Application Update 192

# Carbohydrate Determination of Biofuel Samples

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**Key Words**
Dionex CarboPac SA10 Carbohydrate Column, 62 mil Gasket, 0.4 µL Injection Valve, Biomass

## Introduction

Biofuels (i.e., fuels derived from plant materials and animal wastes) have emerged as an attractive alternative to fossil fuel.[1,2] The cellulosic biomass—the starches and sugars in plant/animal matter—are typically broken down by the application of heat and/or chemicals (pretreatment) followed by enzymatic digestion and fermentation. To maximize the biofuel yield, it is critical to quantify the released carbohydrates during biofuel production. A large number of samples must be analyzed during optimization of the biofuel production processes. Hence, there is a need for a fast, robust, accurate, and quantitative analytical method for carbohydrate determination of biomass samples.

The sugars in biomass samples are often quantified by high-performance liquid chromatography using refractive index (RI) detection. However, that method has long analysis times (45–60 min), poor resolution of some of the biofuel carbohydrates (e.g., arabinose–mannose, sucrose–cellobiose), and possible coelution with other compounds in the sample.[3]

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) overcomes the limitations of the RI-based method and delivers fast carbohydrate analysis, as documented in Thermo Scientific Application Note 282.[4] However, due to the high concentration of the biofuel samples, a several-fold sample dilution must be performed before analysis and that introduces a possible source of error. In this study, two hardware modifications have been applied to handle high-concentration samples:

- A reduced injection volume from 10 to 0.4 µL (P/N 074699)
- An increase in the thickness of the spacer gasket to 62 mil (from 15 mil used in the earlier document) in the electrochemical flow cell to reduce detection sensitivity (P/N 085324)

The combination of a smaller sample injection and reduced sensitivity at the point of detection reduces the degree of dilution required when determining carbohydrates in samples derived from biomass. Overall, the new method is fast, provides good sensitivity, has consistent response, and can be routinely used to determine carbohydrates in biofuels.

## Goal

To reduce the amount of sample dilution required for a rapid HPAE-PAD method to determine the carbohydrates of interest in biomass samples with high carbohydrate concentrations (e.g., acid-hydrolyzed corn stover)

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## Equipment, Software, and Consumables

- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ Ion Chromatography (IC) system, including:
  - SP Single or DP Dual Pump, Gradient or Isocratic, with the vacuum degas option installed (P/N 063353)*
  - EG Eluent Generator
  - DC Detector/Chromatography Compartment
  - High-Pressure Injection Valve with 0.4 µL Internal Sample Loop (P/N 074699)
  - Electrochemical Detector (P/N 079830) and Cell
  - Gold on PTFE Disposable Electrode (P/N 066480)
  - pH, Ag/AgCI Reference Electrode (P/N 061879)
  - High Concentration Carbohydrate Analysis Kit (includes 62 mil PTFE gasket and modified spacer block) (P/N 085324)
- Thermo Scientific Dionex AS-AP Autosampler
- Thermo Scientific Dionex Potassium Hydroxide Eluent Generator Cartridge (EGC III KOH) (P/N 074532)
- Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column (P/N 060477)
- Thermo Scientific Dionex™ Chromeleon™ Chromatography Data System (CDS) software
- EO Eluent Organizer, including 2L plastic bottles and pressure regulator
- Vial Kit, 0.3 mL Polyprop. with Caps and Septa (P/N 055428)
- Thermo Scientific™ Nalgene™ MF75™ Series Sterile Disposable Tissue Culture Filter Units, 1000 mL, 0.2 µm (Fisher Scientific P/N 09-740-46)

* Refer to Dionex ICS-3000 EG Vacuum Degas Conversion Kit Installation Instructions (Document No. 065067) for more information.

## Reagents and Standards

### Reagent

Deionized (DI) water, Type I reagent grade, 18 M -cm resistivity or better, filtered through a 0.2 µm filter immediately before use

### Standards

- L(–)-Fucose (Fisher Scientific P/N AC22588-0010)
- D-Galactose (Fisher Scientific P/N S25334)
- D(+)-Mannose (Fisher Scientific P/N AC15060-1000)
- D-Fructose (Fisher Scientific P/N L96-500)
- D-Xylose (Fisher Scientific P/N X9-25)
- Sucrose (Fisher Scientific P/N S5500)
- D-Glucose (Fisher Scientific, P/N D16-1)
- D-Arabinose (Fisher Scientific P/N S25650)
- D(+)-Cellobiose (Fisher Scientific P/N AC108460250)

| Conditions | |
|---|---|
| Columns: | Thermo Scientific™ Dionex™ CarboPac™ SA10 Guard, 4 × 50 mm (P/N 074902)<br>Dionex CarboPac SA10 Analytical, 4 × 250 mm (P/N 074641) |
| Eluent: | 1 mM Potassium Hydroxide (KOH) |
| Eluent Source: | Dionex EGC III KOH Eluent Generator Cartridge with Dionex CR-ATC Continuously Regenerated Anion Trap Column |
| Flow Rate: | 1.5 mL/min |
| Injection Volume: | 0.4 µL (full loop) |
| Column Temperature: | 40 °C |
| Cell Temperature: | 30 °C |
| Backpressure: | 2500 psi |
| Detection: | PAD |
| Background: | 30–70 nC |
| Working Electrode: | Gold on PTFE Disposable Electrode |
| Electrochemical Cell Gasket: | 62 mil |
| Reference Electrode: | pH, Ag/AgCl |
| Mode: | Ag/AgCl mode |
| Noise: | 30–60 pC |

## Carbohydrate Waveform

### Carbohydrate 4-Potential Waveform for the Electrochemical Detector

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

* Settings required in the Dionex ICS-3000/5000 IC systems but not used in older Dionex IC systems

Reference electrode in Ag/AgCl mode

## Preparation of Solutions and Reagents

### Eluent Solution

#### 1 mM KOH

Generate the KOH eluent on line by pumping high-quality degassed DI water through the Dionex EGC II KOH cartridge. Chromeleon CDS software tracks the amount of KOH used and calculates the remaining cartridge lifetime. Although eluents can be prepared manually, running this application with eluents prepared by an eluent generator is strongly recommended; using manually prepared eluents is not recommended. Consistent preparation of a 1 mM or a 10 mM hydroxide eluent (if proportioning is used) is difficult due to variable carbonate contamination. The impact of carbonate contamination is significant when using low-concentration hydroxide eluents. If eluents must be prepared manually, use sodium hydroxide rather than KOH and prepare according to the general instructions for hydroxide eluents in Thermo Scientific Technical Note 71.[5] For this application, electrolytic eluent generation delivers superior performance; the use of manually prepared eluents is strongly discouraged and the results are not guaranteed.

### Stock Standard Solutions

Dissolve solid standards in DI water to prepare a 200 g/L stock solution for each carbohydrate (Table 1). Maintain the stock solution at -20 °C until needed.

### Working Standard Solutions

Prepare working standards in DI water by diluting the stock solutions. Store working standards at 4 °C. Prepare all dilutions gravimetrically to ensure high accuracy.

## Sample Preparation

### Corn Stover and Wood Hydrolysates

Centrifuge corn stover and wood hydrolysate samples at 16,000 g for 10 min to ensure elimination of particulates, then inject at dilutions of 1/10 or 1/50, respectively, with DI water for analysis.

Acid-hydrolyzed corn stover was donated by the National Renewable Energy Laboratory in Boulder, Colorado.

### Precautions

The treated biomass samples have high concentrations of sugars such as xylose, glucose, and galactose, which can cause carryover. A syringe flush of 500 µL DI water is recommended between samples to reduce carryover. A column wash at 100 mM KOH for 20 min is recommended if retention time shifting is observed. The application of 100 mM KOH changes the system equilibrium; re-equilibration at 1 mM for 30 min is recommended to achieve high precision. Replace the reference electrode every six months and replace the disposable working electrode every four weeks.

Table 1. Precisions and calibration for biofuel sugars.

| Analyte | RT (min) | Retention Time (RT) Precision (RSD)[a] | Peak Area (nC* min) | Peak Area Precision (RSD)[a] | Coefficient of Determination ($r^2$) |
|---|---|---|---|---|---|
| Sucrose | 3.359 | 0.16 | 3.6493 | 1.04 | 0.99729 |
| Arabinose | 3.700 | 0.19 | 4.6796 | 1.01 | 0.99832 |
| Galactose | 3.925 | 0.18 | 5.9078 | 1.21 | 0.99853 |
| Glucose | 4.309 | 0.16 | 6.3237 | 0.96 | 0.99754 |
| Xylose | 4.750 | 0.14 | 5.6679 | 0.95 | 0.99645 |
| Mannose | 5.025 | 0.21 | 5.4818 | 1.27 | 0.99450 |
| Fructose | 5.350 | 0.13 | 6.6884 | 2.47 | 0.99913 |
| Cellobiose | 7.851 | 0.18 | 4.2204 | 0.92 | 0.98452 |

[a] Relative standard deviation, n = 6

## Results and Discussion

### Separation

Figure 1, Chromatogram A shows the separation of sugars in a 2.5 g/L standard mix (except fucose, which is used as an internal standard at 200 ng/mL): fucose, sucrose, arabinose, galactose, glucose, xylose, mannose, fructose, and cellobiose. All nine sugars elute in <9 min. Figure 1, Chromatogram B shows the sugars in an acid-hydrolyzed corn stover sample (dilution 10-fold). The sugars present in this sample were glucose, xylose, and minor amounts of arabinose and galactose (fucose was used as an internal standard). The resolution of these sugars in a short run time demonstrated that this method is suitable for on-line monitoring of samples for biofuel production.

### Linear Range

In biomass samples, some of the sugars are present in concentrations ranging from 50 to 100 g/L, whereas the minor components are present in the 0.1 to 10 g/L range. Using the modifications suggested in this study (i.e., reduced sample volume and a thicker gasket at the electrode), samples can be analyzed after a 10- or 50-fold dilution.

The linearity of the method was determined by injecting calibration standards in triplicate, ranging from 0.1 to 3 g/L. A representative calibration plot is shown in Figure 2 for galactose and the calibration data for all the sugars are summarized in Table 1. The coefficients of determination obtained from the calibration curves were between 0.98452 and 0.99913, using linear least squares regression analysis.

Mean response factors (normalized peak area/concentration for each carbohydrate with fucose used as the internal standard) observed between 0.1 and 3.0 g/L for galactose are shown as ◆ in Figure 3. The solid horizontal lines indicate the plus and minus 10% of the average of the observed response factors (average response factor indicated by the dotted horizontal line). Similar behavior was observed for each of the other sugars.

### Precision

Short-term peak area and retention time precisions were determined for six replicate injections of a mixture of sugar standards. The concentration used for precision injections was 1.0 mg/mL for each of the biofuel sugars. The retention time precisions (RSD) ranged from <0.01 to 0.12%. The peak area precisions were in the range of 1.7–2.7%. The high retention time precisions are attributed to consistent generation of high-purity KOH using the eluent generator. With manually prepared mobile phases, the precisions—especially retention time precision—will almost certainly not be as low as with eluent prepared by an eluent generator.

| | | | |
|---|---|---|---|
| Columnns: | Dionex CarboPac SA10, Guard (4 × 50 mm) | Peaks: | 1. Fucose |
| | Dionex CarboPac SA10, Analytical (4 × 250 mm) | | 2. Sucrose |
| Eluent: | 1 mM KOH | | 3. Arabinose |
| Eluent Source: | Dionex EGC III KOH cartridge | | 4. Galactose |
| Flow Rate: | 1.5 mL/min | | 5. Glucose |
| Temp: | 40 °C | | 6. Xylose |
| Detection: | PAD (Au) | | 7. Mannose |
| Samples: | A. Mix of sugars | | 8. Fructose |
| | B. Corn stover sample, 1/10 dilution | | 9. Cellobiose |

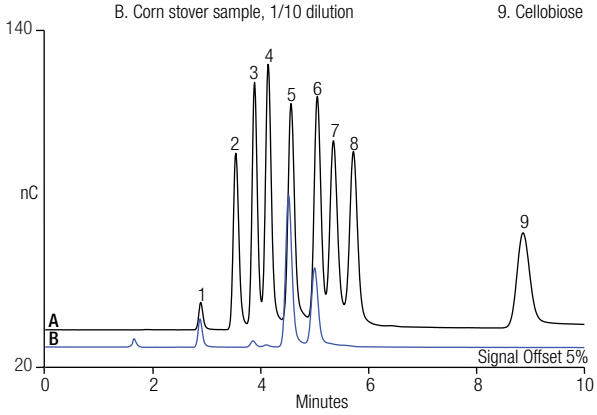


Figure 1. Separation of biofuel sugars (A) and an acid-hydrolyzed (diluted 10-fold) corn stover sample (B) using the Dionex CarboPac SA10 column.

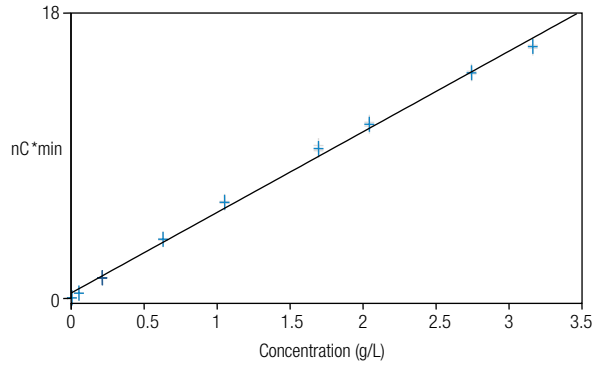


Figure 2. Calibration plot for galactose in the concentration range of 0.1–3 g/L.

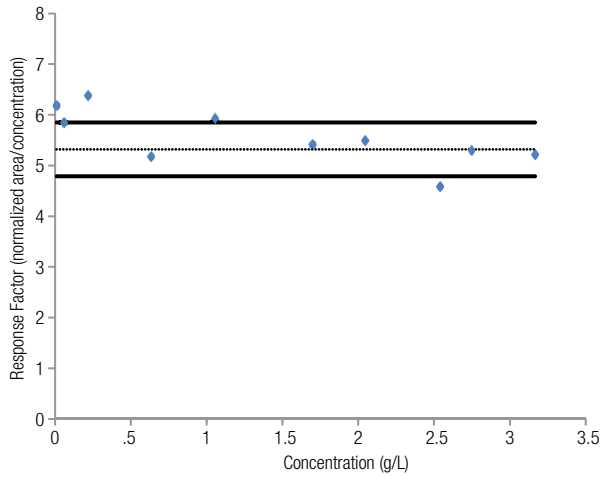


Figure 3. Detector response plot for galactose in the concentration range of 0.1–3 g/L.

## Robustness

Method robustness was tested by evaluating the peak area and retention time stabilities of injections of a 1 g/L mix of standards interspersed with biofuel sample injections. Table 2 summarizes the retention time and peak area precisions for the 1 g/L standard over 315 injections (including 245 corn stover samples and 73 standards injections). A 1.5% decrease in retention time was observed over 80 injections (from 4.06 to 3.96 min for galactose). A column wash (as described in the Precautions section) will restore the retention time. The robustness data shows that the method can be routinely used for handling a large number of biomass samples with high concentrations of carbohydrates.

Table 2. Summary of robustness data.

| Analyte | RT (RSD) | Peak Area (RSD) |
|---|---|---|
| Fucose | 0.97 | 2.77 |
| Sucrose | 1.72 | 2.63 |
| Arabinose | 1.48 | 2.27 |
| Galactose | 1.65 | 2.26 |
| Glucose | 1.75 | 2.62 |
| Xylose | 1.85 | 2.27 |
| Mannose | 1.90 | 3.00 |
| Fructose | 2.10 | 4.36 |
| Cellobiose | 3.18 | 2.57 |

## Comparison of the 62 mil Gasket to the 15 mil Gasket

The thicker gasket extends the linear calibration range to higher concentrations. Increasing the gasket thickness decreases the linear flow rate at the electrode. Some of the consequences of using a thicker gasket that influence the calibration outcome are:

- Increased coulombic efficiency
- Longer residence time at the electrode
- Lower proportion of an analyte reaching the electrode surface by mass transport

Table 3 summarizes the decrease in response of the biofuel sugars with the 62 mil gasket relative to the 15 mil gasket. Due to the increased linear range of the method, carbohydrates present in major and minor amounts can generally be measured using a 10-fold dilution instead of the increased-fold dilution (or multiple dilutions) needed with the 15 mil gasket.

Table 3. Response with the 62 mil gasket relative to the 15 mil gasket.

| Sample | Fucose | Sucrose | Arabinose | Galactose | Glucose | Xylose | Mannose | Fructose | Cellobiose |
|---|---|---|---|---|---|---|---|---|---|
| Mix of Sugars 0.1 g/L | 62% | 55% | 57% | 51% | 51% | 54% | 67% | 63% | 43% |
| Mix of Sugars 1.0 g/L | 63% | 70% | 66% | 64% | 64% | 65% | 77% | 73% | 48% |
| Mix of Sugars 2.0 g/L | 62% | 77% | 69% | 71% | 69% | 69% | 81% | 78% | 51% |
| Mix of Sugars 3.0 g/L | 62% | 84% | 72% | 78% | 74% | 72% | 84% | 84% | 54% |

## Conclusion

This study describes a rapid and robust HPAE-PAD method for the accurate determination of common sugars in acid-hydrolyzed biomass samples. The method uses the Dionex CarboPac SA10 column with electrolytically generated hydroxide eluent, reduced sample size, and a thicker gasket for the working electrode. The method is shown to have a linear range suitable for handling high-concentration biomass samples with minimal sample treatment.

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