Rapid Method for the Estimation of Total Free Monosaccharide Content of Corn Stover Hydrolysate Using HPAE-PAD

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Application Note 225

Introduction

Compositional carbohydrate analysis of biocrop feedstock is essential to the efficient production of cellulosic ethanol.[1] Corn stover is the leaf, husk, stalk, and cob remaining in the field after harvest and makes up about half the yield of a crop of corn. It can also contain weeds and grasses collected during the harvest of corn stover. Along with other lignocellulosic biomass, corn stover provides 1.3 billion tons of raw material annually and is a common feedstock for fermentation systems used in biofuel production.[2]

Corn stover is acid-hydrolyzed to release a water-soluble mixture of carbohydrates, typically consisting of arabinose, glucose, galactose, mannose, xylose, fructose, and cellobiose among other noncarbohydrate substances in 0.5 to 1.5% (w/w) sulfuric acid.[3,4] The concentration of these sugars can vary with the feedstock and the hydrolysis conditions. Knowledge of the monosaccharide content of acid-hydrolyzed corn stover allows an evaluation of the effectiveness of a new hydrolysis process and/or an estimation of the product yield (for example, ethanol, methanol, hydrogen). Although determination of the individual monosaccharide concentrations is possible, the analysis time is typically long, and may involve multiple methods. For many processes, monosaccharide speciation may not be required, and only an estimate of the total monosaccharide concentration is needed.

Monosaccharides lack a good chromophore and, therefore, require high concentrations to be detectable using UV absorbance. Many noncarbohydrate ingredients of acid-hydrolyzed corn stover are chromophoric and can interfere with the direct detection of these carbohydrates by absorbance, especially when concentrated samples must be analyzed. Refractive index (RI) detection has similar limitations.[4] Pulsed amperometric detection (PAD) has a broad linear range for monosaccharides and most other carbohydrates, and is selective for compounds that can be detected under a given set of electrochemical conditions.[5,6] Consequently, many noncarbohydrate compounds that would be detected by UV or RI, remain undetected.

High-performance anion-exchange (HPAE) chromatography can separate glucose, galactose, arabinose, xylose, mannose, fructose, cellobiose, and other carbohydrates.[7–9]

The Thermo Scientific™ Dionex™ CarboPac™ PA1 anion-exchange column rapidly elutes corn stover carbohydrates, resolving the monosacccharides from the unretained and undetected noncarbohydrate components of acid-hydrolyzed corn stover. Under these conditions, disaccharides and trisaccharides are also resolved, with a total elution time less than 10 min. Thus, the sum of all the carbohydrate peaks is a useful measure of the energy production potential of acid-hydrolyzed corn stover.

The carbohydrate content of acid-hydrolyzed corn stover can be very high, and for some carbohydrates can even approach their saturation concentration in an aqueous solution. When used for saccharide determinations, PAD with a gold working electrode is a sensitive detection method, but like other detection techniques it does not have an unlimited linear range. We offer two accessories that allow PAD detection at carbohydrate concentrations found in undiluted acid-hydrolyzed corn stover: 1) an injection valve with 0.2 µL internal injection loop to decrease the amount of undiluted sample loaded on the column, and 2) a thicker (15 mil) working electrode gasket for the electrochemical detector cell.

Compared to the conventional 2 mil gasket, the thicker gasket effectively reduces the electrochemical response by about fourfold and extends the linear range. The combined use of the 0.2 µL injection loop and the 15 mil gasket produces an approximate 400-fold decrease in peak area for a given sample concentration, and allows a linear response for sugars in their concentration ranges in undiluted acid-hydrolyzed corn stover. These accessories are compatible with the Thermo Scientific™ Dionex™ ICS-3000 chromatography system.

In this application note, we estimate the total free carbohydrate content in an undiluted acid-hydrolyzed corn stover. We evaluate performance, including precision, accuracy, and linearity.

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Equipment

- Dionex ICS-3000 system consisting of:
  - DP or SP Gradient or Isocratic Pump, with vacuum degas option
  - DC Detector Compartment with 6-port high-pressure inject valve, single or dual zone temperature control
  - Electrochemical Detector with Carbohydrate Certified (Au) PTEF Disposable Electrodes (P/N 066480, package of six), or conventional gold working electrode (P/N 079850)
  - Combination pH/Ag/AgCl reference electrode (P/N 061879)
  - Thermo Scientific™ Dionex™ AS Autosampler
- High carbohydrate concentration accessories, consisting of:
  - 0.2 µL injection loop (Stator Face Seal Kit, P/N 068383), and
  - Amperometry cell gasket, 15 mil thickness (P/N 057364).
  - EO1 Eluent Organizer, including one or more 2 L plastic bottles and pressure regulator
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System
- Helium 4.5 grade, 99.995%, < 5 ppm oxygen; or Nitrogen 5.0 ultrahigh purity grade, 99.999%, < 1 ppm oxygen (Praxair)
- Filter unit, 0.2 µm Nylon (Nalgene® 90 mm Media Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter)
- Vacuum pump (Gast P/N DOA-P104-AA or equivalent)
- 0.3 mL Polypropylene injection vials with caps (Vial Kit, P/N 055428)

Reagents and Standards

Reagents

- Deionized water, 18 MΩ-cm resistance or higher
- 50% sodium hydroxide (w/w; Fisher Scientific, P/N SS254-500 recommended)

Standards

- D-Glucose, monohydrate (J.T. Baker, Inc., Cat# 1910-01)
- D(–)-Fructose (J.T. Baker, Inc., Cat# M556-05)
- D(+)-Mannose (Sigma-Aldrich, Cat# M-6020)
- D(+)-Galactose (Sigma-Aldrich, Cat# G-0625)
- D(+)-Cellobiose (Sigma-Aldrich, Cat# C-7252)
- D(–)-Arabinose (Sigma-Aldrich, Cat# A-3131)
- D-Xylose (Sigma-Aldrich, Cat# X107-5)

Conditions

Method

Columns:	Dionex CarboPac PA1 Analytical, 4 × 250 mm (P/N 035391)
Flow Rate:	1.0 mL/min
Inj. Volume:	0.2 µL (full loop)
Temperature:	30 °C
Detection:	Pulsed amperometry, gold working electrode, 15 mil gasket
pH:	12.5–12.8
Background:	19–36 nC
Typical System Operating Backpressure:	1050–1070 psi
Method:	200 mM NaOH; isocratic, 10 min run time, or longer if needed.

Carbohydrate Waveform for the ED

Time (s)	*Potential (V)*	*Integration*	*Gain Region**	*Ramp**
0.00	+0.1		Off	On
0.20	+0.1	Begin	On	On
0.40	+0.1	End	Off	On
0.41	–2.0		Off	On
0.42	–2.0		Off	On
0.43	+0.6		Off	On
0.44	–0.1		Off	On
0.50	–0.1		Off	On

Reference electrode in Ag/AgCl mode.

* Additional waveform commands used in the Dionex ICS-3000; not used in our older systems.

Preparation of Solutions and Reagents

Eluents

It is essential to use high-quality water of high-resistivity (18 MΩ-cm) and containing as little dissolved carbon dioxide as possible. Biological contamination should be absent. Source water must be obtained using a water purification system consisting of filters manufactured without electrochemically active surfactants or other compounds (for example, glycerol). Prior filtration through 0.2 µm porosity nylon under vacuum is recommended to remove particulates and reduce dissolved air. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of helium or high-purity nitrogen at all times to reduce diffusion of atmospheric carbon dioxide and opportunistic microorganisms. Dionex (now part of Thermo Scientific) Technical Note 71[10] provides details for good eluent preparation practices.

200 mM Sodium Hydroxide

Combine 20.8 mL of 50% sodium hydroxide with 1979 g of vacuum filter-degassed water to make 2 L.

Stock Standards

Solid carbohydrate standards were placed in plastic vials and dissolved in deionized water to a 200 mg/mL concentration, except xylose (400 mg/mL). These solutions were combined and diluted with water to yield 13.9 mg/mL arabinose, 37.7 mg/mL glucose, 209 mg/mL xylose, 1.31 mg/mL mannose, 5.94 mg/mL galactose, 3.99 mg/mL fructose, and 0.28 mg/mL cellobiose. This mix was diluted 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9; v:v in water to make the concentrations used for calibration and chosen for each monosaccharide standard, to encompass the expected concentrations for each in acid-hydrolyzed corn stover. All dilutions were made gravimetrically to ensure high accuracy, and maintained frozen at –40 °C until needed. Although a 10-point calibration was used in this note to detect any nonlinearity, if present, it is not needed for routine use. (Carbohydrates are hygroscopic. Significant errors due to moisture content in the solids used to make standards may occur. Drying solids under high vacuum and slightly elevated heat [for example, 40–50 °C] can significantly reduce this error for some carbohydrates.)

Sample Preparation

Acid-hydrolyzed corn stover acid (pH ~1.8) was donated by the National Renewable Energy Laboratory (NREL).[2] This sample was centrifuged at 16000 × g for 10 min to ensure elimination of particulates. The supernatant was directly injected into the HPAE-PAD system for analysis. To assess recovery, the supernatant was combined with the highest concentration standard mix in the proportion of 9:1, 8:2, 7:3; v:v of supernatant to standard.

Column:	Dionex CarboPac PA1, 4 mm
Eluent:	200 mM NaOH
Flow Rate:	1.0 mL/min
Column Temp:	30 °C
Inj. Volume:	0.2 µL
Detection:	PAD, Au working electrode, 15 mil gasket, four-potential carbohydrate waveform.
Samples:	1. Carbohydrate standard mixture (consisting of 10 mg/mL arabinose, 4 mg/mL galactose, 27 mg/mL glucose, 1 mg/mL mannose, 3 mg/mL fructose, 0.2 mg/mL cellobiose, 150 mg/mL xylose) 2. Undiluted acid-hydrolyzed corn stover
Peaks:	1. Arabinose 2. Mannose, glucose, xylose, galactose 3. Fructose* 4. Cellobiose*

*And unidentified substances for the acid-hydrolyzed corn stover

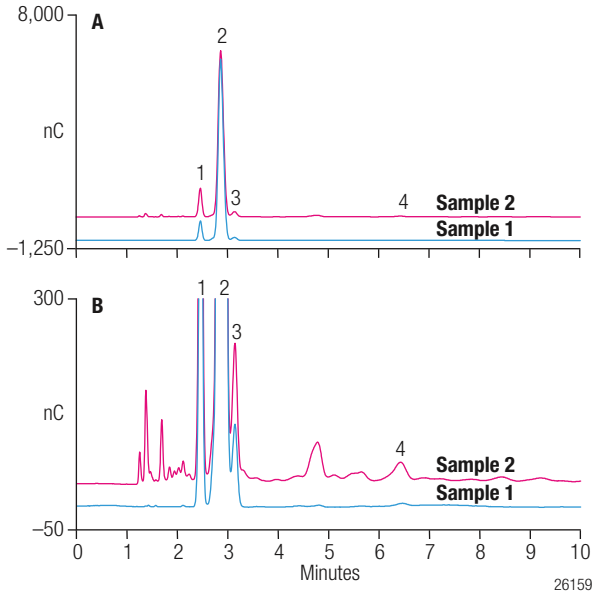

Figure 1. Separation of undiluted acid-hydrolyzed corn stover sugars.

Results and Discussion

Separation

Figure 1 shows the rapid analysis of acid-hydrolyzed corn stover for arabinose, glucose, galactose, xylose, mannose, fructose, cellobiose, and other carbohydrates using a Dionex CarboPac PA1 column. Panel A shows the full display of the major carbohydrate peaks for both the carbohydrate standard mix (chromatogram 1) and the undiluted acid-hydrolyzed corn stover (chromatogram 2). Peak 1 is arabinose, peak 2 is a mixture of glucose, xylose, galactose, and mannose, peak 3 is fructose, and peak 4 is cellobiose. Panel B reduces the range of the signal axis to view minor peaks, with alditols, glycols, alcohols, and other compounds eluting before arabinose, and oligosaccharides eluting after fructose. The identities of these minor peaks were not determined; however, Table 1 presents the retention times for many common carbohydrates found in plant-derived biomass or fermentation broths using this method. This isocratic method was optimized for throughput, and thus coelution of carbohydrate species was intended for simplification of the quantification described below. Both fructose (peak 3) and cellobiose (peak 4) coelute with other unknown substances and accurate measurements are not expected for these substances.

Table 1. Retention times* of carbohydrates found in plant-derived biomass or fermentation systems.

Analyte	Retention Time (min)	Analyte	Retention Time (min)
Void	1.2	Glucose	2.9
Propylene Glycol	1.3	Xylose	2.9
Glycerol	1.4	Galactose	2.9
myo-Inositol	1.4	Fructose	3.2
Erythritol	1.5	Sorbose	3.2
Xylitol	1.6	Ribose	3.3
Sorbitol	1.8	Melibiose	3.7
Galactitol	1.9	Lactose	4.5
Trehalose	2.2	Sucrose	5.4
Mannosamine	2.3	Turanose	6.2
Galactosamine	2.4	Gentiobiose	6.3
Glucosamine	2.4	Cellobiose	6.5
Arabinose	2.5	Palatinose	6.7
N-Acetylglucosamine †	2.6	Raffinose	8.5
Lyxose	2.7	Maltose	9.2
Mannose	2.7	Maltotriose	27.1
Arabitol	1.7		
Ribitol	1.9		
Fucose	1.9		
Rhamnose	2.1		

* Dionex CarboPac PA1 column, 200 mM NaOH, 1.0 mL/min, 30 °C column temperature. Retention times can vary slightly from this table for system-to-system, or day-to-day operation.

† Not expected in acid-hydrolyzed biomass, but may be present following enzymatic digestion.

Detection

Linearity

A calibration curve was produced for total carbohydrates injected, using a mixture of carbohydrates having the same relative concentrations found in acid-hydrolyzed corn stover, and having total concentrations above and below that found in this sample. The r^2 for total peak area (peaks 1–4) plotted against the total monosaccharide standard concentration injected was 0.9997. The percent errors found for the standards measured against the calibration curve ranged from 0–2%, consistent with a high correlation coefficient.

Accuracy

Three different concentrations of a mixture of carbohydrate standards were spiked into acid-hydrolyzed corn stover. Analyte recoveries were determined from the ratio of a measured increase to the theoretical concentrations spiked. The recoveries ranged from 80–120% for each of the first four carbohydrate peaks (1–4, Figure 1). Peaks of unknown identity in the sample, resolved at lower hydroxide eluent concentrations, coelute with fructose (peak 3), and cellobiose (peak 4), and prevent accurate measure of these carbohydrates. When the total peak area of these four peaks was considered along with the total carbohydrate amounts spiked into acid-hydrolyzed corn stover, the percent recoveries ranged from 85–96%.

The high recovery indicates that in spite of the poor accuracy of peaks 3 and 4, their minor contribution to the total amounts of monosaccharide in the corn stover hydrolysate still enables acceptable estimations of monosaccharide (in addition to cellobiose) concentration in these samples. Because peak 2 contains more than one monosaccharide, and the electrochemical response for coeluting sugars will not be a simple sum of the responses for individual sugars, we evaluated a simple mixture to estimate the error. When the ratios of glucose:xylose were 0:1, 1:2, 1:1, 2:1, 1:0 (w:w; at a fixed 100 mg/mL total sugar concentration), the peak area response factors (area/µg injected) were 27.8 ± 0.2, 28.9 ± 0.2, 30.0 ± 0.2, 30.2 ± 0.4, 30.5 ± 0.1 nC*min per µg, respectively (n = four injections in each treatment). Galactose (100 mg/mL) was 30.8 ± 0.1 nC*min per µg.

The variation in response factors between a glucose and xylose mixture, and galactose, shows that this rapid method will only provide an estimate of the sugar content of the acid-hydrolyzed corn stover. Accurate determinations of each acid-hydrolyzed corn stover carbohydrate requires complete separation (not presented here). The total monosaccharide content of the corn stover hydrolysate sample used in this study was estimated to be 199 mg/mL. The reported concentration for this sample was 137 mg/mL, without the mannose or fructose that was included in our determinations.[11] The total monosaccharide content for this sample, analyzed using a method designed to resolve these carbohydrates, was 190 mg/mL, or 184 mg/mL with mannose and fructose omitted.

Precision

The peak area and retention time RSDs were determined for nine injections of a mixture of standards at a concentration of 193 g total carbohydrates/L over one day, when interspersed with 20 injections of undiluted acid-hydrolyzed corn stover. Retention time RSDs ranged from 0.16–0.26%, and peak area RSDs for the four carbohydrate peaks in acid-hydrolyzed corn stover ranged from 0.7–1.3% (4.8% for cellobiose). No loss in retention time or peak area was observed for the standards or sample over these 29 injections.

Method Considerations

The Dionex CarboPac PA1 guard column (4 × 50mm, P/N 043096) was omitted from this study to reduce run times. Although high performance of the analytical column was maintained through the duration of this study for acid-hydrolyzed corn stover, when other types of samples containing lipids, large amounts of soluble polysaccharides, or other highly retained substances are analyzed, the addition of the guard column is recommended to help protect the analytical column from damage. Periodic column washes (using the procedures described in the column manual) will help restore retention times if significant shifts are observed that are not due to eluent preparation errors. Continuous operation at low flow rates of 0.1 mL/min with the cell turned on is the preferred standby mode. For extensive shutdown periods longer than several weeks, the column should be stored in 200 mM NaOH, the system should be flushed

with deionized water, and the electrochemical cell disassembled with the reference electrode stored in its original or freshly prepared potassium chloride solution (3.5 M) and shipping container. The reference electrodes must be replaced every six months, and disposable gold working electrodes replaced every two weeks, or with cell reassembly following system shutdown. The estimated lower limit of detection and limit of quantification for glucose was 0.7 ppm and 2.3 ppm, respectively.

Conclusion

The HPAE-PAD system with high-carbohydrate concentration accessories enables a fast (10 min) estimation of total free monosaccharides in undiluted acid-hydrolyzed corn stover. This allows the scientist to quickly estimate the product yield.

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List of Suppliers

J.T. Baker Incorporated, 222 Red School Lane, Phillipsburg, New Jersey, 08865, USA, 1-800-582-2537.

Fisher Scientific, 711 Forbes Avenue, Pittsburgh, Pennsylvania, 15219-4785, USA, 1-800-766-7000.

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, USA, 1-800-325-3010.

Praxair, 39 Old Ridgebury Road, Dansbury, CT 06810-5113, USA. Tel: 1-877-772-9247.

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