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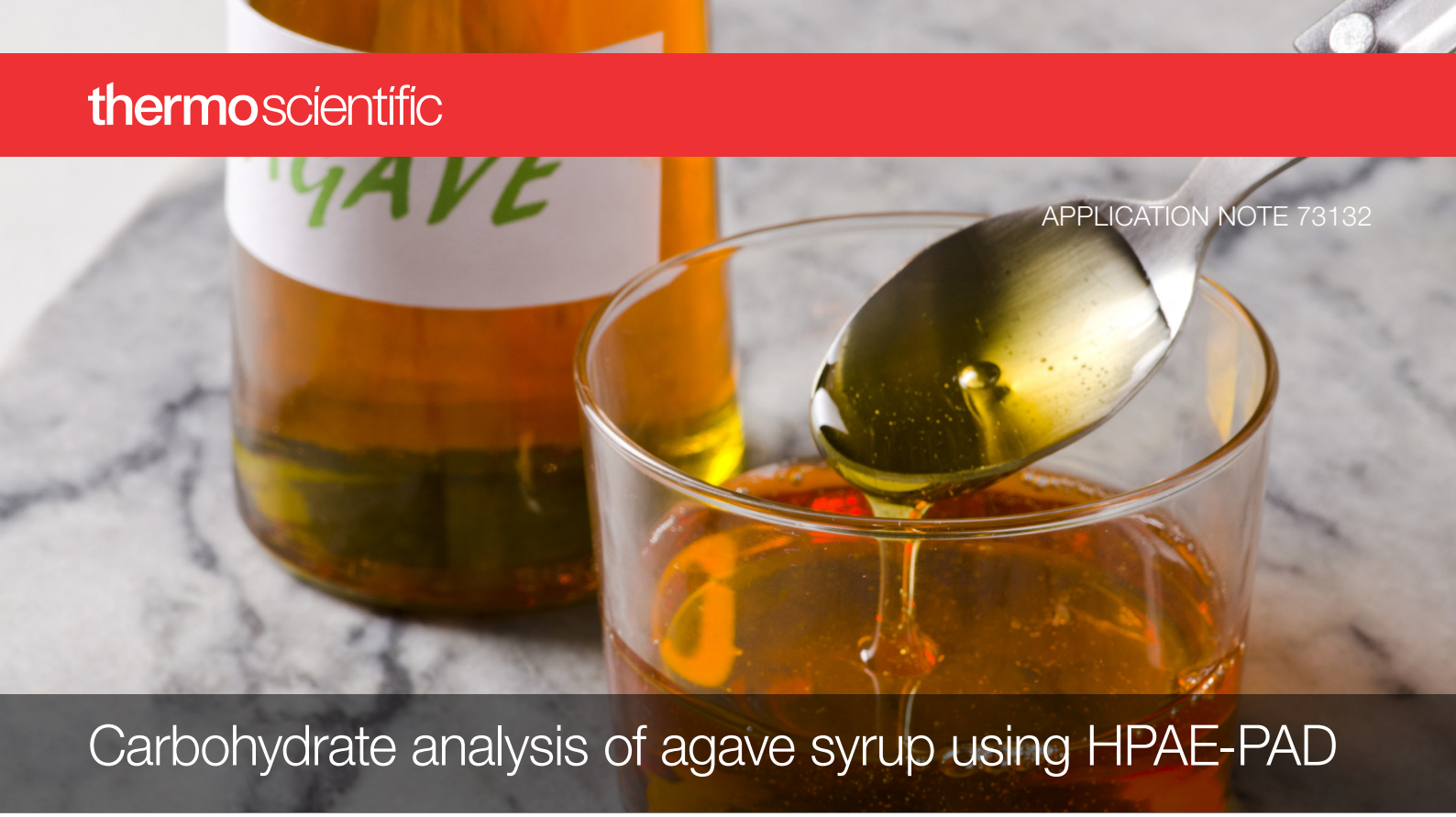

APPLICATION NOTE 73132

Carbohydrate analysis of agave syrup using HPAE-PAD

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Keywords

Dionex CarboPac PA1-2mm column, Dionex ICS 5000+ HPIC system, carbohydrate, polyols, oligosaccharides, fructan, adulteration, Mexico NOM, PAD, electrochemical detection, Dionex ICS-6000 HPIC system

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™ PA1-2mm column

Introduction

Agave syrup is a recent food product from Mexico. It 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 (17–27) when compared to honey (55) and sucrose (68).[1-2] The glycemic index (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

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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). HPAE-PAD is a direct-detection technique 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), polyol (sorbitol, mannitol), and 5-hydroxymethyfural (HMF), and detection of adulterations using HPAE-PAD. According to NOM, agave syrup is diluted with water and analyzed before and after amyloglucosidase and fructanase enzymatic hydrolysis using a Dionex CarboPac PA1 IC column (250 × 4 mm) and pulsed amperometric detection (PAD). The content of the sugars as well as the content of fructan is calculated.

In this application note, the NOM method was evaluated with a Dionex CarboPac PA1 IC column (250 × 2 mm). The 2 mm column requires a flow rate approximately four times less than the 4 mm column, and thus reduces eluent consumption. Key performance parameters were evaluated including separation, linearity, limits of detection, and precision. Three samples were analyzed and the main sugars, polyol, and HMF in those sample were determined. Inositol was also determined because it was reported to be present in agave syrup.[4] Adulteration was evaluated with amyloglucosidase treatment. Total fructan was determined with fructanase treatment.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC system including*:
  - Dionex ICS-5000⁺ DP Pump module
  - Dionex ICS-5000⁺ DC Detector/Chromatography module with ED Electrochemical Detector
  - Dionex AS-AP Autosampler with sample tray cooling, 250 µL sample syringe (P/N 074306), 1200 µL buffer line (P/N 074989), and 1.5 mL vial trays (P/N 074936)
- Thermo Scientific™ Dionex™ ICS-5000⁺ ED Electrochemical Detector Cell (P/N 072044)
- Gold on PTFE Disposable working electrode with 2 mil gaskets (P/N 066480)
- Reference electrode pH, Ag/AgCl (P/N 061879)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software, version 7.2.5

*This method can be run on a single Dionex ICS-5000⁺ HPIC or single Dionex ICS-6000 HPIC system.

Assemble the cell following the Dionex ICS 5000⁺ Operator's Manual[5] and Dionex ED User's Compendium for Electrochemical Detection[6].

Consumables

- Thermo Scientific™ Nalgene™ Syringe Filter, PES membrane, 0.2 µm (Fisher Scientific P/N 725-2520)
- Polypropylene autosampler vials, 1.5 mL with caps and split septa (P/N 079812)
- Thermo Scientific™ Nalgene™ Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (Thermo Scientific P/N 164-0020)
- Microcentrifuge tube, 2 mL (Fisher Scientific P/N 05-408-138)
- Helium, ultrahigh purity grade from Airgas

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Sodium acetate salt, electrochemical grade (Thermo Scientific Dionex P/N 059326)
- Sodium hydroxide, 50% (w/w) (Fisher P/N SS254-500)
- 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)
- Hydromethylfurfural (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

Columns:*	Dionex CarboPac PA1 Guard, 2 × 50 mm (P/N 057179) Dionex CarboPac PA1 Separation, 2 × 250 mm (P/N 057178)
Eluent:	Gradient (Table 1)
Flow rate:	0.25 mL/min
Column temp.:	30 °C
Injection volume:	2.5 µL (Full loop)
Autosampler temp.:	5 °C
Reference electrode:	Ag/AgCl
Working electrode:	Disposable electrode gold, with a 2 mil gasket
Detection:	Pulsed Amperometric Detector (Electrochemical Detection)
Detection compartment temp.:	30 °C
Detection waveform:	Gold, Carbohydrates, 4-Potential (Table 2)
System backpressure:	~1200 psi
Run time:	50 min

*The Mexican official method describes the column as follows: "HPAEC 250 × 4 mm with guard column (50 × 4 mm) with anion exchange specific resin. Dionex CarboPac PA1 or equivalent column." The diameter of the PA1 column is 2 mm. Therefore, the flow rate was adjusted from 1 mL/min (Mexican NOM method condition) to 0.25 mL/min. The injection volume was adjusted from 10 µL (Mexican method condition) to 2.5 µL.

Table 1. Eluent gradient

Time (min)	A (%) 200 mM NaOH	B (%) 200 mM NaOH+ 700 mM NaOAc	C (%) H_2O
-20		Equilibrium	
-20	50	0	50
-10	50		50
-9.9	50	1	49
0		Run	
6	50	1	49
12	50	4	46
30	50	20	30
40	50	40	10
40.1	100	0	0
50		End	

Table 2. Carbohydrates, 4-potential waveform

Time (s)	Potential (V)	Integration
0	0.1	Off
0.2	0.1	On
0.4	0.1	Off
0.41	-2.0	Off
0.42	-2.0	Off
0.43	0.6	Off
0.44	-0.1	Off
0.5	-0.1	Off

Preparation of solutions and reagents

Eluent preparation

Eluent A (200 mM sodium hydroxide)

This eluent is prepared as described in the NOM method.

- Degas 2 L of DI water by sparging helium gas in a plastic eluent bottle for at least 15 min.
- Add 900 mL of degassed water into a 1 L volumetric flask, add 10.4 mL or 16 g of 50% (w/w) sodium hydroxide solution and bring to volume.
- Mix well and transfer eluent into a 2 L eluent bottle, continue bubbling helium for 30 min, and blanket with helium gas at 5–8 psi.

Eluent B (200 mM sodium hydroxide + 700 mM sodium acetate)

- Degas 2 L of DI water as indicated for eluent A.
- Dissolve 57.4 g of sodium acetate in 800 mL of degassed DI water. Vacuum filter this solution through a 0.2 µm Nalgene 1 L nylon filter to remove particles from the sodium acetate that can damage parts of the pump.
- Transfer the solution to a 1 L volumetric flask, add 10.4 mL or 16 g of 50% (w/w) NaOH, and bring to volume.
- Mix well and transfer eluent into a 2 L eluent bottle, continue bubbling helium for 30 min and blanket with helium gas at 5–8 psi.
- For additional details on eluent preparation refer to Thermo Scientific Technical Note 71.[7]

Eluent C (Water)

- Degas 2 L of DI water as indicated for eluent A.

Standards preparation

Stock standard solutions

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

Working standard solutions

- Prepare the stock calibration standard mixture and then prepare calibration standards levels 1–5 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 solution to 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 the solution to 2 mL vials and keep 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 dissolve completely.
- Filter 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 (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.

Table 3. Calibration standards

Analyte	Stock concentration (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

Table 4. Sample enzyme treatment

Code	Sample stock (µL)	Amyloglucosidate (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

Results and discussion

Separation

The Dionex CarboPac PA1 column is a general-purpose column for the separation of mono, di, and some oligosaccharides by HPAE-PAD. It is recommended for analyzing common sugars in food samples.[8]

Figure 1 shows a separation of main sugars (fructose, glucose, and sucrose), polyol (sorbitol, mannitol, inositol), and HMF. Inositol, sorbitol, mannitol, HMF, fructose, and glucose are well resolved. The resolution between all the components is >2.0.

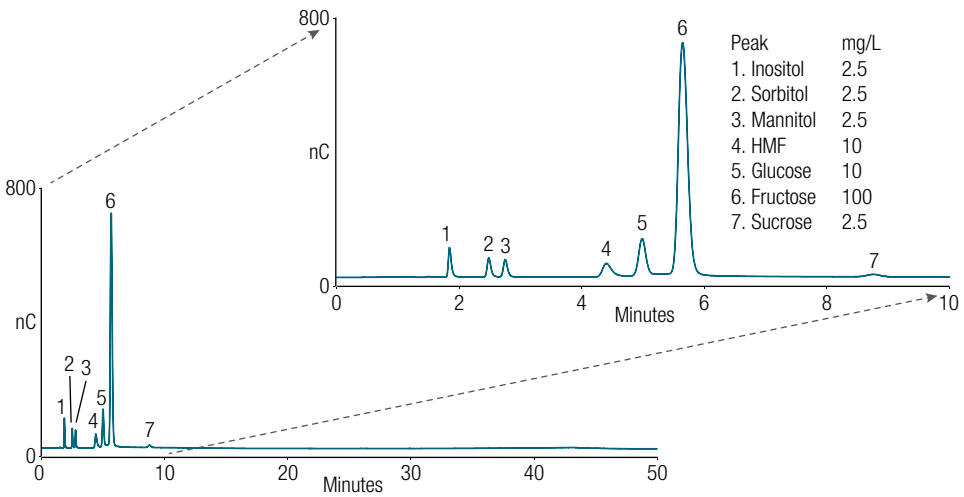

Figure 1. Separation of seven agave sugar standards using a Dionex CarboPac PA1-2mm column

Calibration

The calibration standard mixture was prepared with individual carbohydrate concentrations that are typical for agave syrup.

Calibration curves with five concentration levels were constructed for the seven-carbohydrate standard. For fructose, the calibration curves show 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 2 shows the calibration curves. Table 6 summarizes the calibration data. The coefficient of determination (r^2) was greater than 0.999 for each component.

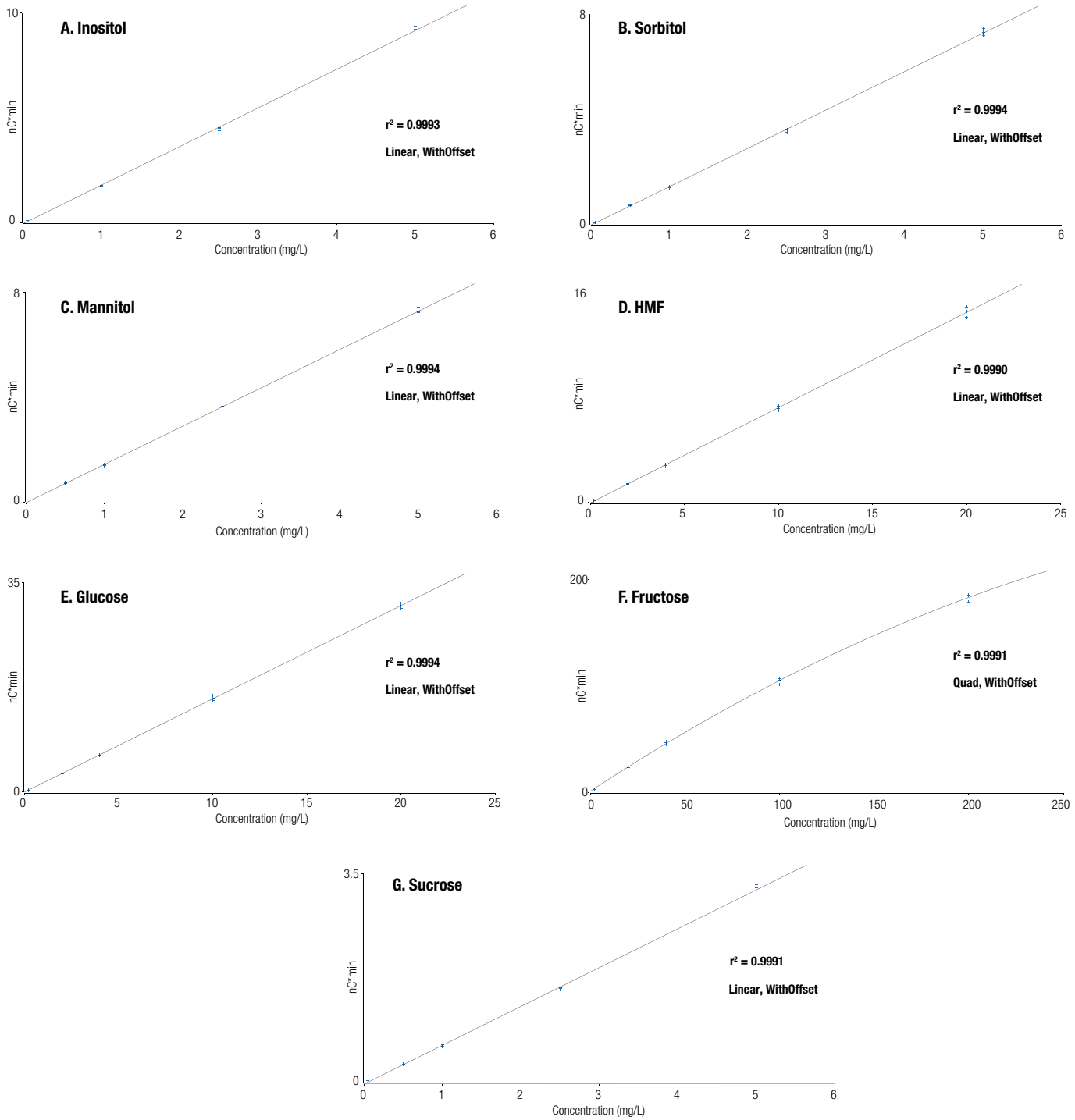

Figure 2. Seven carbohydrate calibration curves, linear fitting except fructose

Table 6. Calibration

Standard	Range (mg/L)	Calibration type	Coefficient of determination (r^2)
Inositol	0.05–5	Linear	0.9993
Sorbitol	0.05–5	Linear	0.9994
Mannitol	0.05–5	Linear	0.9994
HMF	0.2–20	Linear	0.9990
Glucose	0.2–20	Linear	0.9994
Fructose	2–200	Quadratic	0.9991
Sucrose	0.05–5	Linear	0.9991

Limit of detection and limit of quantification

Determination of the S/N ratio is performed by comparing measured signal from standard with low concentrations of analyte with those of blank samples and 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).

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 3). Sample A7 was used for the determination of glucose and fructose in agave syrup (Figure 4). Table 8 summarizes the results of carbohydrate and polyol analysis. The major carbohydrate found in the three agave syrup samples was fructose with a concentration range of 70–72%. The other major carbohydrate identified was glucose with a concentration range of 1.3–1.6%.

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	1.40	4.68	0.936	3.12
Sorbitol	1.38	4.58	0.917	3.06
Mannitol	1.79	5.96	1.19	3.97
HMF	9.89	33.0	6.59	22.0
Glucose	3.57	11.9	2.38	7.94
Fructose	4.17	13.9	2.78	9.26
Sucrose	15.3	51.1	10.2	34.1

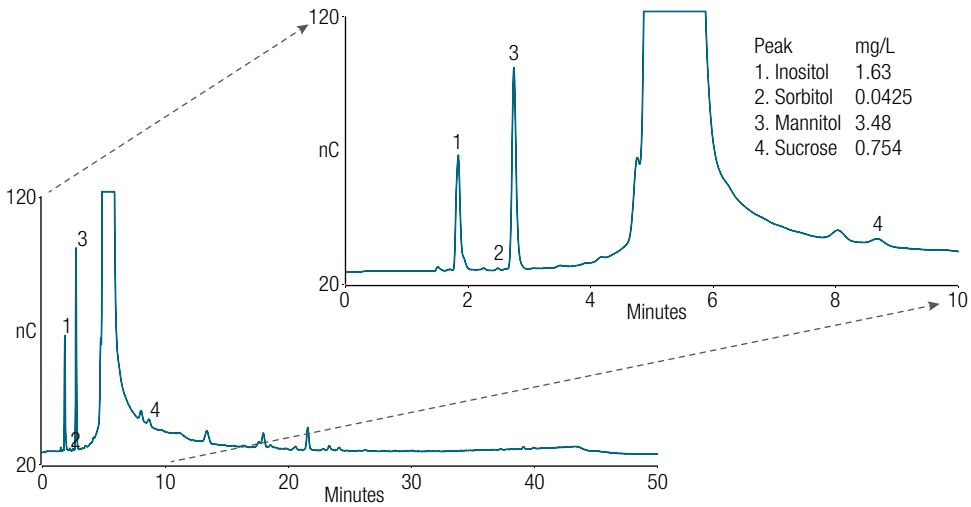

Figure 3. Carbohydrate profile of Sample 1-A1

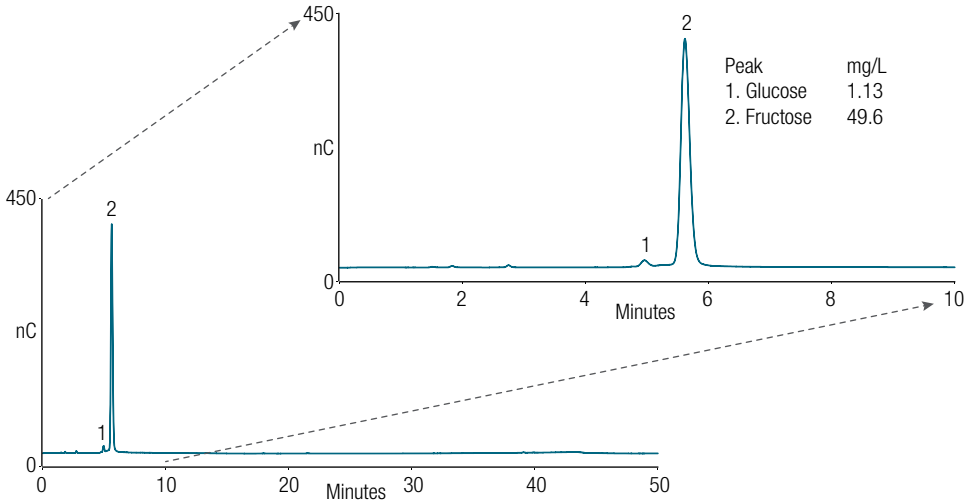

Figure 4. Glucose and fructose in Sample 1-A7

Table 8. Carbohydrate content (g/100g)

Sample	Inositol	Sorbitol	Mannitol	HMF	Glucose	Fructose	Sucrose
Sample 1	0.108	0.00295	0.227	ND	1.39	73.2	0.0334
Sample 2	0.0708	0.00292	0.104	ND	1.51	71.2	0.0660
Sample 3	0.0608	0.00221	0.213	ND	1.40	72.0	0.0857

The major polyols identified in the agave syrup sample were mannitol and inositol, with concentration ranges of 0.1–0.3% and 0.06–0.11%, respectively.

The oligosaccharide profile of the three agave syrup samples was examined by HPAE-PAD, and a representative chromatogram is shown in Figure 3. Figure 5 shows the oligosaccharide profile before and after hydrolysis with amyloglucosidase. The oligosaccharide profile changes little after amyloglucosidase hydrolysis. Figure 6 shows a slight increase in the glucose content after hydrolysis with amyloglucosidase. This indicates that the agave syrup sample was not adulterated with HFCS or corn syrup.

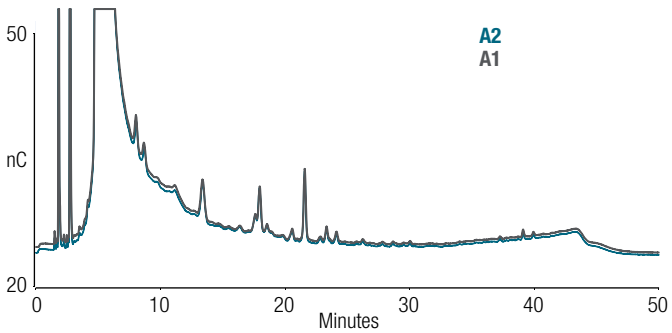

Figure 5. Oligosaccharide profile of Sample 1 before (A1) and after amyloglucosidase hydrolysis (A2)

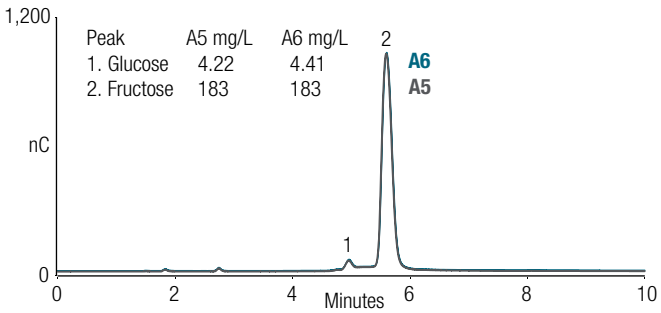

Figure 6. Glucose and fructose of Sample 1 before (A5) and after amyloglucosidase hydrolysis A6 (zoom in 10 min)

Figure 7 shows the oligosaccharide profile before and after hydrolysis with fructanase. Some fructoligosaccharides (FOS) between RT 17 and 30 min were removed after hydrolysis with fructanase. Figure 8 shows that the glucose and fructose increase slightly after hydrolysis with fructanase. This suggests that the sample contains a small amount of fructan.

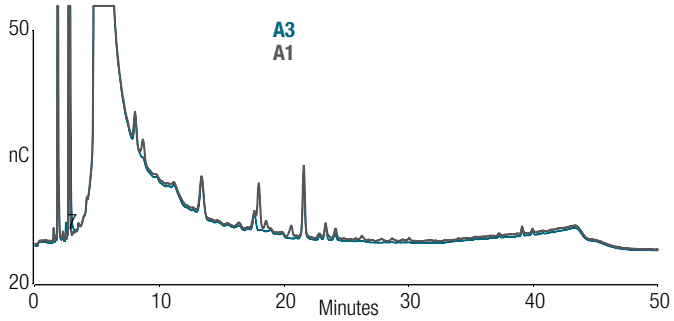

Figure 7. Oligosaccharide profile of Sample 1 before (A1) and after fructanase hydrolysis (A3)

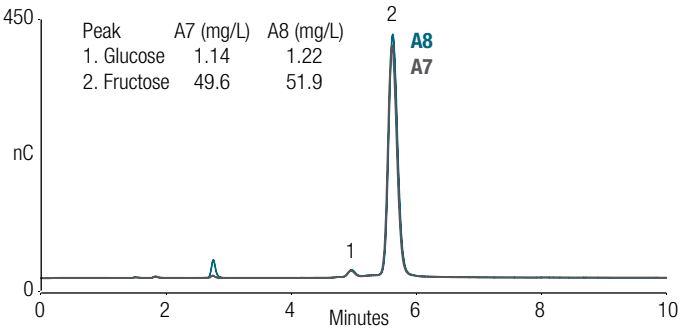

Figure 8. Glucose and fructose of Sample 1 before (A7) and after fructanase hydrolysis A8 (zoom in 10 min)

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 content results.

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

Sample	Corrected glucose	Corrected fructose	Total fructan
1	0.110	3.06	2.86
2	0.131	2.38	2.27
3	0.173	3.35	3.19

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

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

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

CG = 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 sample (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 85% to 115%, 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 a week. As shown in Table 11, the calculated peak area precision varied from 1.06% to 2.82% with retention time precision < 1% for all target carbohydrates.

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	0.0410	1	99.3	0.0430	1	94.6	0.0332	1	101
Glucose	1.12	1	94.2	1.12	1	85.5	1.07	1	89.5
Sucrose	0.754	1	103	0.977	1	87.2	1.26	1	114

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Table 11. Retention time and peak area precisions

Analyte	Inositol	Sorbitol	Mannitol	HMF	Glucose	Fructose	Sucrose
Retention Time RSD	0.260	0.330	0.460	0.380	0.880	0.920	1.22
Peak Area RSD	1.50	1.74	2.54	2.46	2.82	2.46	1.06

Conclusion

This application note demonstrated that the Mexican NOM method for agave syrup carbohydrate analysis could be successfully executed with a Dionex CarboPac PA1-2mm column. The separation, linearity, reproducibility, and sensitivity were excellent. This method is reliable and can be used for major sugars, polyol, and HMF determination in agave syrup. The carbohydrate profile after enzymatic hydrolysis can be used to detect adulteration.

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