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APPLICATION NOTE 72610

Determination of carbohydrates in algal biofuel samples

Authors

Sachin Patil,[1] Mary Tran-Gyamfi,[2] Ryan W. Davis,[2] Jeffrey Rohrer[1]

[1]Thermo Fisher Scientific, Sunnyvale, CA

[2]Biomass Science and Conversion Tech, Sandia National Laboratories, Livermore, CA

Keywords

Dionex ICS-5000+, RFIC system, Dionex CarboPac PA20-4µm column, microalgae, biofuel, uronic acid, sugar

Goal

To demonstrate two methods for the determination of key sugars present in acid-hydrolyzed algal biomass

Introduction

Microalgae have received significant attention not only because of their potential to be a viable feedstock for bio-based production of transportation fuels[1] but also because of the nutritional value and health benefits of algae-based foods.[2] The breakdown of fermentable carbohydrates present in algal biomass is used for monitoring the efficiency of biomass-to-biofuel conversion, and is directly related to target biofuel yield and process economics. In addition, a complete characterization of the carbohydrate breakdown products in the algae is essential for efficient nutrient recycling to determine which sugars are best absorbed by the algae. Carbohydrate content in microalgal biomass often contains complex mixtures of C5 and C6 sugars, requiring a separation technique with high resolution and high sensitivity. Hence, the determination of carbohydrates in algal biomass is a crucial step in designing a production process. However, the development of robust analytical methods remains a challenge.

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High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) can be used to determine carbohydrates in these samples. HPAE-PAD has been shown to deliver fast carbohydrate determinations in lignocellulosic biomass hydrolysate samples in Thermo Scientific Application Note 1089.[3] Unfortunately, this method is unable to resolve the key amino sugars galactosamine and glucosamine from other sugars present in the algae biomass.

The current work describes two methods that use a Thermo Scientific™ Dionex™ CarboPac™ PA20-4µm column for the determination of key sugars present in acid-hydrolyzed algal biomass. The first is a short method that separates twelve common carbohydrate sugars in less than fifteen minutes. This method does not separate uronic acids, which are key components that are present at low concentrations. Because the uronic acids bind to the column strongly, acetate ions are used as a pusher, which results in elongation of the total method run time. Therefore, a second longer method was designed that separates the two uronic acids in addition to the twelve other sugars separated by the shorter method. When a quick quantification of the sugars present in the samples is desired, the short method can be used. Whereas, when a detailed sugar composition calculation is desired, the long method can be used. Both methods can be conveniently run at the same time on a dual ion chromatography system such as the Thermo Scientific™ Dionex™ ICS-5000+ IC system used here. Both methods were validated for precision, accuracy, and robustness during routine use.

Experimental

Conditions short method

Columns:	Dionex CarboPac PA20-4µm, 2 × 100 mm (P/N 302749) Dionex CarboPac PA20-4µm, 2 × 30 mm Guard (P/N 302750)
Column Temp.:	32 °C
Compartment Temp.:	30 °C
Flow Rate:	0.27 mL/min
Working Electrode:	Gold disposable on PTFE (P/N 066480)
Electrochemical Cell Gasket:	2 mil
Reference Electrode:	pH, Ag/AgCl
Sampler Tray Temp.:	4 °C
Inj. Volume:	2.5 µL (full loop mode)
Eluent Source:	Thermo Scientific™ Dionex™ EGC 500 KOH Eluent Generator Cartridge (P/N 075778) with Thermo Scientific™ Dionex™ CR ATC 500 Continuously Regenerated Anion Trap Column (P/N 075550)
Typical Backpressure:	3500 psi
Run Time:	20.5 min

Elution Conditions:

Time (min)	*Elution*
0	1.5 mM potassium hydroxide
9.9	1.5 mM potassium hydroxide
9.91	100 mM potassium hydroxide
14.5	100 mM potassium hydroxide
14.51	Re-equilibrium, 1.5 mM potassium hydroxide
20.5	End

Carbohydrate Waveform:

Time (s)	*Potential (V)*	*Integration*
0.00	+0.10	
0.20	+0.10	Begin
0.40	+0.10	End
0.41	-2.0	
0.42	-2.0	
0.43	+0.6	
0.44	-0.1	
0.50	-0.1	

Conditions long method

Columns:	Dionex CarboPac PA20-4µm, 2 × 100 mm (P/N 302749) Dionex CarboPac PA20-4µm, 2 × 30 mm Guard (P/N 302750)
Column Temp.:	32 °C
Compartment Temp.:	30 °C
Flow Rate:	0.22 mL/min
Eluent:	A) DI water B) 0.1 M sodium acetate in 0.1 M sodium hydroxide C) 0.2 M sodium hydroxide
Working Electrode:	Gold disposable on PTFE (P/N 066480)
Sampler Tray Temp.:	4 °C
In. Volume:	2.5 µL (full loop mode)
Typical Backpressure:	2700 psi
Run Time:	39 min
Elution Conditions:	Table 1

Carbohydrate Waveform:	*Time (s)*	*Potential (V)*	*Integration*
	0.00	+0.10	
	0.20	+0.10	Begin
	0.40	+0.10	End
	0.41	-2.0	
	0.42	-2.0	
	0.43	+0.6	
	0.44	-0.1	
	0.50	-0.1	

Table 1. Elution conditions for the long method

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)	Elution
0	99	0	1*	2 mM sodium hydroxide
8	99	0	1	2 mM sodium hydroxide
13	50	0	50	100 mM sodium hydroxide
13	0	30	70	30 mM sodium acetate in 170 mM sodium hydroxide
19	0	100	0	100 mM sodium acetate in 100 mM sodium hydroxide
22	0	100	0	100 mM sodium acetate in 100 mM sodium hydroxide
22	0	0	100	200 mM sodium hydroxide
28	0	0	100	200 mM sodium hydroxide
28	99	0	1	Re-equilibrium
39	99	0	1	end

*The proportioning level used here is right at the performance limit of the proportioning valve. The beginning of a valve failure, which would not be observed with many methods, would likely be recognized by this method.

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free Ion Chromatography (RFIC™) system was used in this work. The Dionex ICS-5000+ IC system is a modular ion chromatograph that includes:
  - SP Single Pump module (P/N 061707) or DP Dual Pump module (P/N 061712) with degas option
  - DC Detector Compartment (P/N 061767) with single-temperature zone
  - Electrochemical Detector (P/N 061719) and cell (P/N 061757)
  - pH-Ag/AgCl Reference Electrode (P/N 061879)
  - Carbohydrate Disposable Au Working Electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 066480)
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Thermo Scientific™ Dionex™ VP vacuum pump package (P/N 066463)
- Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt™ P/N 72.692.005)
- Thermo Scientific™ Nalgene™ Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Syringe filter, 0.2 µm GHP, 13 mm (Waters® P/N WAT097962)

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- L(–)-Fucose (Sigma®, P/N F2543)
- D-Galactose (Sigma, P/N G0625)
- D(+)-Mannose (Sigma P/N M6020)
- D-Fructose (Sigma P/N F2543)
- D-Xylose (Aldrich® P/N X-10705)
- D-Glucose (P/N 1910-01)
- D(-)-Arabinose (Sigma P/N A3131)
- D(+)-Cellobiose (Sigma P/N C7252)
- D(+)-Galacturonic acid (Sigma P/N 48280)
- D-Mannuronic acid (Sigma P/N SMB00280)
- D(-)-Ribose (Sigma P/N R7500)
- D-Mannitol (Sigma P/N M4125)
- D(+)-Galactosamine Hydrochloride (Sigma P/N G0500)
- D(+)-Glucosamine Hydrochloride (Sigma P/N G4875)

Samples

Samples of *Microchloropsis salina* CCMP 1776 (formerly *Nannochloropsis salina*)[4] biomass were produced at the Sandia Algae Testbed Facility in 1000 L (4.2 m^2 surface area) pilot-scale environmentally simulated open raceway ponds (ORP). Inocula were generated in 80 L air bubble column photobioreactors under constant illumination and transferred to ORPs at an optical density of 0.8 at 10% v/v in L1-enriched artificial seawater (Table 2). Following inoculation, the ORPs were subjected to air sparging (300 L/min) and paddle wheel mixing (9 RPM). Diurnal light/temperature cycling corresponded to <25–1400 µmol/photons/m^2/s and 18–23 ± 1 °C for night and day, respectively. Under semi-continuous harvesting conditions, the net productivity of the cultures was 250 ± 25 mg/L on the ash-free dry weight basis. Biomass for analysis was dewatered using a continuous flow centrifuge operating at 6000 RPM (bowl radius 12", ~1 L/min throughput) to achieve a 24% solids paste, which was stored at -80 °C prior to hydrolysis.

Acid hydrolysis

Individual hydrolysis reactions were set in screw cap vials by mixing 30 mg of acid neutralized and freeze dried algae biomass sample with 1 mL of 2 M TFA. All vials were incubated for 24 h at 102 °C in an oven. The experiment also included a negative control. Instead of acid, 1 mL DI water was added to the negative control sample. After 24 h all samples were allowed to cool for 10 min at room temperature and were centrifuged at 10,000 RPM for 3 min. The samples were then neutralized by adding 2 mL of 1 M NaOH to increase the sample pH to between 7 and 8. All the samples were filtered using 0.2 µm GHP syringe filters. This solution was brought to 10 mL with DI water. The samples were diluted 10-fold further with DI water to yield a 1000-fold total dilution. A 2.5 µL sample was injected directly onto the column.

Table 2. Chemical composition of L1-enriched artificial seawater medium

Medium Component	Concentration
Artificial seawater	
NaCl	362.7 mM
Na_2SO_4	25.00 mM
KCl	8.030 mM
$NaHCO_3$	2.067 mM
KBr	0.725 mM
H_3BO_3	0.433 mM
NaF	0.0657 mM
$MgCl_2 \cdot 6H_2O$	47.18 mM
$CaCl_2 \cdot 2H_2O$	9.134 mM
$SrCl_2 \cdot 6H_2O$	0.0214 mM
Nutrients	
$NaNO_3$	0.882 mM
Na_2HPO_4	0.0362 mM
Trace metals	
$Na_2EDTA \cdot 2H_2O$	11.7 µM
$FeCl_3 \cdot 6H_2O$	11.7 µM
$MnSO_4 \cdot 4H_2O$	0.909 µM
$ZnSO_4$	0.080 µM
$CoSO_4$	0.050 µM
$CuSO_4 \cdot 5H_2O$	0.010 µM
$Na_2MoO_4 \cdot 2H_2O$	0.082 µM
Na_2SeO_3	0.010 µM
$NiSO_4 \cdot 6H_2O$	0.010 µM
Na_3VO_4	0.010 µM
K_2CrO_4	0.010 µM
Vitamins	
Thiamine	296 nM
Vitamin B12	0.369 nM
Biotin	2.05 nM

Results and discussion

Separation

A representative chromatogram of a standard mix containing twelve of the common biofuel sugars at 0.4 mg/L each obtained using the short method on a Dionex CarboPac PA20-4µm 2 mm column set is shown in Figure 1A. For the short method, ten of the twelve peaks were well resolved with each injection requiring 20.5 min. To enable separation of uronic acids along with monosaccharides a longer method was designed. Figure 2 shows separation of a TFA-hydrolyzed algae sample using the long method. Galacturonic acid and mannuronic acid are two of the common uronic acids present in algae acid-hydrolyzed samples, although the samples prepared in this study did not show their presence. The total method run time is 39 min. The smaller particle size and shorter column format results in a faster run time. A method similar to the short method used to resolve fewer monosaccharides was 32 min.[6] The smaller column dimensions of the column used here also result in lower eluent consumption and improved process economy.

Linearity and precision

The linearity of monosaccharide determination was studied by generating response curves for ten sugars for the short method and twelve sugars for the long method. The sugar concentrations ranged from 0.02 mg/L to 2.0 mg/L. The linearity ranges and correlation coefficients are shown in Tables 3A and 3B for both methods, respectively. A linear curve fit was used for all of the sugars except glucosamine and galactosamine, for which a second order polynomial curve fit was used.

The method precision was determined at three injections of the 0.4 mg/L sugar mix. The results included in Tables 3A and 3B show excellent short-term peak area as well as retention time precision for all three concentration levels tested with all RSD values below 3%.

Accuracy

Accuracy of the assay was determined by spiking known amounts of sugars into the acid hydrolysates prepared for the algae samples used in the study. The sugar concentrations spiked were 0.2 and 0.5 mg/L. The spike levels and recoveries calculated using the short as well as long method are included in Tables 4A and 4B, respectively. The results show excellent recoveries of the spiked monosaccharides with all the recoveries falling between 80% and 120% for the majority of the sugars. The short method showed significantly lower recoveries for mannitol. This could possibly be due to an impurity that could not be separated from mannitol by the short method.

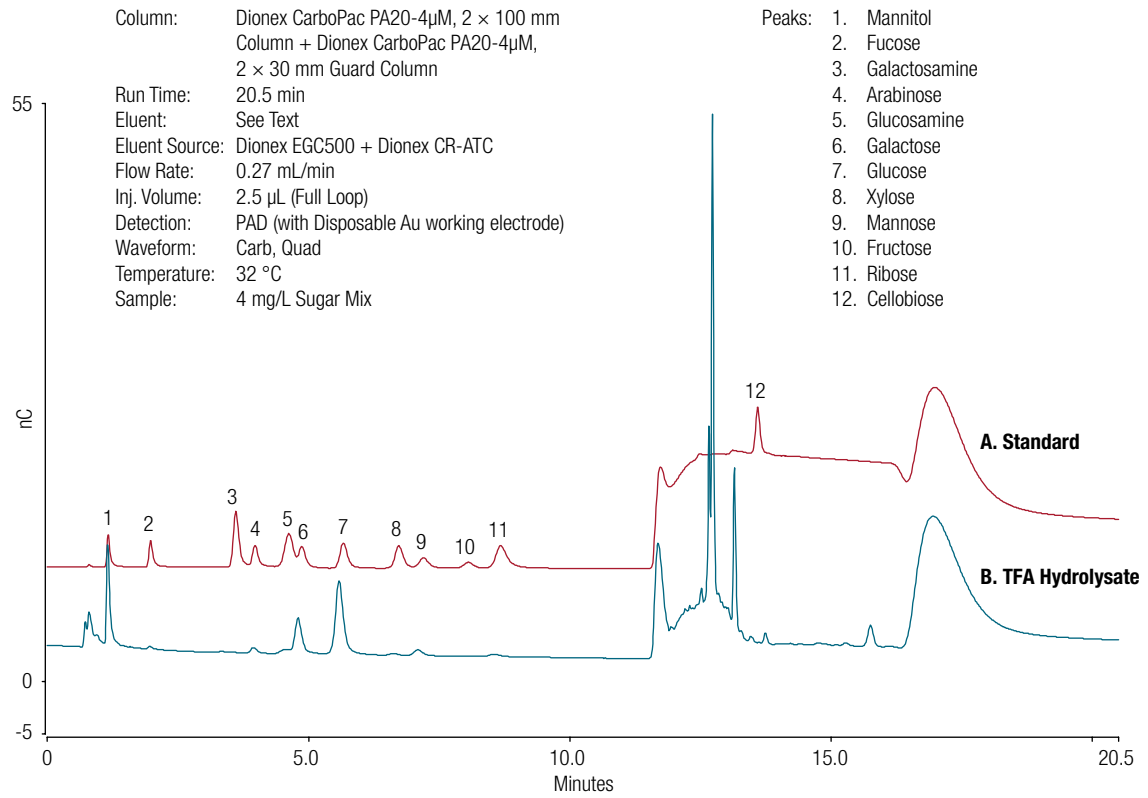

Figure 1. Short method for determination of sugars in TFA hydrolysed algae samples

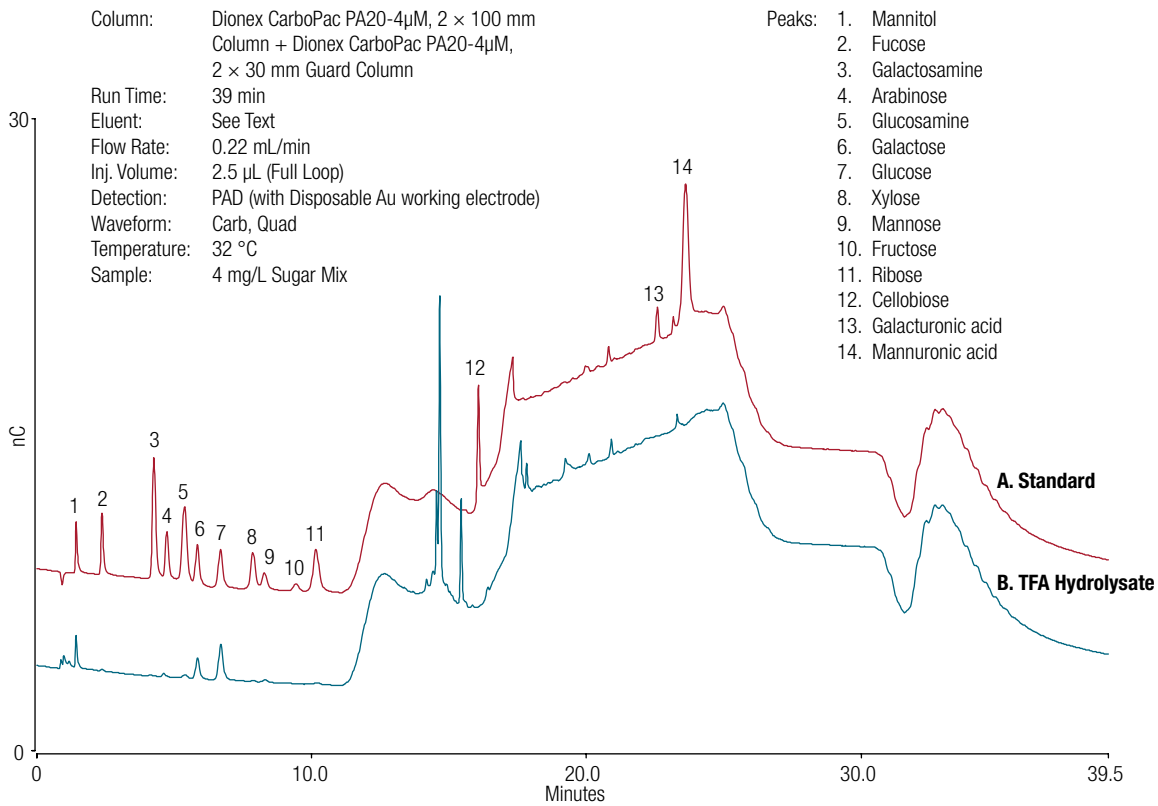

Figure 2. Long method for determination of sugars in TFA hydrolysed algae samples

Table 3A. Calibration and precision data for the short method (n=3)

Sugar Name	Conc. Range (mg/L)	Coefficient of Determination	Resolution	RT RSD	Peak Area RSD
Mannitol	0.02–1	0.997	6.86	0.41	1.14
Fucose	0.02–1	0.999	10.18	0.24	0.58
Galactosamine**	0.02–1	0.999	2.01	0.22	0.56
Arabinose	0.02–1	0.997	2.79	0.23	0.85
Glucosamine**	ND	ND	1.13	0.1	1.94
Galactose	ND	ND	3.39	0.16	2.67
Glucose	0.02–1.5	0.996	3.87	0.22	0.97
Xylose	0.02–1	0.997	1.54	0.25	0.67
Mannose	0.02–1	0.998	2.73	0.23	0.94
Fructose	0.02–1	1	1.78	0.27	0.43
Ribose	0.02–1	1	17.6	0.25	0.54
Cellobiose	0.02–1	0.999	-	0.1	1.60

Table 3B. Calibration and precision data for the long method (n=3)

Sugar Name	Conc. Range (mg/L)	Coefficient of Determination	Resolution	RT RSD	Peak Area RSD
Mannitol	0.02 to 2	0.998	6.63	0.34	1.61
Fucose	0.02 to 2	0.999	10.08	0.20	1.20
Galactosamine**	0.02 to 2	1.000	2.08	0.11	0.52
Arabinose	0.02 to 2	1.000	2.41	0.10	2.11
Glucosamine**	0.02 to 2	1.000	1.47	0.09	1.45
Galactose	0.02 to 2	0.998	3.03	0.16	2.85
Glucose	0.02 to 2	0.997	3.85	0.07	0.81
Mannose	ND	ND	1.27	0.11	1.18
Xylose	ND	ND	3.16	0.10	1.81
Fructose	0.02 to 1	0.996	1.85	0.09	2.46
Ribose	0.02 to 2	0.999	20.87	0.05	1.75
Cellobiose	0.02 to 2	1.000	41.61	0.00	1.72
Galacturonic acid	0.02 to 2	1.000	4.04	0.02	1.64
Mannuronic acid	0.02 to 2	0.999	-	0.02	0.25

** A second-order polynomial curve fit was used for galactosamine and glucosamine calibration curves. ND-Not determined

Table 4A (Part 1). Recovery of algae sugar spikes into the algae acid hydrolysates using the short method (n=3)

Spike Level (mg/L)	Mannitol		Fucose		Galactosamine		Arabinose	
	Avg Amount (mg/L)	%Recovery	Avg Amount (mg/L)	%Recovery	Avg Amount (mg/L)	%Recovery	Avg Amount (mg/L)	%Recovery
0.5	0.81	59	0.02	95	0.01	96	0.11	98
0.2	0.84	65	0.02	90	0.01	98	0.11	120

Table 4A (Part 2). Recovery of algae sugar spikes into the algae acid hydrolysates using the short method (n=3)

Spike Level (mg/L)	Glucose		Xylose		Mannose		Ribose	
	Avg Amount (mg/L)	%Recovery	Avg Amount (mg/L)	%Recovery	Avg Amount (mg/L)	%Recovery	Avg Amount (mg/L)	%Recovery
0.5	1.13	91	0.01	100	0.18	95	0.03	100
0.2	1.11	95	0.01	110	0.18	110	0.04	100

Table 4B (Part 1). Recovery of algae sugar spikes into the algae acid hydrolysates using the long method (n=3)

Spike Level (mg/L)	Mannitol		Fucose		Galactosamine		Arabinose	
	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery
0.5	0.76	83	0.02	87	0.00	94	0.08	110
0.2	0.85	100	0.03	91	0.00	87	0.08	92

Table 4B (Part 2). Recovery of algae sugar spikes into the algae acid hydrolysates using the long method (n=3)

Spike Level (mg/L)	Glucosamine		Galactose		Glucose	
	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery
0.5	0.03	94	0.74	100	1.16	96
0.2	0.04	100	0.90	120	1.42	88

Table 4B (Part 3). Recovery of algae sugar spikes into the algae acid hydrolysates using the long method (n=3)

Spike Level (mg/L)	Fructose		Ribose		Cellobiose	
	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery
0.5	0.00	96	0.01	100	0.00	87
0.2	0.00	97	0.01	110	0.00	97

Table 4B (Part 4). Recovery of algae sugar spikes into the algae acid hydrolysates using the long method (n=3)

Spike Level (mg/L)	Galacturonic acid		Mannuronic acid	
	Amount (mg/L)	%Recovery	Amount (mg/L)	%Recovery
0.5	0.00	99	0.00	86
0.2	0.00	99	0.00	90

Robustness

Assay robustness for both methods was determined on two columns. The robustness was studied by introducing ±10% variation in common chromatographic method parameters. The parameters varied in this study were: initial eluent concentration, final eluent concentration, column temperature, and flow rate. Method performance under these conditions was evaluated by calculating percent difference in three key chromatographic parameters: retention time, peak asymmetry, and resolution. The results for the robustness studies using the short method are included in Tables 5 (A–C) and 6 (A–C) for columns 1 and 2, respectively. For both columns, none of the experimental variations tested here resulted in significant disruption of the three target chromatography parameters. The highest impact was observed for cellobiose, which showed significant change in retention at 110 mM final eluent concentration. This is because manually prepared eluent was used for these conditions and this led to increased time for concentration change during the elution.

Table 5A. Results of retention time robustness study performed for the short method on column 1 using 0.4 mg/L standard containing ten sugars (n=3)

Condition	Retention Time %Difference									
	Mannitol	Fucose	Galactosamine	Arabinose	Glucose	Mannose	Xylose	Fructose	Ribose	Cellobiose
28.5 °C Temperature	1.89	3.53	8.73	6.05	8.48	8.94	9.76	7.99	9.64	2.46
35.2 °C Temperature	-1.46	-2.26	-6.41	-3.67	-5.58	-5.95	-6.76	-4.80	-6.36	-1.52
0.3 mL/min Flow Rate	-10.07	-10.01	-10.43	-10.19	-10.26	-10.27	-10.34	-10.43	-10.46	-3.69
0.24 mL/min Flow Rate	5.02	12.68	12.67	12.86	12.92	12.93	13.02	13.21	13.12	4.59
1.35 mM Initial Eluent Conc	0.00	0.00	0.07	0.08	0.20	0.37	0.47	0.48	0.38	-0.04
1.65 mM Initial Eluent Conc	0.00	-0.41	-0.92	-0.64	-0.95	-0.96	-1.12	-1.15	-1.16	-0.25
90 mM Final Eluent Conc	-0.03	-0.14	-0.23	-0.08	-0.05	-0.04	-0.04	-0.11	-0.10	1.18
110 mM Final Eluent Conc	-0.29	-0.27	-0.84	-0.71	-0.85	-0.92	-1.09	-1.08	-1.04	21.63*

*Manually prepared eluent was used to achieve 110 mM eluent concentration and the resultant longer flow path lead to an increase in cellobiose retention time.

Table 5B. Results of peak asymmetry robustness study performed for the short method on column 1 using 0.4 mg/L standard containing ten sugars (n=3)

Condition	Asymmetry %Difference									
	Mannitol	Fucose	Galactosamine	Arabinose	Glucose	Mannose	Xylose	Fructose	Ribose	Cellobiose
28.5 °C Temperature	6.20	0.37	-6.35	1.04	1.86	5.49	1.61	7.89	8.00	-3.13
35.2 °C Temperature	2.27	-0.19	13.54	0.52	2.66	-0.61	2.69	-4.73	1.18	1.70
0.3 mL/min Flow Rate	-2.87	-2.43	5.80	1.04	0.53	1.22	0.81	2.21	3.06	-1.70
0.24 mL/min Flow Rate	0.98	0.37	9.67	6.74	4.52	4.27	0.54	4.10	5.41	12.78
1.35 mM Initial Eluent Conc	-0.61	4.10	4.97	8.03	5.32	2.13	3.23	2.52	4.24	7.39
1.65 mM Initial Eluent Conc	-1.97	5.41	8.56	1.04	6.12	1.22	1.34	3.79	3.29	4.55
90 mM Final Eluent Conc	-3.63	0.93	4.42	-1.04	-1.86	-1.22	-1.61	-0.32	0.94	-0.28
110 mM Final Eluent Conc	0.91	-6.34	1.38	-1.04	-1.33	-0.91	0.81	0.95	3.76	-7.10

Table 5C. Results of peak resolution robustness study performed for the short method on column 1 using 0.4 mg/L standard containing ten sugars (n=3)

Condition	Resolution %Difference									
	Mannitol	Fucose	Galactosamine	Arabinose	Glucose	Mannose	Xylose	Fructose	Ribose	Cellobiose
28.5 °C Temperature	0.73	7.85	-24.61	ND	-0.17	8.44	-16.54	18.13	-17.39	–
35.2 °C Temperature	-2.49	-6.78	29.32	-29.00	-0.26	-11.11	22.84	-20.00	14.83	–
0.3 mL/min Flow Rate	-3.66	-3.78	-0.35	-3.80	-2.57	-4.32	-3.83	-2.24	19.45	–
0.24 mL/min Flow Rate	-0.34	1.30	2.97	0.58	1.37	1.85	2.59	0.00	-19.56	–
1.35 mM Initial Eluent Conc	-2.10	-0.87	-1.05	1.84	-0.17	0.00	-0.49	-1.87	-1.18	–
1.65 mM Initial Eluent Conc	-1.95	0.30	4.36	-2.99	-0.26	-3.09	-0.74	-0.56	2.70	–
90 mM Final Eluent Conc	-0.54	-0.37	1.57	0.23	-0.09	-0.21	-0.49	0.00	0.84	–
110 mM Final Eluent Conc	0.15	-0.63	1.57	-2.76	-0.43	-3.09	-0.37	-0.19	41.89*	–

*Manually prepared eluent was used to achieve 110 mM eluent concentration and the resultant longer flow path leads to an increase in cellobiose retention time.

Table 6A. Results of retention time robustness study performed for the short method on column 2 using 0.4 mg/L standard containing ten sugars (n=3)

Condition	Retention Time %Difference									
	Mannitol	Fucose	Galactosamine	Arabinose	Glucose	Mannose	Xylose	Fructose	Ribose	Cellobiose
28.5 °C Temperature	1.83	2.92	7.62	4.50	6.74	7.21	8.01	5.88	7.53	1.74
35.2 °C Temperature	-1.64	-2.77	-7.32	-5.05	-6.93	-7.25	-8.05	-6.52	-7.87	-2.12
0.3 mL/min Flow Rate	-10.58	-10.55	-11.10	-11.21	-11.34	-11.33	-11.36	-11.77	-11.71	-4.23
0.24 mL/min Flow Rate	12.19	12.10	11.76	11.47	11.52	11.44	11.55	11.43	11.40	4.04
1.35 mM Initial Eluent Conc	-0.73	-0.82	-1.29	-1.57	-1.63	-1.58	-1.36	-1.81	-1.77	-0.85
1.65 mM Initial Eluent Conc	-0.73	-1.38	-2.65	-2.87	-3.30	-3.43	-3.50	-4.05	-4.00	-1.28
90 mM Final Eluent Conc	-1.19	-1.65	-2.65	-2.94	-3.25	-3.35	-3.24	-3.85	-3.84	-0.18
110 mM Final Eluent Conc	-1.44	-2.10	-3.56	-3.77	-4.16	-4.35	-4.45	-4.98	-4.87	15.94*

Table 6B. Results of peak asymmetry robustness study performed for the short method on column 2 using 0.4 mg/L standard containing ten sugars (n=3)

Condition	Asymmetry %Difference									
	Mannitol	Fucose	Galactosamine	Arabinose	Glucose	Mannose	Xylose	Fructose	Ribose	Cellobiose
28.5 °C Temperature	-1.66	-4.05	-16.59	-2.63	-4.30	0.27	0.98	2.19	15.48	0.00
35.2 °C Temperature	-1.66	-0.53	10.28	5.49	1.13	-5.35	2.93	-7.12	-2.98	-8.40
0.3 mL/min Flow Rate	-6.20	2.82	-1.64	2.15	0.68	-2.41	0.49	0.00	9.33	-5.77
0.24 mL/min Flow Rate	-3.78	-8.27	3.50	3.10	-8.60	7.49	-6.34	-2.19	1.79	-9.97
1.35 mM Initial Eluent Conc	-0.15	0.88	-1.40	4.53	3.17	1.87	3.90	1.10	-1.79	0.00
1.65 mM Initial Eluent Conc	-4.08	-1.76	-2.34	2.39	1.36	-1.34	0.73	0.55	0.20	-9.71
90 mM Final Eluent Conc	-2.72	0.00	-3.50	2.15	2.04	0.80	0.98	0.27	2.58	-1.84
110 mM Final Eluent Conc	-8.62	-9.68	7.71	2.39	-9.28	-1.60	1.46	3.01	6.55	-0.79

Table 6C. Results of peak resolution robustness study performed for the short method on column 2 using 0.4 mg/L standard containing ten sugars (n=3)

Condition	Resolution %Difference									
	Mannitol	Fucose	Galactosamine	Arabinose	Glucose	Mannose	Xylose	Fructose	Ribose	Cellobiose
28.5 °C Temperature	1.02	4.86	-27.12	N.D.	0.79	8.30	-18.87	17.21	-16.56	0.00
35.2 °C Temperature	-1.93	-8.13	22.97	-21.81	0.44	-10.64	17.88	-17.62	19.05	0.00
0.3 mL/min Flow Rate	2.49	-4.08	-4.15	-3.10	-2.81	-3.40	-6.41	-3.07	20.50	0.00
0.24 mL/min Flow Rate	-0.25	0.54	-2.94	2.19	1.14	0.21	-0.49	-0.20	-18.92	0.00
1.35 mM Initial Eluent Conc	-3.67	-2.65	-4.49	0.65	-1.32	0.85	-5.55	-1.84	0.85	0.00
1.65 mM Initial Eluent Conc	-2.34	-2.76	-3.28	-1.16	-1.50	-2.13	-5.30	-0.41	7.74	0.00
90 mM Final Eluent Conc	-2.85	-2.99	-4.49	8.77	-1.93	0.00	-6.66	-2.25	6.96	0.00
110 mM Final Eluent Conc	-1.37	-3.44	-5.70	-4.65	-2.99	-7.02	-9.37	-4.71	45.19*	0.00

The robustness data for the long method is included in Tables 7 (A–C) and 8 (A–C). The long method also showed no significant disruption of chromatographic parameters under the conditions tested, except for eluent concentration variation during step 3 of the gradient program. It is possible that this is due to the complex elution program and less than adequate equilibration time for this step change. However, eluent concentration changes at all other steps resulted in no significant change in the chromatographic parameters.

Table 7A (Part 1). Results of retention time robustness study performed for the long method on column 1 using 0.4 mg/L standard containing fourteen sugars (n=3)

Condition	RT %Difference						
	Mannitol	Fucose	Galactosamine	Arabinose	Glucosamine	Galactose	Glucose
30 °C Temperature	1.00	1.18	3.51	1.89	4.04	2.19	2.92
35.2 °C Temperature	-1.75	-2.33	-6.52	-4.12	-7.30	-4.82	-5.88
0.2 mL/min Flow Rate	10.13	9.95	9.90	9.94	9.83	9.98	9.89
0.24 mL/min Flow Rate	-8.34	-8.53	-8.79	-8.77	-8.75	-8.89	-8.84
45 mM Step 2 Eluent Conc	0.02	0.13	0.06	0.06	0.16	0.00	0.09
55 mM Step 2 Eluent Conc	0.00	0.00	-0.08	-0.06	0.00	-0.10	-0.05
0.9 mM Eluent Conc	-0.19	0.35	0.77	0.76	1.13	0.66	0.96
1.1 mM Initial Eluent Conc	-0.19	-0.34	-0.65	-0.53	-0.73	-0.62	-0.71
180 mM Step 4 Eluent Conc	0.02	0.13	0.19	0.24	0.37	0.24	0.38
220 mM Step 4 Eluent Conc	-0.37	-0.34	-0.45	-0.47	-0.36	-0.57	-0.50

Table 7A (Part 2). Results of retention time robustness study performed for the long method on column 1 using 0.4 mg/L standard containing fourteen sugars (n=3)

Condition	RT %Difference						
	Mannose	Xylose	Fructose	Ribose	Cellobiose	Galacturonic Acid	Mannuronic Acid
30 °C Temperature	3.09	3.38	2.17	3.17	0.88	0.15	0.52
35.2 °C Temperature	-6.11	-6.65	-5.14	-6.57	-1.47	-0.26	-0.45
0.2 mL/min Flow Rate	11.83	8.03	9.93	9.93	3.84	3.46	3.66
0.24 mL/min Flow Rate	-8.85	-8.91	-9.03	-9.02	-3.86	-3.07	-3.24
45 mM Step 2 Eluent Conc	0.18	0.23	0.27	0.20	1.07	0.00	-0.02
55 mM Step 2 Eluent Conc	-0.04	-0.04	0.00	-0.03	-1.08	-0.19	-0.15
0.9 mM Eluent Conc	1.25	1.59	1.55	1.46	0.28	-0.09	-0.11
1.1 mM Initial Eluent Conc	-0.78	-0.95	-0.98	-0.97	-0.19	-0.10	-0.10
180 mM Step 4 Eluent Conc	0.46	0.54	0.51	0.47	0.42	0.00	-0.07
220 mM Step 4 Eluent Conc	-0.39	-0.34	-0.44	-0.47	-0.04	-0.07	0.00

Table 7B (Part 1). Results of peak asymmetry robustness study performed for the long method on column 1 using 0.4 mg/L standard containing fourteen sugars (n=3)

Condition	Asymmetry %Difference						
	Mannitol	Fucose	Galactosamine	Arabinose	Glucosamine	Galactose	Glucose
30 °C Temperature	-2.03	0.18	-7.45	-0.73	-11.31	-4.40	-8.99
35.2 °C Temperature	-0.62	-3.99	7.93	-4.87	11.92	5.16	-4.39
0.2 mL/min Flow Rate	-3.74	0.91	0.72	3.65	2.26	0.38	-5.04
0.24 mL/min Flow Rate	-2.96	0.36	-1.68	0.73	-3.47	-2.14	-8.99
45 mM Step 2 Eluent Conc	-4.84	-8.17	-1.20	-2.19	-1.96	0.88	-11.40
55 mM Step 2 Eluent Conc	-4.52	-5.26	1.92	-4.14	1.36	0.75	-12.50
0.9 mM Eluent Conc	-0.78	-6.72	-0.24	-0.97	-2.26	1.38	-3.95
1.1 mM Initial Eluent Conc	-4.06	-2.90	2.40	-2.92	0.15	-0.13	-8.77
180 mM Step 4 Eluent Conc	1.40	-1.27	0.96	1.70	-0.45	-0.38	-8.77
220 mM Step 4 Eluent Conc	-2.34	-0.54	-1.92	1.95	-1.36	-0.88	-1.32

Table 7B (Part 2). Results of peak asymmetry robustness study performed for the long method on column 1 using 0.4 mg/L standard containing fourteen sugars (n=3)

Condition	Asymmetry %Difference						
	Mannose	Xylose	Fructose	Ribose	Cellobiose	Galacturonic Acid	Mannuronic Acid
30 °C Temperature	-2.65	-3.65	-2.96	-10.00	-5.85	1.78	-4.86
35.2 °C Temperature	-5.31	1.56	-16.09	-4.13	7.46	10.36	-5.71
0.2 mL/min Flow Rate	0.88	-7.03	-5.28	-21.74	16.08	-4.44	-0.86
0.24 mL/min Flow Rate	-3.54	0.00	-6.82	1.52	-2.63	-3.55	6.00
45 mM Step 2 Eluent Conc	-3.54	2.60	-15.83	-4.35	-2.63	13.31	0.57
55 mM Step 2 Eluent Conc	-2.65	5.08	-15.06	-5.22	-2.05	15.38	1.14
0.9 mM Eluent Conc	-0.29	-2.60	-1.42	-6.74	-2.05	6.21	2.86
1.1 mM Initial Eluent Conc	-4.13	-2.08	-10.94	-3.48	-4.97	20.41	1.14
180 mM Step4 Eluent Conc	-3.24	3.65	-13.77	-5.00	-4.97	13.91	0.29
220 mM Step4 Eluent Conc	-2.65	-2.08	1.42	-4.57	-1.46	26.33	1.71

Table 7C (Part 1). Results of resolution robustness study performed for the long method on column 1 using 0.4 mg/L standard containing fourteen sugars (n=3)

Condition	Resolution %Difference						
	Mannitol	Fucose	Galactosamine	Arabinose	Glucosamine	Galactose	Glucose
30 °C Temperature	0.67	2.60	-16.32	16.27	-20.13	6.27	-0.79
35.2 °C Temperature	-2.28	-8.78	23.42	-24.12	36.06	-8.96	0.62
0.2 mL/min Flow Rate	0.93	1.38	2.10	0.98	4.65	1.46	3.00
0.24 mL/min Flow Rate	0.31	-1.15	-0.48	-0.98	-2.43	-0.11	0.35
45 mM Step 2 Eluent Conc	2.18	0.54	0.65	1.68	-0.44	1.68	2.11
55 mM Step 2 Eluent Conc	2.18	0.68	0.48	0.84	-0.22	1.12	0.79
0.9 mM Eluent Conc	2.02	0.74	0.16	3.93	-4.42	2.91	1.67
1.1 mM Initial Eluent Conc	2.13	0.41	2.10	-0.84	2.88	0.11	1.41
180 mM Step4 Eluent Conc	0.98	-0.37	-0.65	0.42	-0.88	0.67	0.62
220 mM Step4 Eluent Conc	-0.88	-2.23	-2.26	-0.70	-3.54	-1.57	-1.41

Table 7C (Part 2). Results of resolution robustness study performed for the long method on column 1 using 0.4 mg/L standard containing fourteen sugars (n=3)

Condition	Resolution %Difference						
	Mannose	Xylose	Fructose	Ribose	Cellobiose	Galacturonic Acid	Mannuronic Acid
30 °C Temperature	6.51	-9.20	10.73	-8.49	-10.55	2.74	–
35.2 °C Temperature	-5.21	17.66	-19.45	16.08	5.45	-12.78	–
0.2 mL/min Flow Rate	5.21	3.34	1.64	-8.38	2.36	1.89	–
0.24 mL/min Flow Rate	2.34	-0.63	-2.00	8.62	-1.29	1.63	–
45 mM Step 2 Eluent Conc	4.69	2.82	0.36	6.61	-2.07	-1.29	–
55 mM Step 2 Eluent Conc	0.26	-0.84	0.00	-2.73	-4.02	2.92	–
0.9 mM Eluent Conc	9.11	2.19	-0.91	-1.80	-3.52	-1.29	–
1.1 mM Initial Eluent Conc	3.13	0.31	-2.73	0.94	-5.27	0.51	–
180 mM Step4 Eluent Conc	4.43	0.10	-1.64	0.06	-4.27	-3.60	–
220 mM Step4 Eluent Conc	2.34	-0.21	-2.73	-2.52	-4.66	-3.43	–

Table 8A (Part 1). Results of resolution robustness study performed for the long method on column 2 using 0.4 mg/L standard containing twelve sugars (n=3)

Condition	RT %Difference						
	Mannitol	Fucose	Galactosamine	Arabinose	Glucosamine	Galactose	Glucose
30 °C Temperature	0.80	1.53	4.40	2.67	5.02	3.06	4.00
35.2 °C Temperature	-1.54	-2.22	-5.84	-3.56	-6.51	-4.20	-5.16
0.2 mL/min Flow Rate	9.96	9.85	10.05	10.03	9.94	10.14	10.06
0.24 mL/min Flow Rate	-8.16	-8.21	-8.50	-8.52	-8.51	-8.60	-8.54
45 mM Step 2 Eluent Conc	-0.37	-0.11	-0.20	-0.24	-0.31	-0.24	-0.25
55 mM Step 2 Eluent Conc	-0.19	-0.24	-0.27	-0.25	-0.31	-0.24	-0.25
0.9 mM Eluent Conc	-5.43	-5.51	-5.52	-5.55	-5.50	-5.58	-5.49
1.1 mM Initial Eluent Conc	0.00	-0.35	-0.60	-0.60	-0.82	-0.58	-0.66
180 mM Step 4 Eluent Conc	0.00	0.23	0.31	0.28	0.25	0.37	0.33
220 mM Step 4 Eluent Conc	-0.19	-0.34	-0.72	-0.76	-0.82	-0.90	-0.83

Table 8A (Part 2). Results of resolution robustness study performed for the long method on column 2 using 0.4 mg/L standard containing twelve sugars (n=3)

Condition	RT %Difference						
	Mannose	Xylose	Fructose	Ribose	Cellobiose	Galacturonic Acid	Mannuronic Acid
30 °C Temperature	4.23	4.61	3.43	4.53	0.93	0.07	0.37
35.2 °C Temperature	-5.25	-5.57	-4.01	-5.55	-1.01	-0.45	-0.94
0.2 mL/min Flow Rate	10.08	9.98	10.16	10.18	-1.62	3.61	3.89
0.24 mL/min Flow Rate	-8.54	-8.65	-8.72	-8.65	-2.26	-2.98	-3.16
45 mM Step 2 Eluent Conc	-0.28	-0.43	-0.35	-0.35	0.81	-0.07	-0.07
55 mM Step 2 Eluent Conc	-0.25	-0.40	-0.38	-0.35	-1.15	-0.20	-0.13
0.9 mM Eluent Conc	-5.50	-5.51	-5.58	-5.51	-1.01	-2.03	-2.15
1.1 mM Initial Eluent Conc	-0.81	-1.06	-1.08	-0.98	-0.14	0.01	0.01
180 mM Step 4 Eluent Conc	0.35	0.20	0.23	0.29	0.41	-0.10	-0.11
220 mM Step 4 Eluent Conc	-0.92	-1.03	-1.14	-1.08	-0.15	-0.81	-0.45

Table 8B (Part 1). Results of resolution robustness study performed for the long method on column 2 using 0.4 mg/L standard containing twelve sugars (n=3)

Condition	Asymmetry %Difference						
	Mannitol	Fucose	Galactosamine	Arabinose	Glucosamine	Galactose	Glucose
30 °C Temperature	6.17	-3.10	-7.11	-1.06	-7.10	1.12	-0.28
35.2 °C Temperature	-2.11	-4.01	5.26	-4.24	7.74	2.53	1.42
0.2 mL/min Flow Rate	-0.32	-0.91	-0.26	3.98	2.58	-0.56	1.99
0.24 mL/min Flow Rate	-5.68	-9.11	-1.58	-0.27	-0.32	0.84	0.28
45 mM Step 2 Eluent Conc	5.68	-2.19	-1.05	0.53	1.61	0.28	-0.57
55 mM Step 2 Eluent Conc	4.38	-6.92	0.53	-1.59	0.97	0.00	1.14
0.9 mM Eluent Conc	1.30	-1.09	0.00	3.45	0.65	-1.97	5.68
1.1 mM Initial Eluent Conc	1.95	-2.00	-0.26	0.53	1.94	1.40	5.40
180 mM Step 4 Eluent Conc	8.44	-1.09	-0.53	1.33	1.94	0.28	4.55
220 mM Step 4 Eluent Conc	-8.60	-3.10	1.32	0.00	1.61	1.12	1.14

Table 8B (Part 2). Results of resolution robustness study performed for the long method on column 2 using 0.4 mg/L standard containing twelve sugars (n=3)

Condition	Asymmetry %Difference						
	Mannose	Xylose	Fructose	Ribose	Cellobiose	Galacturonic Acid	Mannuronic Acid
30 °C Temperature	-1.63	1.46	1.68	-9.33	-19.11	-4.58	4.68
35.2 °C Temperature	-0.33	3.21	-5.70	15.89	19.43	4.58	26.62
0.2 mL/min Flow Rate	-0.33	7.29	-8.39	-16.91	20.38	18.30	21.58
0.24 mL/min Flow Rate	-1.31	10.20	-13.76	7.00	-2.87	7.52	12.59
45 mM Step 2 Eluent Conc	-1.63	1.17	-11.74	1.17	0.00	1.96	2.16
55 mM Step 2 Eluent Conc	-2.29	5.54	-2.01	8.45	0.64	-5.56	2.16
0.9 mM Eluent Conc	0.33	1.75	-13.42	2.04	-10.19	6.21	9.35
1.1 mM Initial Eluent Conc	-2.61	2.92	1.01	7.00	-2.55	-2.29	0.36
180 mM Step4 Eluent Conc	-1.31	2.04	-4.36	0.00	1.91	-1.31	1.44
220 mM Step4 Eluent Conc	-0.65	5.54	-5.37	4.66	-3.18	14.71	10.07

Table 8C (Part 1). Results of resolution robustness study performed for the long method on column 2 using 0.4 mg/L standard containing twelve sugars (n=3)

Condition	Resolution %Difference						
	Mannitol	Fucose	Galactosamine	Arabinose	Glucosamine	Galactose	Glucose
30 °C Temperature	0.94	4.32	-15.35	16.80	-21.65	8.49	0.16
35.2 °C Temperature	-2.21	-6.22	22.95	-21.14	33.40	-6.69	1.07
0.2 mL/min Flow Rate	-0.09	1.63	1.46	0.81	3.71	0.85	1.64
0.24 mL/min Flow Rate	-1.60	-2.41	-1.75	-2.44	-3.30	-1.06	-1.15
45 mM Step 2 Eluent Conc	0.71	0.19	0.00	-0.95	0.82	0.42	0.25
55 mM Step 2 Eluent Conc	0.42	0.16	0.73	-0.41	1.03	0.11	0.08
0.9 mM Eluent Conc	-1.55	-1.28	-1.32	-1.63	-2.47	-0.64	-1.40
1.1 mM Initial Eluent Conc	0.09	0.22	0.58	-1.49	3.30	-0.21	-0.33
180 mM Step 4 Eluent Conc	-0.47	0.06	0.00	-0.27	1.65	0.00	0.41
220 mM Step 4 Eluent Conc	0.19	-0.38	0.15	-0.54	-0.82	1.27	0.25

Table 8C (Part 2). Results of resolution robustness study performed for the long method on column 2 using 0.4 mg/L standard containing twelve sugars (n=3)

Condition	Resolution %Difference						
	Mannose	Xylose	Fructose	Ribose	Cellobiose	Galacturonic Acid	Mannuronic Acid
30 °C Temperature	4.81	-11.65	12.48	-4.50	4.68	2.03	0.94
35.2 °C Temperature	-5.72	15.62	-20.45	14.35	9.66	-14.12	-2.21
0.2 mL/min Flow Rate	-0.92	-0.18	4.85	-13.64	-1.91	0.00	-0.09
0.24 mL/min Flow Rate	-3.66	-4.34	-2.77	8.71	-1.43	-0.62	-1.60
45 mM Step 2 Eluent Conc	-2.29	-0.55	-1.91	5.87	5.82	0.88	0.71
55 mM Step 2 Eluent Conc	-2.75	0.74	0.00	-4.55	-0.74	2.12	0.42
0.9 mM Eluent Conc	-0.92	-2.68	-1.56	-5.38	-0.84	-0.62	-1.55
1.1 mM Initial Eluent Conc	-4.12	-1.11	-0.35	0.14	-0.27	2.29	0.09
180 mM Step 4 Eluent Conc	-2.29	-1.39	-1.21	0.54	1.44	2.12	-0.47
220 mM Step 4 Eluent Conc	-1.60	-2.50	-1.56	0.00	0.61	10.24	0.19

Conclusions

Two methods for sugar determination in acid-hydrolyzed algae samples are presented here. For the first short method, ten of the twelve peaks are well resolved and the total run time is 20.5 min; whereas the long method resolved 14 peaks in 39 min. Both methods are linear at sugar concentrations between 0.05 to 2 mg/L. Both methods show excellent retention time as well as peak area method precision. The short method was able to withstand the majority of the deliberate ±10% variations in method conditions as measured by changes in key chromatography parameters. The long method also showed no major disruption on key chromatography parameters except the variation of eluent concentration during the uronic acid elution phase. Due to the complex nature of eluent changes needed for uronic acid elution, the long method showed retention time variation during this phase. Using these methods, carbohydrates present in TFA-hydrolyzed algae biomass samples were quantified. Finally, the 2 × 100 mm column used here allows for reduced eluent consumption, and thereby improves the overall process economics. In summary, the methods proposed here are convenient, precise, and robust for algal biomass sugar analysis, which will improve the reliability of biomass-to-biofuel efficiency calculations.

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