

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

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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™ PA200-1 mm column using a KOH/KMSA eluent produced electrolytically using dual eluent generation cartridge (Dual EGC) mode

Introduction

Agave syrup, a recent food product from Mexico, is produced from the sap of the agave plant. This product has gained popularity as an alternative to traditional sweeteners, such as table sugar 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 carbohydrates 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-hydroxymethylfurfural (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 pulsed amperometric detection (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 manually prepared sodium hydroxide/sodium acetate eluents, reducing eluent consumption four-fold.⁴ We also showed that this method could be successfully executed with a Dionex CarboPac PA1 column (250 × 1 mm) using HPAE-PAD in Dual EGC mode. This reduces eluent consumption and eliminates the manual preparation of the sodium hydroxide/sodium acetate eluents. The Dionex CarboPac PA200 column has been specially developed to provide high-resolution separations of charged and neutral oligosaccharides and is the recommended column for oligosaccharide applications.⁵ In this application note, the NOM method was evaluated with a Dionex CarboPac PA200 column (250 × 1 mm) using HPAE-PAD in Dual EGC mode. 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 samples were determined. Inositol was also determined because it was reported to be present in agave syrup.⁶ The possibility of adulteration was evaluated with amyloglucosidase enzymatic hydrolysis. Total fructan was determined with fructanase treatment. Compared to the Dionex CarboPac PA1 column, the Dionex CarboPac PA200 column provides better separation for oligosaccharides. As a result, the oligosaccharide profiles before and after enzymatic hydrolysis can be more clearly observed and used for adulteration detection.

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 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 with 1 mil gasket, Pack of 6 (P/N 303397).
- Reference electrode, pH, Ag/AgCl (P/N 061879)

Assemble the cell following the Dionex ICS-6000 operator manual⁷ and Dionex ED User’s Compendium for Electrochemical Detection.⁸

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)

Software

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software, version 7.2.9

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 M9546)
- Sorbitol (Sigma-Aldrich, P/N S1876)
- Inositol (Sigma-Aldrich, P/N I5125)
- Hydroxymethylfurfural (Fisher Scientific, P/N AC1214)
- Amyloglucosidase powder (36,000 U/g) (Megazyme, P/N E0AMGDFFD)
- 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 PA200 Guard, 1 × 50 mm (P/N 302862) Dionex CarboPac PA200 Separation, 1 × 250 mm (P/N 302861)
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	0	50
0	0	50
6	0	50
12	10	80
30	35	80
40	70	80
40.1	0	50
50	0	50

Table 2. Carbohydrates, 4-potential waveform

Time (s)	Voltage (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. Confirm vacuum status by pressing the commands (F8), select pump_1, right-click on the properties panel and check 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 a 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 (35–55 kPa). 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. 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, remove the backpressure

tubing, install a Dionex CarboPac PA200-1mm column set, set KMSA and KOH to the desired concentrations 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.

Working standard solutions

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

Table 3. Calibration standards

Analyte	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

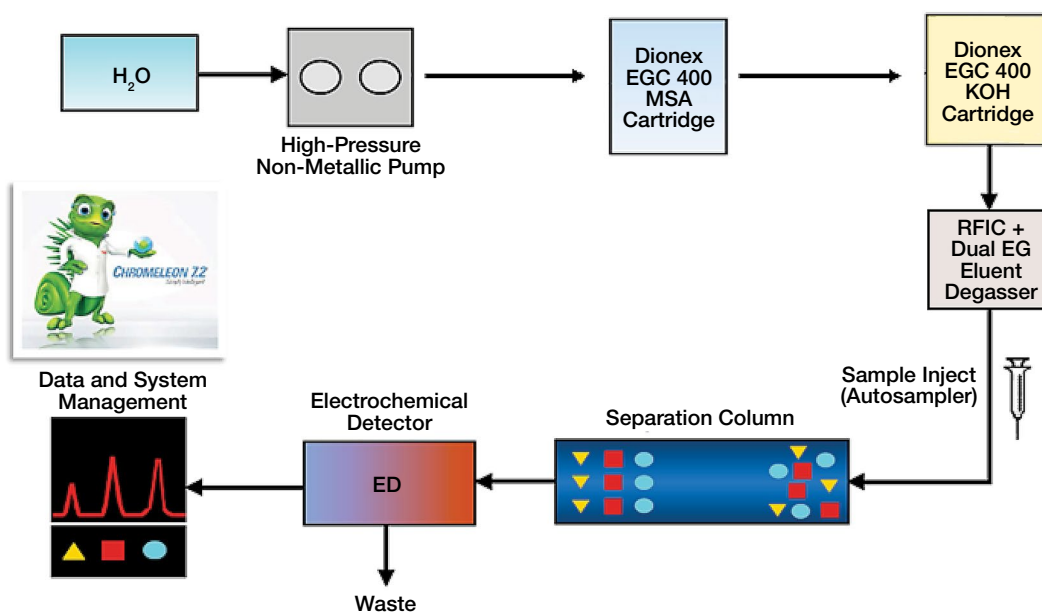


Figure 1. Dionex ICS-6000 HPIC system in Dual EGC mode

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. Aliquot this solution into 2 mL vials and store at -20 °C.

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 the following samples for enzymatic hydrolysis in 2 mL centrifuge tubes (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 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 PA200 column has been developed to provide high-resolution separations of charged and neutral oligosaccharides and is the recommended column for these applications.⁹ Figure 2 shows a separation of the 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 are well resolved. The resolution between all the components is >2.0 .

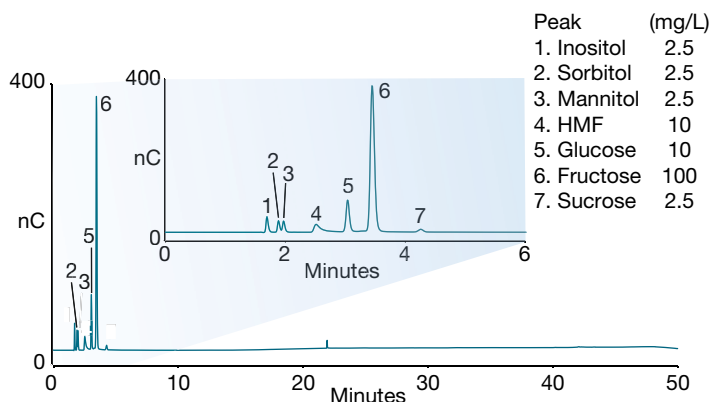


Figure 2. Separation of seven agave sugar standards using a Dionex CarboPac PA200-1 mm column set

Table 4. Sample enzyme treatment

Code	Sample stock (μL)	Amyloglucosidase (AG) (μL)	Fructanase (FN) (μL)	H ₂ O (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 ₂ O (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 by using 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 were 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.

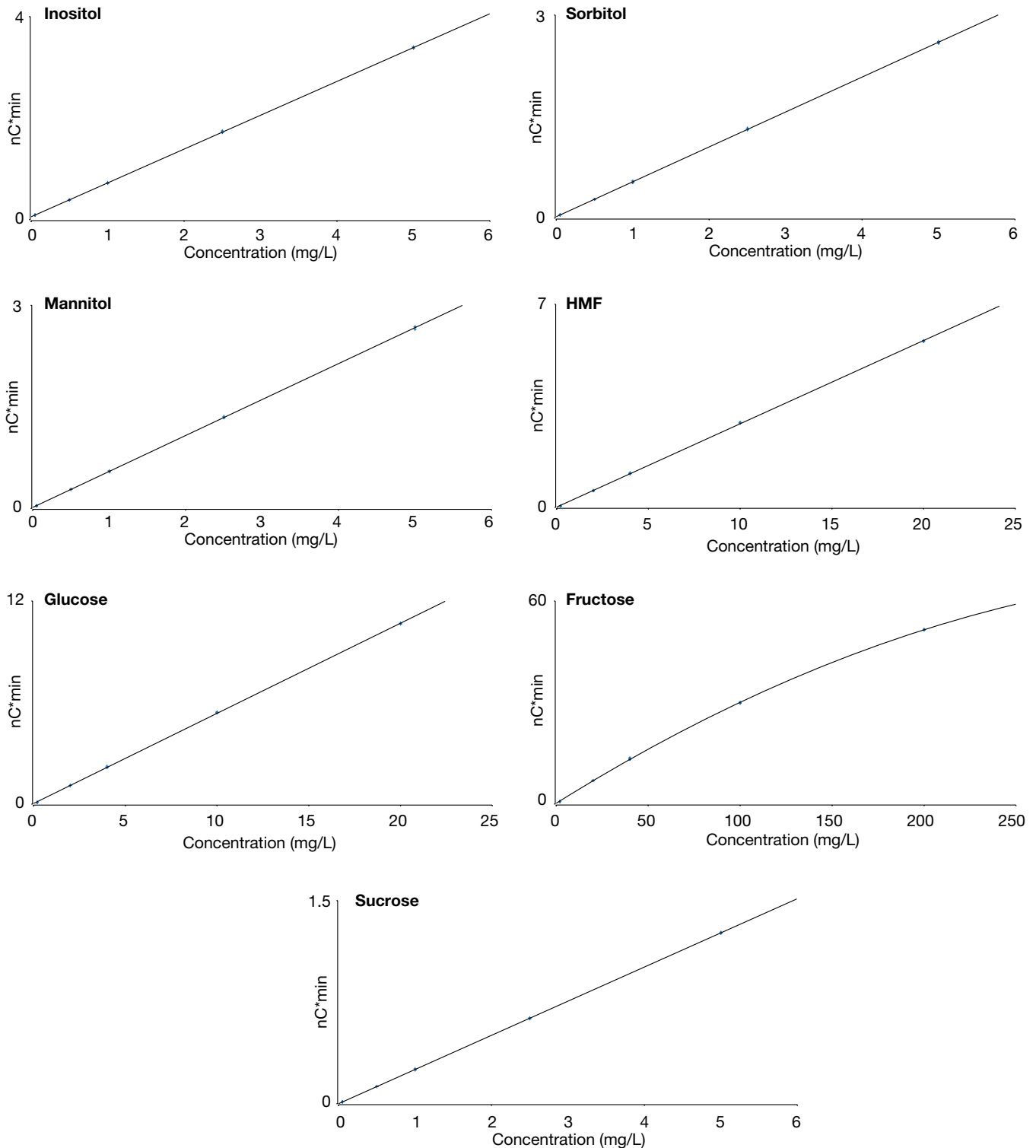


Figure 3. Seven carbohydrate calibration curves, linear fitting except fructose

Table 6. Calibration

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

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

Determination of the S/N ratio is performed by comparing the measured signal from a standard with a low concentration to a blank sample and establishing the minimum concentration at which the analyte can be reliably detected. A S/N of 3 is used for estimating the detection limit (LOD), and a S/N of 10 is used for estimating the quantification limit (LOQ).¹⁰ 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 based on the sample weight (250 mg) and sample volume (50 mL) (Table 7).

Table 7. LOD and LOQ

Analyte	LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/L}$)	LOD in agave syrup ($\mu\text{g/g}$)	LOQ in agave syrup ($\mu\text{g/g}$)
Inositol	2.90	9.65	1.930	6.43
Sorbitol	1.15	3.83	0.767	2.56
Mannitol	1.38	4.59	0.919	3.06
HMF	11.56	38.5	7.70	25.7
Glucose	5.23	17.4	3.49	11.6
Fructose	1.50	5.00	1.00	3.33
Sucrose	14.6	48.6	9.72	32.4

Table 8. Carbohydrate content (g/100 g)

Analyte	Inositol	Sorbitol	Mannitol	HMF	Glucose	Fructose	Sucrose
Sample 1	0.112	0.00262	0.247	ND	1.48	73.1	0.0421
Sample 2	0.0699	0.00250	0.102	ND	1.35	70.7	0.0604
Sample 3	0.0670	0.00214	0.232	ND	1.31	74.8	0.0826

Sample analysis

Three brands of agave syrup were obtained from a local supermarket. Sample A1 was used to determine 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 71–75% of the total carbohydrate. The other major carbohydrate identified was glucose, with 1.3–1.5% of the total carbohydrate.

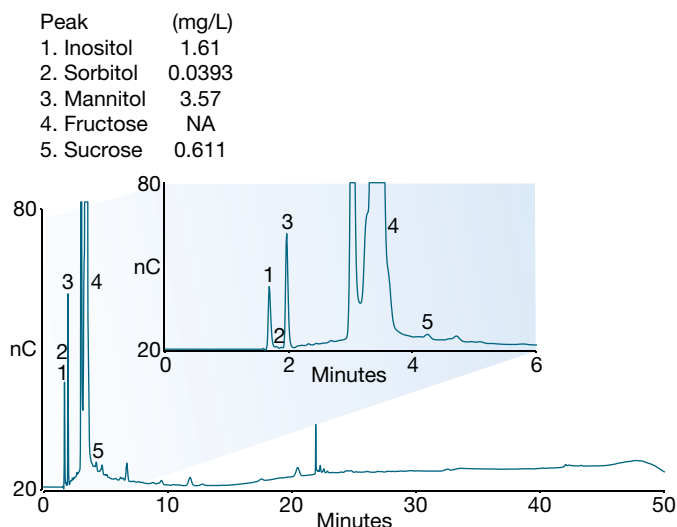


Figure 4. Carbohydrate profile of Sample 1-A1

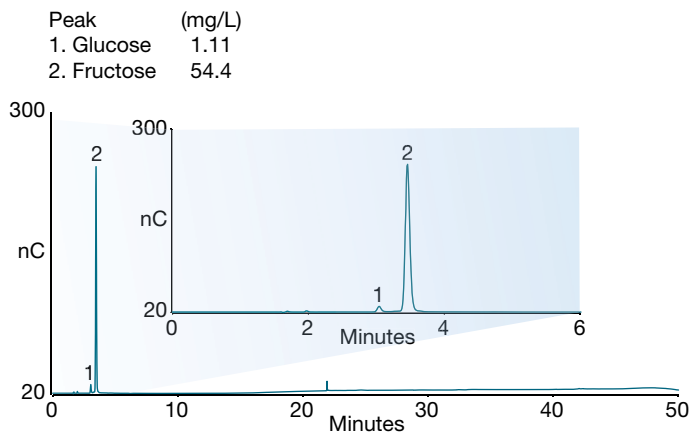


Figure 5. Glucose and fructose in Sample 1-A7

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 PA1-2 mm 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 hydrolysis 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.

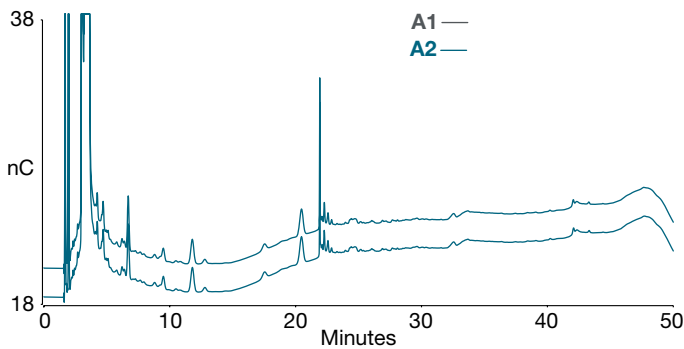


Figure 6. Oligosaccharide profile of Sample 1 before (A1) and after amyloglucosidase hydrolysis (A2)

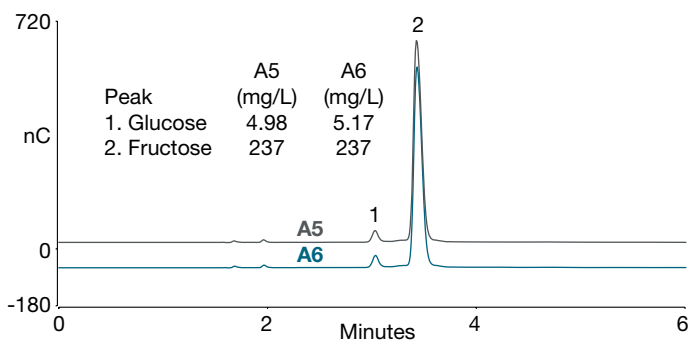


Figure 7. Glucose and fructose of Sample 1 before (A5) and after amyloglucosidase hydrolysis A6 (zoom in 6 min)

Figure 8 shows the oligosaccharide profile before and after hydrolysis with fructanase. Some fructooligosaccharides (FOS) eluting between 10 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.

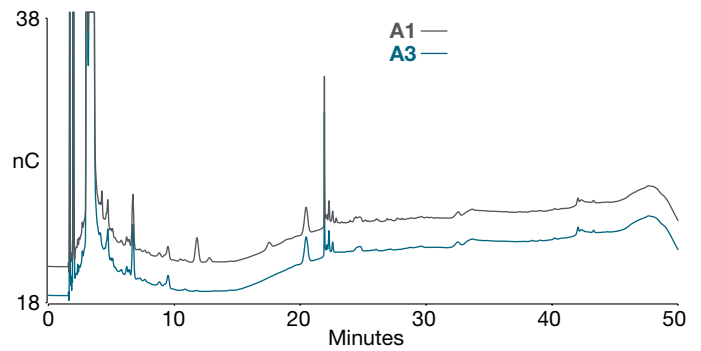


Figure 8. Oligosaccharide profile of Sample 1 before (A1) and after fructanase hydrolysis (A3)

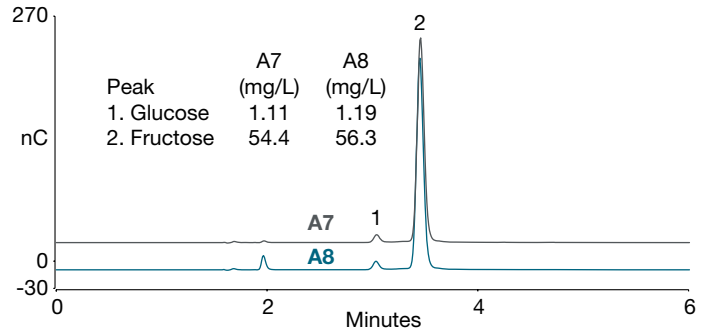


Figure 9. Glucose and fructose of Sample 1 before (A7) and after fructanase hydrolysis A8 (zoom in 6 min)

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 the uptake of water during fructan hydrolysis. Table 9 summarizes the samples' fructan results.

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

Where

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 the uptake of water during fructan hydrolysis

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

	Corrected glucose	Corrected fructose	Total fructan
Sample 1	0.121	2.63	2.48
Sample 2	0.168	3.50	3.31
Sample 3	0.114	1.43	1.40

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 these three carbohydrates ranged from 97.2 to 105%, 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 0.85 to 1.66% with retention time precision <0.28% for all target carbohydrates. The precision results were better than the results obtained with the Dionex CarboPac PA1-2 mm 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.

Conclusion

This application note demonstrates that the NOM method for agave syrup carbohydrate analysis could be successfully executed with a Dionex CarboPac PA200-1 mm column using HPAE-PAD in EGC 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.

Table 10. Recoveries of carbohydrates 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	0.0487	1	102	0.0375	1	99.5	0.0321	1	101
Glucose	1.11	1	103	1.02	1	101	0.981	1	97.2
Sucrose	0.632	1	105	0.905	1	101	1.24	1	105

Table 11. Retention time and peak area precision

	Analyte						
	Inositol	Sorbitol	Mannitol	HMF	Glucose	Fructose	Sucrose
Retention time RSD	0.28	0.00	0.00	0.00	0.16	0.14	0.11
CD peak area RSD	0.85	1.66	1.42	1.05	1.14	1.30	0.95

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