Carbohydrate analysis of agave syrup using HPAE-PAD in dual eluent generation cartridge mode

Authors: Jingli Hu and Jeffrey Rohrer
Thermo Fisher Scientific, Sunnyvale, CA

Keywords: Dionex CarboPac PA1-1mm column, Dionex ICS-6000 HPIC system, polyols, oligosaccharides, fructan, adulteration, Mexico NOM, electrochemical detection

Goal

To demonstrate that the Norma Oficial Mexicana (Official Mexican Standard) method for agave syrup carbohydrate analysis can be executed with a Thermo Scientific™ Dionex™ CarboPac™ PA1-1mm column using KOH/KMSA eluent produced electrolytically using Dual Eluent Generation Cartridge (Dual EGC) mode

Introduction

Agave syrup is a recent food product from Mexico that is produced from the sap of the agave plant. This product has gained popularity as an alternative to traditional sweeteners, such as table sugar (sucrose) and honey, partially due to its low glycemic index (GI) (17–27) when compared to honey (55) and sucrose (68)[1-2]. The GI is a relative ranking of carbohydrate in foods according to how they affect blood glucose levels. Carbohydrates with a low GI value (55 or less) are more slowly digested, absorbed, and metabolized, and cause a lower and slower rise in blood glucose and, therefore, insulin levels. Agave syrup has a low GI primarily because almost all the sugar in it is fructose, and it has very little glucose. The high fructose content also makes it sweeter than syrups containing appreciable levels of glucose or sucrose so that less agave syrup can be used to achieve the same level of sweetness, thus decreasing calorie intake.

Knowledge of the chemical composition of foods is important not only for human health but also for authenticity. Due to the increasing popularity of agave syrup as a tabletop sweetener and as a food ingredient, it has become an adulteration target. The fact that agave

syrup is primarily composed of carbohydrates results in the relatively simple and economically viable adulteration of this material with less expensive nutritive sweeteners such as high fructose corn syrup (HFCS). One way to detect this type of adulteration is by oligosaccharide profiling using high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD). This technique features direct detection and therefore eliminates errors associated with analyte derivatization.

As the main producer of agave, Mexico has recently created a governmentally approved paper "NOM-003-SAGARPA-2016" as an official guideline for the characterization of pure agave syrup.[3] The core part of the method in the Norma Oficial Mexicana (NOM) is the determination of the main sugars (fructose, glucose, and sucrose), polyols (sorbitol, mannitol), and 5-hydroxymethyfural (HMF), and detection of adulterations using HPAE-PAD. According to the NOM, agave syrup is diluted with water and analyzed before and after amyloglucosidase and fructanase enzymatic hydrolysis using a Dionex CarboPac PA1 column (250 × 4 mm) and PAD. The content of the sugars as well as the content of fructan is calculated.

The NOM method for agave syrup carbohydrate was successfully executed with a Dionex CarboPac PA1 column (250 × 2 mm) using the manually prepared sodium hydroxide/sodium acetate eluents and thus reducing eluent consumption four-fold.[4] In this application note, the NOM method was evaluated with a Dionex CarboPac PA1 column (250 mm × 1 mm) using HPAE-PAD in Dual EGC mode. The 1 mm column requires a flow rate approximately four times lower than the 2 mm column, which further reduces eluent consumption. Dual EGC mode replaces the manual preparation of the sodium hydroxide/sodium acetate eluents. This mode uses a methanesulfonic acid (MSA) eluent generation cartridge (EGC) and a potassium hydroxide (KOH) EGC in series to generate an extremely reproducible and accurate KOH/KMSA eluent gradient needed for separating complex carbohydrates. Key performance parameters were evaluated including separation, linearity, limits of detection, and precision. Three samples were analyzed and the main sugars, polyols, and HMF in those sample were determined. Inositol was also determined because it was reported to be present in agave syrup.[5] The possibility of adulteration was evaluated with amyloglucosidase enzymatic hydrolysis. Total fructan was determined with fructanase enzymatic hydrolysis.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-6000 HPIC system including:
  - Dionex ICS-6000 DP Pump module or SP Pump module
  - Dionex ICS-6000 EG Eluent Generator module
  - Dionex Dual EG Eluent degasser (P/N 22181-60951)
  - Dionex ICS-6000 DC Detector/Chromatography module with ED Electrochemical Detector
  - Dionex AS-AP Autosampler with sample tray cooling, (P/N 074926)
  - 4-port valve rebuild kit (P/N 074699), which includes a 0.4 µL injection loop
- Thermo Scientific™ Dionex™ ICS-6000 ED Electrochemical Detector Cell (P/N 072044)
- Gold on PTFE Disposable working electrode including four 2 mil gaskets (P/N 066480) Note: The 2 mil gaskets are not used in this application.
- Working electrode gasket, 1 mil (P/N 072161)
- Reference electrode, pH, Ag/AgCl (P/N 061879)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software, version 7.2.9

Assemble the cell following the Dionex ICS-6000 operator's manual[6] and Dionex ED User's Compendium for Electrochemical Detection.[7]

Consumables

- Thermo Scientific™ Nalgene™ Syringe Filter, PES membrane, 0.2 µm (P/N 725-2520)
- Thermo Scientific™ Nalgene™ Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Microcentrifuge tube, 2 mL (Fisher Scientific P/N 05-408-138)
- Nitrogen, ultrahigh purity grade from Airgas
- Thermo Scientific™ Dionex™ EGC 400 KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 302766)
- Thermo Scientific™ Dionex™ EGC 400 MSA Methanesulfonic Acid Eluent Generator Cartridge (P/N 302767)

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Glucose (Sigma-Aldrich P/N G8270)
- Fructose (Sigma-Aldrich, P/N F2543)
- Sucrose (Sigma-Aldrich P/N S7903)
- Mannitol (Sigma-Aldrich, P/N M-9546)
- Sorbitol (Sigma-Aldrich, P/N S-1876)
- Inositol (Sigma-Aldrich, P/N I-5125)
- Hydroxymethylfurfural (Fisher Scientific, P/N AC1214)
- Amyloglucosidase powder (36000 U/g) (Megazyme, P/N E0AMGDFPD)
- Fructanase Mixture (Purified-powder) (exo-inulinase 20,000 U, endo-inulinase 1000 U) (Megazyme, P/N E-FRMXPD)

Samples

Three agave syrup samples were purchased from a local supermarket.

Chromatographic conditions

Parameter	Value
Columns	Dionex CarboPac PA1 Guard, 1 × 50 mm (P/N 303273) Dionex CarboPac PA1 Separation, 1 × 250 mm (P/N 303272)
Eluent	Gradient (Table 1)
Flow rate	0.063 mL/min
Column temperature	30 °C
Injection volume	0.4 µL
Autosampler temperature	5 °C
Reference electrode	Ag/AgCl
Working electrode	Disposable electrode gold, with a 1 mil (25.4 µm) gasket
Detection	Pulsed Amperometric Detector (Electrochemical Detection)
Detection compartment temperature	30 °C
Detection waveform	Gold, Carbohydrates, 4-Potential (Table 2)
System backpressure	~3300 psi (100 psi = 0.6894 MPa)
Run time	70 min

Table 1. Eluent gradient

Time (min)	KMSA (mM)	KOH (mM)
-20	3	102
0	3	102
6	3	102
12	14	108
30	70	130
40	100	100
40.1	3	102
50	3	102

Table 2. Carbohydrates, 4-potential waveform

Time (s)	Potential (V) vs Ag/AgCl	Integration
0	0.1	Off
0.2	0.1	On
0.4	0.1	Off
0.41	-2	Off
0.42	-2	Off
0.43	0.6	Off
0.44	-0.1	Off
0.5	-0.1	Off

System installation and precautions

Install the system according to Figure 1. To ensure a stable baseline and low background noise, it is crucial to have sufficient removal of the hydrogen and oxygen gases formed with the EGC generated eluents. Connect the vent of the Dionex Dual EG Eluent degasser to the vacuum port located at the back of the Dionex DP module and confirm that the vacuum status is OK before proceeding to the startup of the chromatography system. An operational vacuum degasser pump (part of the analytical pump) is important to the success of this and other electrochemical detection methods. This pump can be accidentally turned off during a system restart or instrument configuration. If the vacuum degasser pump is not running, poor baseline performance and loss of column compacity can be observed. Always ensure that the vacuum degas pump is on before running a sequence. Press F8 or click the “gear” command button on the main Chromeleon instrument panel and locate the pump and then the degasser status. Make sure the degasser is “On” and “DegasserVaccum” is “OK”. If they are “OFF” and “NOT OK”, turn on the degasser by selecting “On” from the drop-down menu and wait until “DegasserVacuum” turns to “OK”. Confirm the vacuum status by pressing the commands (F8), select pump_1, right click on the

properties panel, and choose expert mode from the three modes available “Normal, Advanced, Expert.” If the value reads NOT OK, check for leaks at all the connections for vacuum. Install PEEK backpressure tubing (P/N 22181-20031) as needed to achieve an instrument pressure value above 3000 psi. To ensure the best system performance, only turn on the EGC power when the system pressure exceeds 3000 psi. Fill a 2 L eluent bottle with degassed DI water. Connect the eluent bottle to the pump and keep the eluent blanket under an insert gas (helium or nitrogen) at 5-8 psi. Turn on the pump and pump DI water through the Dionex EGC 400 MSA cartridge for 15 min at a flow rate of 1 mL/min. Then condition the Dionex EGC 400 MSA cartridge for 30 min using 100 mM MSA at a flow rate of 0.1 mL/min. Connect the Dionex EGC 400 KOH and pump DI water through the Dionex EGC 400 KOH cartridge for 15 min at a flow rate of 1 mL/min. Then condition the Dionex EGC 400 KOH cartridge for 30 min using 100 mM KOH at a flow rate of 0.1 mL/min. After conditioning the EGCs, install a Dionex CarboPac PA1-1mm column set, set KMSA and KOH to the desired concentration for the application, and keep the flow on for 60 min.

Preparation of solutions and reagents

Standards preparation

Stock standard solutions

Prepare individual stock standard solutions (1000 mg/L) of inositol, sorbitol, mannitol, hydroxymethylfurfural, glucose, and sucrose in DI water. Prepare a 5000 mg/L stock standard solution of fructose in DI water.

Table 3. Calibration standards

Standards	Stock conc. (mg/L)	L1 (mg/L)	L2 (mg/L)	L3 (mg/L)	L4 (mg/L)	L5 (mg/L)
Inositol	50	0.05	0.5	1	2.5	5
Sorbitol	50	0.05	0.5	1	2.5	5
Mannitol	50	0.05	0.5	1	2.5	5
HMF	200	0.2	2	4	10	20
Glucose	200	0.2	2	4	10	20
Fructose	2000	2	20	40	100	200
Sucrose	50	0.05	0.5	1	2.5	5

Working standard solutions

Prepare a seven-sugar stock calibration standard mixture (2nd column Table 3) and then prepare calibration standards by diluting the stock standard mixture (Table 3).

Enzyme preparation

Amyloglucosidase (270 U/mL)

Dissolve 75.2 mg powder in 10 mL of DI water. Aliquot this solution into 2 mL vials, and store at -20 °C.

Fructanase (1400 U ex-inulinase, 70 U endo-inulinase)

Add 10 mL of DI water to the product bottle to prepare 2000 U exo-inulinase, 100 U endo-inulinase. Dilute with DI water to prepare 1400 U exo-inulinase, 70 U endo-inulinase. Transfer this solution to 2 mL vials, and keep at -20 °C.

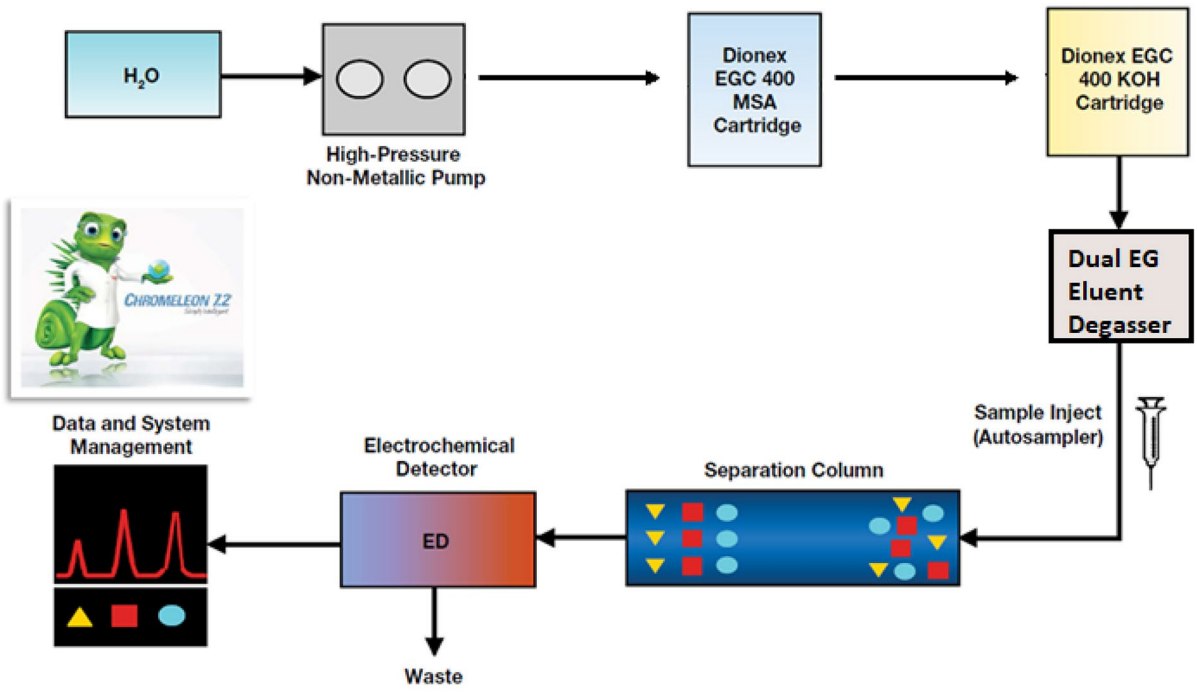

Figure 1. Dionex ICS-6000 HPIC system in Dual EGC mode

Sample preparation

Weigh 250 mg of agave syrup (±0.01 mg) in a 100 mL plastic bottle, add 50 mL of DI water, and mix thoroughly. Filter this sample with a Nalgene Syringe Filter, PES 0.2 µm.

From the stock sample solution, prepare samples for enzymatic hydrolysis in 2 mL centrifuge tubes as shown in Table 4. Close the microcentrifuge tube and vortex for 15 s. Place tubes A2, A3, A4, B1, and B2 in an electric heater at 45 °C for 30 min, then increase the temperature to 80 °C and maintain for 20 min to stop the hydrolysis. Vortex for 15 s. Dilute solutions A1, A2, and A3 with DI water for the determination of glucose and fructose, according to Table 5.

Results and discussion

Separation

The Dionex CarboPac PA1 column is a general-purpose column for the separation of mono, di, and some oligosaccharides by high pH anion-exchange chromatography, coupled with PAD.[8] Figure 2 shows a separation of main sugars (fructose, glucose, and sucrose), polyols (sorbitol, mannitol, inositol), and 5-hydroxymethylfurfural (HMF).

As Figure 2 shows, inositol, sorbitol, mannitol, HMF, fructose, and glucose were well resolved. The resolutions between all the components were >2.0.

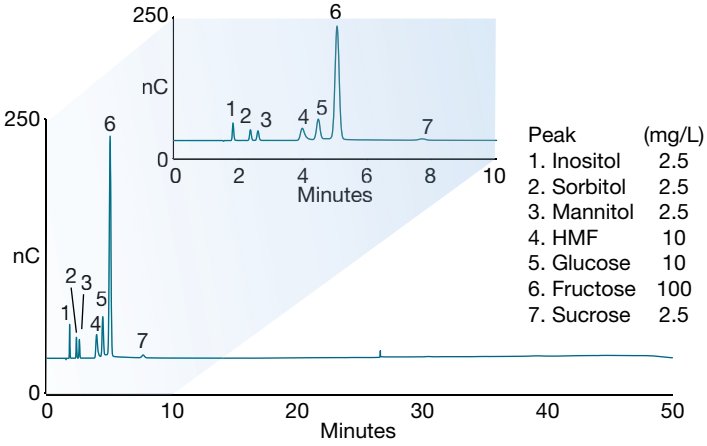

Figure 2. Separation of seven agave sugar standards using a Dionex CarboPac PA1-1mm column

Table 4. Sample enzyme treatment

Code	Sample stock (µL)	Amyloglucosidase (AG) (µL)	Fructanase (FN) (µL)	H_2O (µL)	Note
A1	600			1400	Sample
A2	600	40		1360	Sample after AG
A3	600		40	1360	Sample after FN
A4	600	40	40	1320	Sample after AG and FN
B1		40		1960	AG blank
B2			40	1960	FN blank

Table 5. Sample dilution

Code	Sample	Sample volume (µL)	H_2O (µL)	Dilution fold	Note
A5	A1	200	800	5	Glucose
A6	A2	200	800	5	Glucose after AG
A7	A1	50	950	20	Fructose and glucose
A8	A3	50	950	20	Fructose and glucose after FN

Calibration

The calibration standard mixture was prepared with individual carbohydrate concentrations that could be diluted into the concentration range typical for agave syrup.

Calibration curves with five concentration levels were constructed for each of the seven carbohydrates. For fructose, the calibration curve showed deviation from linearity in the selected calibration range. Therefore, the peak area versus concentration data for fructose was fit using a quadratic regression function. The linear least squares model was used for the other six carbohydrates. Figure 3 shows the calibration curves. Table 6 summarizes the calibration data. The coefficient of determination (r^2) was greater than 0.999 for each component.

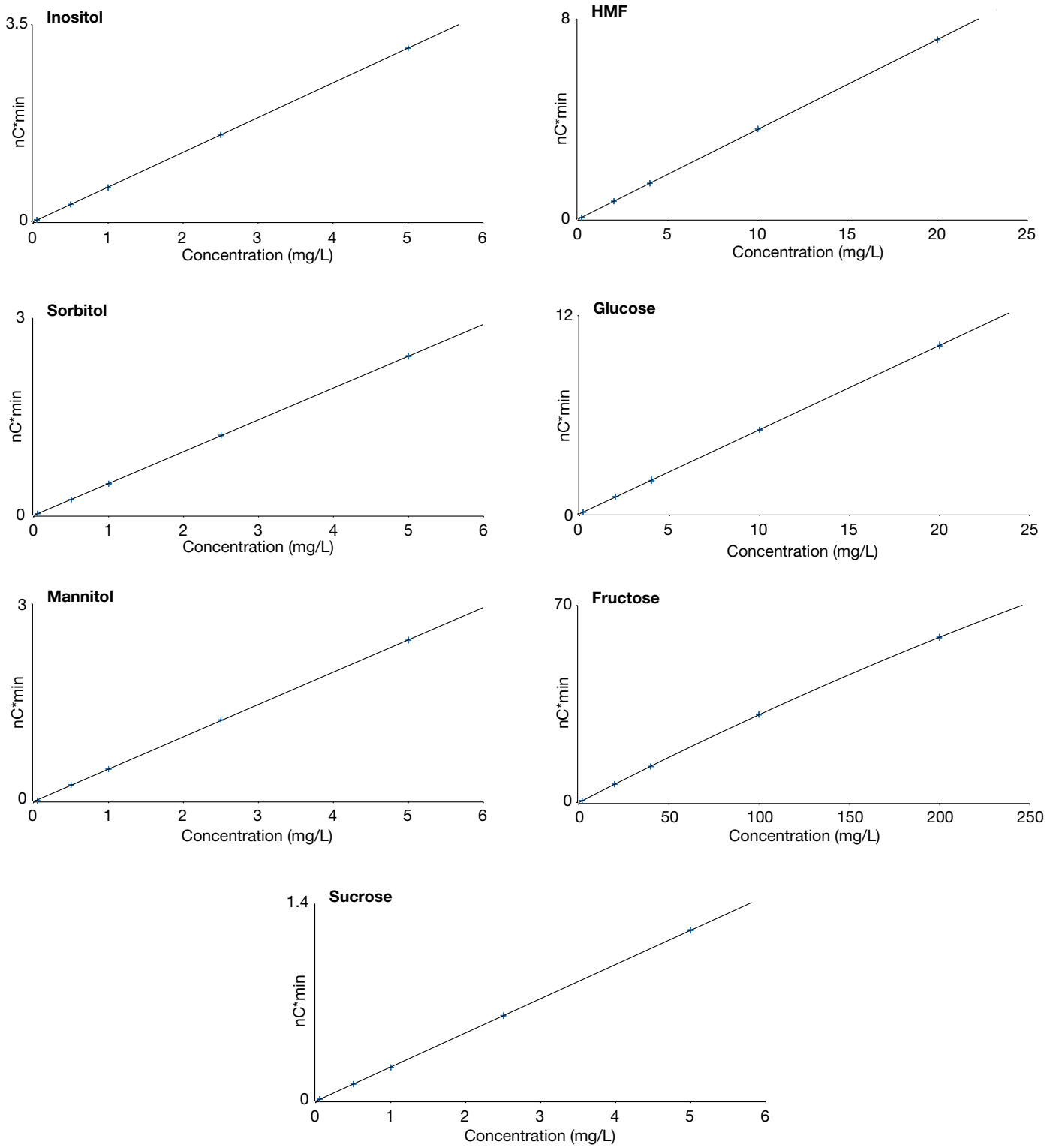

Figure 3. Seven carbohydrate calibration curves

Table 6. Calibration

Standard	Range (mg/L)	Calibration type	Coefficient of determination (r^2)
Inositol	0.05–5	Linear	1.0000
Sorbitol	0.05–5	Linear	1.0000
Mannitol	0.05–5	Linear	0.9999
HMF	0.2–20	Linear	1.0000
Glucose	0.2–20	Linear	0.9999
Fructose	2–200	Quadratic	1.0000
Sucrose	0.05–5	Linear	1.0000

Limit of detection (LOD) and limit of quantification (LOQ)

The determination of the signal-to-noise (S/N) ratio is performed by comparing the measured signal from a standard with a low concentration to a blank sample and thus establishing the minimum concentration at which the analyte can be reliably detected. A S/N=3 is used for estimating the detection limit (LOD), and a S/N=10 is used for estimating the quantification limit (LOQ)[9]. In this study, the baseline noise was first determined by measuring the peak-to-peak noise in a representative 1-min segment of the baseline where no peaks elute, but close to the peak of interest. The signal was determined from the peak height. The LOD and LOQ in the agave syrup sample were calculated on the basis of the sample weight (250 mg) and sample volume (50 mL) (Table 7).

Table 7. LOD and LOQ

Analyte	LOD (µg/L)	LOQ (µg/L)	LOD in agave syrup (µg/g)	LOQ in agave syrup (µg/g)
Inositol	1.25	4.18	0.837	2.79
Sorbitol	1.34	4.47	0.894	2.98
Mannitol	1.35	4.48	0.897	2.99
HMF	7.56	25.2	5.04	16.8
Glucose	3.41	11.4	2.27	7.58
Fructose	4.06	13.5	2.71	9.02
Sucrose	14.3	47.6	9.53	31.8

Sample analysis

Three brands of agave syrup were obtained from a local supermarket. Sample A1 was used for the determination of inositol, sorbitol, mannitol, HMF, and sucrose in agave syrup. (Figure 4). Sample A7 was used for the determination of glucose and fructose in agave syrup. (Figure 5). Table 8 summarizes the results of carbohydrate and polyol analysis. The major carbohydrate found in the three agave syrup samples was fructose, which made up 73–75% of the total carbohydrate. The other major carbohydrate identified was glucose, 1.2–1.4% of the total carbohydrate.

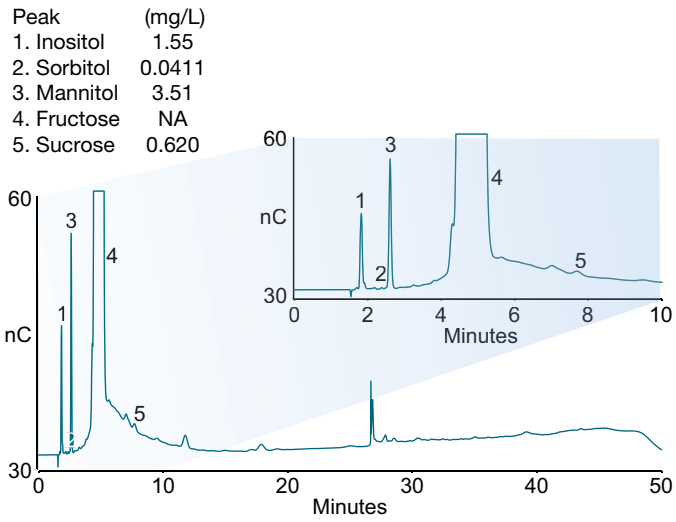

Figure 4. Sample #1 A1

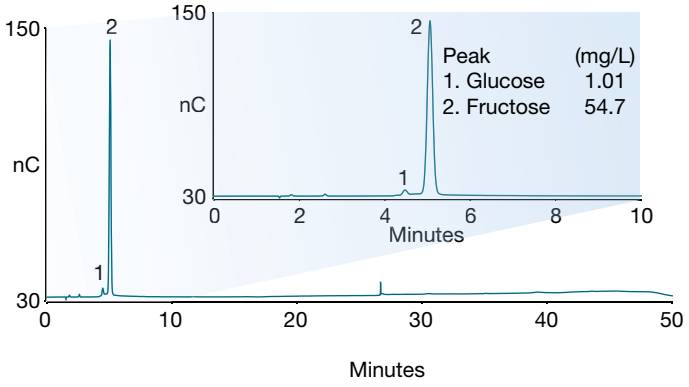

Figure 5. Sample #1 A7

Table 8. Carbohydrate content (g/100 g)

Analyte	Inositol	Sorbitol	Mannitol	HMF	Glucose	Fructose	Sucrose
Sample 1	0.1030	0.00274	0.234	ND	1.35	73.0	0.0413
Sample 2	0.0655	0.00255	0.09981	ND	1.30	73.4	0.0556
Sample 3	0.0596	0.00195	0.224	ND	1.23	74.8	0.0804

The major polyols identified in the agave syrup sample were mannitol and inositol comprising 0.1–0.3% and 0.06–0.11% of the total carbohydrate, respectively. The results were similar to the results obtained with a Dionex CarboPac PA1-2mm column using manually prepared sodium hydroxide and sodium acetate eluent.[5]

The oligosaccharide profile of the three agave syrup samples was examined by HPAE-PAD, and a representative chromatogram is shown in Figure 4. Figure 6 shows the oligosaccharide profile before and after treatment with amyloglucosidase. The oligosaccharide profile changes little after amyloglucosidase hydrolysis. Figure 7 shows the glucose content increases only slightly after hydrolysis with amyloglucosidase. This indicates that the agave syrup sample was not adulterated with HFCS or corn syrup.

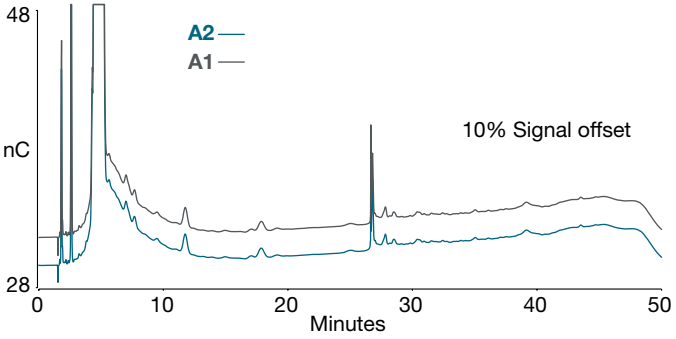

Figure 6. Oligosaccharide profile of Sample 1 before (A1) and after amyloglucosidase hydrolysis (A2)

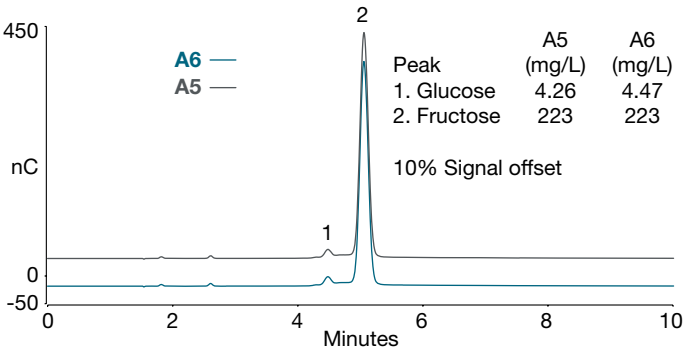

Figure 7. Glucose and fructose of Sample 1 before (A5) and after amyloglucosidase hydrolysis A6 (zoom in 10 min)

Figure 8 shows the oligosaccharide profile before and after treatment with fructanase. Some fructooligosaccharides (FOS) between 17 and 30 min were removed after hydrolysis with fructanase. Figure 9 shows that glucose and fructose increase slightly after hydrolysis with fructanase. This suggests that the sample contains a small amount of fructan.

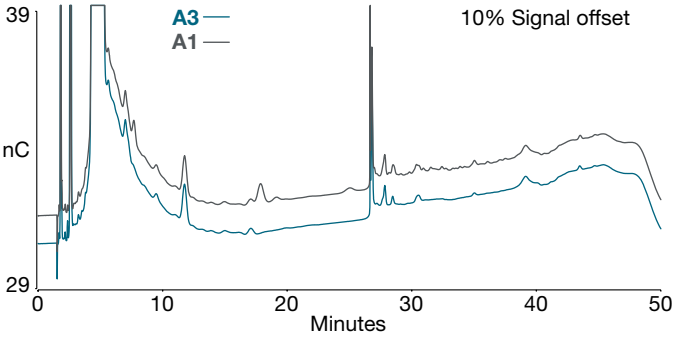

Figure 8. Oligosaccharide profile of Sample 1 before (A1) and after fructanase hydrolysis (A3)

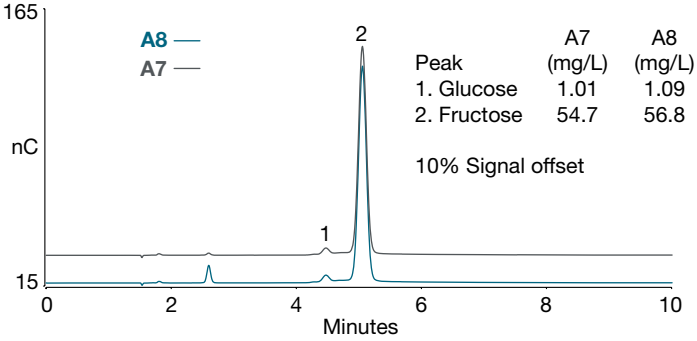

Figure 9. Glucose and fructose of Sample 1 before (A7) and after fructanase hydrolysis A8 (zoom in 10 min)

To determine the amount of fructans, the corrected fructose and glucose were calculated by subtracting the fructose and glucose present in the product. A correction factor of 0.9 is applied to fructose to correct for uptake of water during fructan hydrolysis. Table 9 summarizes the fructan results in the samples.

Table 9. Total fructan in sample (g/100 g)

Analyte	Corrected glucose	Corrected fructose	Total fructan
Sample 1	0.104	2.83	2.65
Sample 2	0.175	3.72	3.52
Sample 3	0.147	1.90	1.86

$TF = (C_F \times 0.9) + C_G$

TF = Total fructan in sample (g/100 g)

C_F = Fructose released from fructan (g/100 g)

C_G = Glucose release from fructan (g/100 g)

0.9 = factor to correct for uptake of water during fructan hydrolysis

Accuracy

The accuracy of the method was evaluated by determining recoveries of sorbitol, glucose, and sucrose in agave syrup samples (Table 10). Sample A1 was used for sorbitol and sucrose spiking experiments. Sample A7 was used for a glucose spiking experiment. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The recovery for three carbohydrates ranged from 95.7 to 104%, indicating this method can accurately determine carbohydrates in agave syrup samples.

Precision

The precision of the method was determined by triplicate injection of the level 4 calibration standard on three separate days over one week. As shown in Table 11, the calculated peak area precision varied from 0.11 to 1.83%, with retention time precision <0.26% for all target carbohydrates. The precision results were better than the results obtained with the Dionex CarboPac PA1-2mm column using manually prepared sodium hydroxide/sodium acetate eluent.[5] The high precision of this method is consistent with results typically obtained with a system using eluent generation.

Table 10. Recoveries of carbohydrate spiked in agave syrup

Analyte	Sample 1			Sample 2			Sample 3		
	Amount found (mg/L)	Amount Added (mg/L)	Recovery (%)	Amount found (mg/L)	Amount added (mg/L)	Recovery (%)	Amount found (mg/L)	Amount added (mg/L)	Recovery (%)
Sorbitol	0.0411	1	102	0.0383	1	101	0.0292	1	101
Glucose	1.01	1	95.8	1.01	1	102	0.920	1	95.7
Sucrose	0.638	1	104	0.902	1	98.3	1.35	1	97.2

Table 11. Retention time and peak area precision

	Analyte						
	Inositol	Sorbitol	Mannitol	HMF	Glucose	Fructose	Sucrose
Retention time RSD	0.26	0.20	0.18	0.0	0.0	0.0	0.26
Peak area RSD	1.75	1.35	0.11	1.12	1.23	1.26	1.83

thermo scientific

Conclusion

This application note demonstrated that the NOM method for agave syrup carbohydrate analysis could be successfully executed with a Dionex CarboPac PA1-1mm column using HPAE-PAD in Dual Eluent Generation Cartridge Mode. The separation, linearity, reproducibility, and sensitivity were excellent. This method is reliable and can be used for major sugars, polyols, and HMF determination in agave syrup. The carbohydrate profile after enzymatic hydrolysis can be used for adulteration detection. Comparison with a traditional HPAE-PAD separation of agave syrup using sodium hydroxide/sodium acetate eluents showed that the Dual EGC method delivers similar resolution of agave carbohydrates but simplifies operation (no eluent preparation) and improves retention time precision.

References

1. Wolever, T.; Vuksan, V.; Jenkins, A. L.; Campbell, J. Determination of glycemic index of: Organic 100% blue agave syrup and prebiotic 100% blue agave syrup; Final report for Glycemic Index Laboratories: Toronto, ON, November 2011.
2. Foster-Powell, K.; Holt, S.H.A.; Brand-Miller, J.C. International table of glycemic index and glycemic load values: 2002. *Am. J. Clin Nutr.* **2002,** *76,* 5–56.
3. NORMA Oficial Mexicana NOM-003-SAGARPA-2016.
4. Thermo Scientific Application Note 73132: Carbohydrate analysis of agave syrup using HPAE-PAD. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-73132-ic-hpae-pad-carbohydrate-agave-syrup-an73132-en.pdf
5. Willems, J.L.; Low, N.H. Major carbohydrate, polyol, and oligosaccharide profiles of agave syrup. Application of this data to authenticity analysis. *J. Agric. Food Chem.* **2012,** *60*(35), 8745–54. doi: 10.1021/jf3027342. Epub 2012 Aug 21.
6. Thermo Scientific Dionex ICS-6000 Ion Chromatography System Operator's Manual. https://assets.thermofisher.com/TFS-Assets/CMD/manuals/man-22181-97002-ics-6000-man2218197002-en.pdf
7. Thermo Scientific Electrochemical Detection User's Compendium, P/N 065340-02, April 2013. http://tools.thermofisher.com/content/sfs/manuals/Man-065340-Electrochemical-Detection-Man065340-EN.pdf
8. Thermo Scientific Dionex CarboPac PA1 Columns Product Manual P/N 065547-01, November 2013. https://assets.thermofisher.com/TFS-Assets/CMD/manuals/Man-065547-CarboPac-PA1-Column-Man065547-EN.pdf
9. ICH Guideline Q2B, Validation of Analytical Procedures, Methodology (CPMP/ICH/281/95), Geneva, Switzerland, November 1996.

Find out more at **thermofisher.com/carbopac**

ThermoFisher SCIENTIFIC