method of analysis of biomass sugars using high-performance liquid chromatography

F.A. Agblevor[1,*], A. Murden[1] & B.R. Hames[2]
[1]*Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA*
[2]*National Renewable Energy Laboratory, Golden, CO 80401, USA*
[*]*Author for correspondence (Fax: +540-231-3199; E-mail: Fagblevo@vt.edu)*

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Abstract

The precise quantitative analysis of biomass derived sugars is a very important step in the conversion of biomass feedstocks to fuels and chemicals. However, the most accurate method of biomass sugar analysis is based on the gas chromatography analysis of derivatized sugars either as alditol acetates or trimethylsilanes. The derivatization method is time-consuming but the alternative HPLC method cannot resolve most sugars found in biomass hydrolysates. We have demonstrated for the first time that by careful manipulation of the HPLC mobile phase, biomass monomeric sugars (arabinose, xylose, fructose, glucose, mannose, and galactose) can be analyzed quantitatively and there is excellent baseline resolution of all the sugars. This was demonstrated for both standard sugars and corn stover hydrolysates. Our method can also be used to analyze dimmeric sugars (cellobiose and sucrose).

Introduction

The analysis of lignocellulosic biomass has been investigated extensively in the past, but methods vary depending on the feedstocks and the end application. The pulp and paper industry uses the TAPPI standards for hardwood and softwood analysis (T249 cm-85, 1995); the forage industry uses the acid and neutral detergent methods (Van Soest & Wine 1967) and the biomass industry uses ASTM standards or combinations thereof (ASTM E1758-01, 2003; ASTM E1821, 2003). The ASTM standards were based on the round robin tests conducted in the 1990s on standard NIST biomass reference samples (Milne *et al.* 1992). These methods are based on 72% H_2SO_4 hydrolysis and conversion to alditol acetates followed by gas chromatographic (GC) analysis. The GC method is particularly accurate for low sugar concentrations; however, it is time-consuming and prone to errors.

The alternative sugar analysis method is based on the high performance liquid chromatography (HPLC) analysis of the hydrolysate. Although this method is relatively fast, it is less sensitive for low sugar concentrations especially when the refractive index detector is used. The pulsed amperiometric detector improves on the detection limits, but suffers from the poor column resolution of the sugar peaks. Another major problem with the HPLC method of sugar analysis is the poor stability of the lead carbohydrate column. Column regeneration is challenging and sample preparation is time-consuming. Furthermore, the resolution of sugars such as fructose and mannose and the detection of dimmers are rather poor. Both the HPLC and GC methods are primary methods which do not need any external calibration.

There has also been extensive research in the past to develop secondary rapid analytical methods such as molecular beam mass spectrometry (Agblevor *et al.* 1994), near-infrared coupled with principal component analysis (Sanderson *et al.* 1996, Hames *et al.* 2003). All these methods require primary analytical methods to determine values for calibration of the methods. Thus, there is a need for the development of a rapid, robust, primary analytical method for the

analysis of biomass sugars. In this communication we present our recent results on the improved HPLC analysis of biomass hydrolysis sugars.

Materials and methods

The standard sugar samples arabinose, xylose, fructose, mannose, galactose, glucose, sucrose, and cellobiose were acquired from Sigma. Prevail carbohydrate ES column (250 × 4.6 mm) packed with 5 μm spherical polymer beads coated with proprietary bonding material. The pre-column (7.5 × 4.6 mm cartridge) and the analytical columns were obtained from Alltech Associates Inc (Deerfield, IL).

Biomass hydrolysate sample was provided by the National Renewable Energy Laboratory, Golden, CO. Acetonitrile and water mixture were used as mobile phase, and a Shimadzu HPLC 10 AVP instrument (Shimadzu Scientific, Colombia, MD) was used for the analysis. A Shimadzu low temperature evaporative light scattering detector (ELSD-LT) was used for compound detection. The ELSD-LT was operated at 40 °C, 250 kPa and air was used as the nebulizing gas. The samples were quantified using Shimadzu CLASS-VP 7 chromatographic software.

Analysis

The standard sugar samples were prepared from 1.5 mg ml^{-1} up to 75 mg ml^{-1} depending on the response of the particular sugar. About 2 ml of standard sugar solutions were filtered through 0.25 μm syringe filter into auto sample vials and loaded into the Shimadzu SIL-10AXL autoinjector. The mobile phase consisted of 100% deionized and degassed water (pump A) and 100% degassed acetonitrile (pump B). The samples were analyzed either in the isocratic or gradient mode. In the isocratic mode, the acetonitrile (85%) and water (15%) were pumped at a total flow rate of 0.6 ml min^{-1} to 1 ml min^{-1}. The specific choice of flow rate depended on the degree of separation desired. For most cases we used 1 ml min^{-1} to reduce the analysis time. The sample injection volume was 5 μl and the run time was 30 min.

For gradient analysis, the sample injection volume was 5 μl; the mobile phase flow rate was 0.6 ml min^{-1}. The initial solvent was 85% acetonitrile with gradual increase in water content to 25% over 20 min.

About 5 ml of corn stover hydrolysate was measured into a test tube and the pH of the solution was adjusted by the addition of 50 mg solid $Ca(OH)_2$ and mixing thoroughly with a Genie mixer to a final pH 4. The sample was centrifuged at 10 000 × g for 10 min and then decanted. The decanted samples were filtered through 0.25 μm syringe filter into 2 ml auto-sampling vials. The samples (5 μl) were injected using the autoinjector.

To analyze the mixture of monomers and dimmers, the chromatogram was initially run in the isocratic mode (acetonitrile:water, 85/15 v/v) for 25 min, and then shifted to gradient mode at the same flow rate of 1 ml min^{-1}. The water content was increased from 15% to 35% for 10 min and then reduced to 15% for 10 min. The total run time was 45 min.

Calibration curves

External calibration curves were developed by analyzing sugars at 1.5, 5, 10, 25 and 50 mg ml^{-1}. Calibration curves were obtained by plotting log area versus log concentration. For all the sugars analyzed, the curves were linear with r^2 values greater than 0.98.

Results and discussion

The analysis of the standard sugars on the Prevail carbohydrate ES column showed a strong dependence on the composition of the mobile phase. For good baseline resolution we discovered that 15% water/85% acetonitrile run isocratically resolved all the biomass derived monomeric sugars such as arabinose, xylose, fructose, glucose, mannose, galactose as shown in the chromatogram (Figure 1). The manufacturer recommendation of 25% water/72% acetonitrile as mobile phase was not suitable for the biomass derived sugars.

In the gradient mode, the best results were obtained when the initial composition was 15% water/85% acetonitrile and gradually increasing to 25% water. The major advantage of the gradient system was shorter analysis time (15 min), but the peaks were not well resolved compared to the isocratic mode (data not shown).

The resolution of the sugars obtained using the Prevail carbohydrate column was superior to those obtained using either Pb^{2+}, Ca^{2+}, or H^+ carbohydrate columns (ASTM E 1758-01 2003, Walter *et al.* 2001). In the Pb^{2+} column, the resolution of xylose and glucose were not baseline. Further, in the Pb^{2+} carbohydrate column, mannose, galactose and fructose are not resolved.

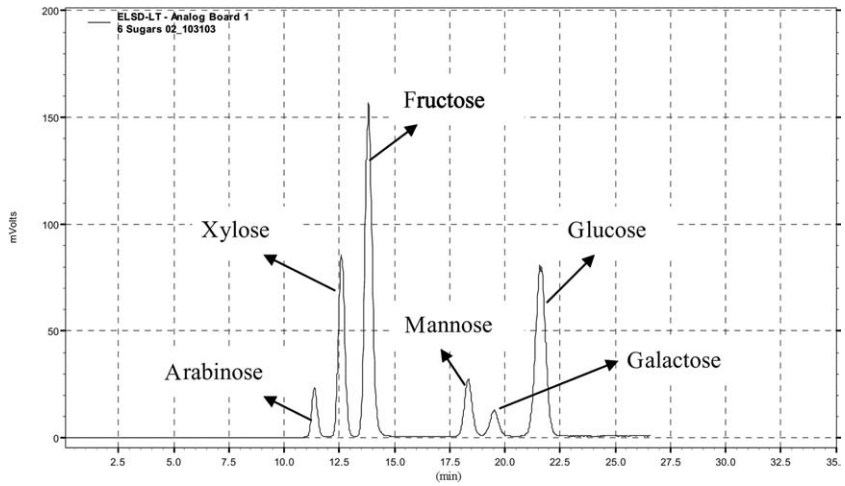

Fig. 1. Chromatogram of a mixture of six standard sugars analyzed isocratically.

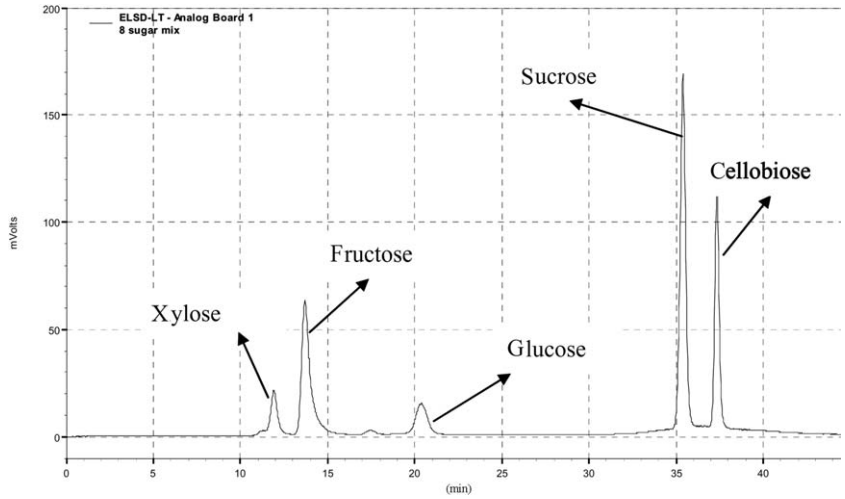

Fig. 2. Chromatogram of a mixture of seven standard sugars analyzed in both isocratic and gradient modes.

In addition to the monomeric sugars, the column could also resolve disaccharides such as sucrose, cellobiose and maltose. The resolution of these sugars also depended very strongly on the composition of the mobile phase. Although the isocratic regime could resolve the monomeric sugars, it was impossible to resolve dimeric sugars. The dimmers were retained on the column, but when the mobile phase was made more polar by increasing the water content, the dimmers eluted and were resolved. The cellobiose and sucrose peaks were well resolved (Figure 2), but the baseline was not flat because of the change in the mobile phase composition.

For all analysis the evaporative light scattering detector was used. The detection limits for fructose, glucose, and galactose were 24, 50, and 85 ng. Mannose and galactose were less sensitive than the other sugars. Because the detection mode of this instrument is based on the light scattering, the dimmers showed a stronger response than the monomeric sugars. The response was also not linear but a plot of log peak area versus log concentration was linear.

For the analysis of the corn stover hydrolysate (liquid fraction of the dilute sulfuric acid pretreated corn stover) the pH of the solution was raised from less than 1 to 4 before the analysis, because the column operates best between pH 2 and 14. The chromatogram in Figure 3 clearly shows that the corn stover hydrolysate contains similar compounds as the standard sugar mixture and the sugars could be effectively resolved. However, there were some unknown compounds, which eluted very early. We investigated lactic

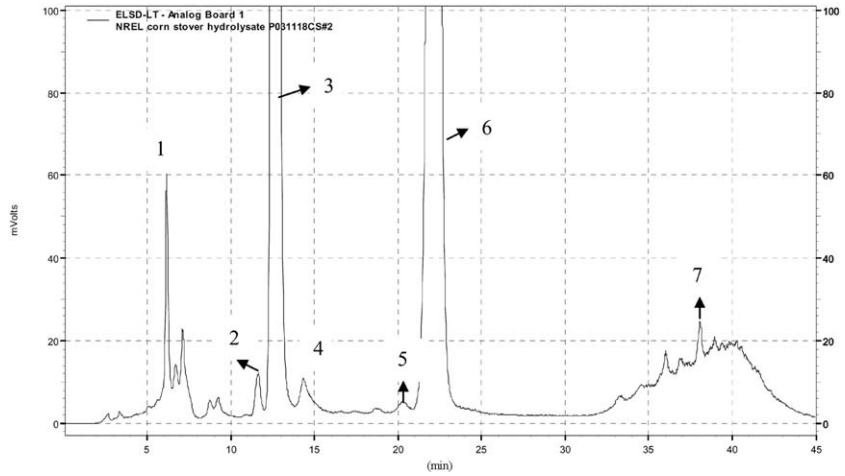

Fig. 3. Chromatogram of corn stover hydrolysate sugars. Peak 1 = unknown; 2 = arabinose; 3 = xylose; 4 = fructose; 5 = galactose; 6 = glucose; 7 = cellobiose.

acid, acetic acid, furfural and 5-hydroxymethyl furfural but these compounds were too volatile and could not be detected using the ELSD-LT.

The corn stover hydrolysate also contained low concentrations of cellobiose and sucrose in addition to the monomeric sugars (arabinose, xylose, fructose, mannose, galactose, glucose). The more interesting sugar in the corn stover hydrolysate was fructose. We postulated that the fructose derived from the sucrose in the corn stover which hydrolyzed into glucose and fructose. The quantitation of the sugars was very reproducible as shown by the small standard deviations observed for repeated runs.

Conclusions

We have demonstrated for the first time that through careful manipulation of the mobile phase, it is possible to achieve baseline resolution of the known monomeric sugars which occur in significant quantities in biomass hydrolysates. These results are clearly superior to those obtained using either lead, calcium or hydrogen carbohydrate columns. Furthermore, dimmeric sugar components could also be detected by this method. Thus, for biomass pretreatment methods, the precise effect of the pretreatment could be followed and evaluated with the method. The preparation of the hydrolysate sample was minimal and therefore shortening the analysis time for the sugars.

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