Determination of low- and non-caloric sweeteners in food and beverages by HPAE-PAD

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Goal

To develop an HPAE-PAD method for the determination of low- and non-caloric sweeteners in food and beverages

Introduction

Artificial sweeteners, used as sugar substitutes in food and beverage products, have increased in popularity in recent years as more consumers are concerned about obesity and dental decay caused by consuming natural sugars. In addition, artificial sweeteners are used for products marketed to diabetics. Because artificial sweeteners are 200–700 times sweeter than table sugar, they can be used in smaller amounts to create the same level of sweetness.

Artificial sweeteners are broadly classified into two categories: non-nutritive and nutritive sweeteners. Aspartame, acesulfame potassium, neotame, saccharin, stevia, and sucralose are commonly used non-nutritive, U.S. Food and Drug Administration (FDA)-approved artificial sweeteners. They may have unfavorable effects on health, including glucose intolerance and failure to cause weight reduction. The nutritive sweeteners include sugar alcohols, such as sorbitol, xylitol, lactitol, mannitol, erythritol, and maltitol.

Recently, naturally occurring rare sugars have emerged as an alternative category of sweeteners. These monosaccharides and their derivatives are found in nature in small quantities and lack significant calories. Allulose is one such low-calorie sweetener that occurs in small amounts in wheat and dried fruits, such as raisins and dried figs. The chemical structure of allulose is similar to fructose, but it is not metabolized/absorbed by the body in the same way and does not contribute the same number of calories. According to the FDA, which recently approved allulose as GRAS (generally recognized as safe), it provides about 0.4 calories per gram versus 4 calories per gram in cane sugar. For labeling purposes, it is important to determine the amount of sweetener used in low-calorie foods and beverages.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for carbohydrate determinations. Basic eluents (pH >12) are required to ionize carbohydrates to form oxyanions for separation. These anions are separated on an HPAE column. Carbohydrates are then detected by PAD with a gold working electrode, and therefore no sample derivatization is required. HPAE-PAD has been used to determine sucralose in sugar-free foods and beverages.[1-4]

In this application note, we describe an HPAE-PAD method to determine three sugar substitutes (erythritol, allulose, and sucralose) in low calorie/zero sugar/sugar-free foods and beverages. These sugar substitutes are often used in combination in food and beverages to create the same level of sweetness as natural sugars as well as minimize less desired flavors of these sugar substitutes. The separation of the sugars was achieved on a Thermo Scientific™ Dionex™ CarboPac™ PA20 column.[5] In this work, we analyzed five commercial sugar-free products for their erythritol, allulose, and sucralose contents. In addition, we demonstrate this application using a PdH reference electrode (RE) and compare the results to using an Ag/AgCl RE. These data extend our previous studies with the PdH RE.[6]

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC™ system* including:
  - Thermo Scientific™ Dionex™ ICS-5000⁺ DP Dual Pump with degas option (P/N 079975)
  - Thermo Scientific™ Dionex™ ICS-5000⁺ DC Detector Chromatography Compartment with dual temperature zones, two injection valves (P/N 075943)
  - Thermo Scientific™ Dionex™ ICS-5000⁺ ED Electrochemical Detector (P/N 072042) and Thermo Scientific™ Dionex™ ICS-5000⁺ ED Electrochemical Detector Cell (P/N 072044)
- Thermo Scientific™ Dionex™ AS-AP Autosampler (P/N 074926) with tray cooling option (recommended)
- Thermo Scientific™ Chromeleon™ Chromatography Data System Software, Version 7.2.9

*This application can be performed on a Dionex ICS-6000 system.

Consumables

- Thermo Scientific™ Dionex™ ICS-5000⁺ ED Electrochemical Detector Ag/AgCl pH Reference Electrode (P/N 061879)
- Thermo Scientific™ Dionex™ ICS-5000⁺ ED Electrochemical Detector PdH Reference Electrode (P/N 072075)
- Thermo Scientific™ Dionex™ ICS-5000⁺ ED Electrochemical Detector Gold on PTFE Disposable Electrodes, pack of 6 (two 2.0 mil gaskets included) (P/N 066480) (1 mil = 25.4 µm)
- Thermo Scientific™ Dionex™ Vial Kit, 10 mL Polystyrene with Caps and Blue Septa (P/N 074228)
- Thermo Scientific™ Nalgene™ Syringe Filters, PES, 0.2 µm (Fisher Scientific, P/N 09-740-61A)
- AirTite™ All-Plastic Norm-Ject™ Syringes, 5 mL, sterile (Fisher Scientific, P/N 14-817-28)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Sodium hydroxide, 50% w/w (Fisher Scientific P/N SS254-500)
- Sodium acetate, anhydrous, electrochemical grade (P/N 059326)
- D-Glucose, Sigma-Aldrich (P/N G8270)
- D-Fructose, Sigma-Aldrich (P/N F2543)
- Sucrose, Sigma-Aldrich (P/N S-9378)
- Sucralose 98.0+%, TCI America (P/N S0839)
- D-Psicose (Allulose) 99.0+%, TCI America (P/N P1699)
- Erythritol, Pfanstiehl Laboratories Inc.

Preparation of solutions and reagents

Eluent solutions

100 mM sodium hydroxide: To make 0.1 M NaOH, add 5.2 mL of 50% (w/w) NaOH to 1 L of degassed DI water by removing the NaOH aliquot from the middle of the 50% solution where sodium carbonate is least likely to have formed. Do not pipet from the bottom where there may be sodium carbonate precipitate, and prepare eluent only from a bottle of 50% sodium hydroxide that still contains at least a third of its original volume. Place the tip of the pipette containing the aliquot of NaOH ~1 in. (2.54 cm) below the surface of the DI water and dispense the NaOH. If properly prepared without stirring, most of the concentrated sodium hydroxide will stay in the lower half of the container and the rate of carbon dioxide adsorption will be much lower than that of a homogenous solution. Seal the container after the sodium hydroxide transfer is complete. Immediately replace the cap on the 50% hydroxide bottle as well. Swirl to mix the contents of the tightly sealed container holding the 0.1 M sodium hydroxide. Keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi) at all times and store for no more than one week.

1 M sodium acetate/100 mM sodium hydroxide: To make 1 L of 100 mM sodium hydroxide containing 1.0 M sodium acetate, dispense approximately 800 mL of DI water into a 1 L volumetric flask. Vacuum degas for approximately 5 min. Add a stir bar and begin stirring. Weigh 82.0 g anhydrous, crystalline sodium acetate. Add the solid acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt has dissolved, remove the stir bar with a magnetic retriever. Add DI water to the flask to bring the volume to the 1 L mark. Vacuum filter the solution through a 0.2 µm nylon filter. This can take some time because the filter may clog with insoluble material from the sodium acetate. Using a plastic volumetric pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide solution from the middle of the bottle. Dispense the sodium hydroxide solution into the acetate solution ~1 in. (2.54 cm) under the surface of the acetate solution and then mix in the same manner as the 100 mM NaOH above. Keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi) at all times and store for no more than one week. See Thermo Scientific Technical Note 71 for detailed information on eluent preparation for HPAE-PAD.[7]

Standard solutions

Stock standard solutions: Prepare individual stock standard solutions (1000 mg/L) of erythritol, allulose, glucose, fructose, sucrose, and sucralose, by dissolving 0.1 g of compound in 100 mL DI water. Maintain the stock solution at -20 °C until needed.

Working standards: Using the respective stock standards, prepare mixed sugar standards by diluting the stock standard solution with DI water to a final volume of 100 mL. Nine calibration standards (concentrations listed in Table 1) were prepared.

Table 1. Working standards

Calibration standard	Sugar			
	Erythritol	Allulose	Sucrose	Sucralose
Std 1 (mg/L)	0.156	0.156	0.156	0.156
Std 2 (mg/L)	0.313	0.313	0.313	0.313
Std 3 (mg/L)	0.625	0.625	0.625	0.625
Std 4 (mg/L)	1.25	1.25	1.25	1.25
Std 5 (mg/L)	2.50	2.50	2.50	2.50
Std 6 (mg/L)	5.00	5.00	5.00	5.00
Std 7 (mg/L)	10.0	10.0	10.0	10.0
Std 8 (mg/L)	20.0	20.0	20.0	n. a
Std 9 (mg/L)	n. a	40.0	n. a	n. a

Sample preparation

The five commercial low sugar/sugar-free products listed in Table 2 were purchased.

Table 2. Samples used in this study

Sample	Sample	Labeled sweeteners
1	Protein bar I	Erythritol, allulose, sucralose,
2	Protein bar II	Erythritol, sucralose,
3	Keto cookie baking mix	Erythritol
4	Zero sugar flavored water	Erythritol
5	Zero sugar sports drink	Acesulfame K, sucrose, sucralose

Protein bar:
Step 1: Crush one full protein bar into a powder using a mixer/blender, mixing thoroughly to ensure homogeneity, and place the powder in a sample container and store it at -20 °C for further use. This is the stock sample.

Step 2: Weigh 1 g of the powder from Step 1 into a 100 mL volumetric flask. Add DI water up to 100 mL mark and mix thoroughly.

Step 3: Centrifuge a portion of the solution at 3000 RPM. Aspirate the supernatant and pass it through a 0.22 µm syringe filter. Dilute the sample as necessary and inject into the ICS system.

Cookie baking mix:
Step 1: Mix/blend the powder in a mixer/blender to ensure homogeneity and place the powder in a sample container as the stock sample. Store it at -20 °C for further use.

Step 2: Weigh 1 g of the powder in 100 mL volumetric flask. Add DI water up to the 100 mL mark and mix thoroughly.

Step 3: Centrifuge a portion of the solution at 3000 RPM. Aspirate the supernatant and pass it through a 0.22 µm syringe filter. Dilute the sample as necessary and inject into the ICS system.

Beverage samples:
Step 1: Weigh 1 g of the sample in 100 mL volumetric flask. Add DI water up to the 100 mL mark and mix.

Step 2: Pass a portion through a 0.22 µm syringe filter. Dilute the sample as necessary and inject into the ICS system.

System preparation

The Dionex ICS 5000+ HPIC system is configured for electrochemical detection, operating under high pressure conditions up to 5000 psi. To install this application, connect the Dionex AS-AP autosampler and Dionex ICS-5000+ system modules. Refer to Technical Note TN73348[6] for details on the preparation of the electrochemical cell and the installation of the Ag/AgCl and PdH REs.

Table 3. Chromatographic conditions

Parameter	Setting
System	Dionex ICS-5000+ HPIC system
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA20 Guard, 3 × 30 mm (P/N 060144) Dionex CarboPac PA20 Analytical, 3 × 150 mm (P/N 060142)
Column temp.	30 °C
Compartment temp.	20 °C
Eluent A	100 mM NaOH
Eluent B	1 M Sodium acetate/100 mM NaOH
Gradient	0–3 min: 0 mM NaOAc/100 mM NaOH 3–10 min: 0–300 mM NaOAc/100 mM NaOH 10–15 min: 300 mM NaOAc/100 mM NaOH 15–25 min: 0 mM NaOAc/100 mM NaOH
Flow rate	0.5 mL/min
Injection volume	10 µL
Inject mode	Push full
Loop overfill factor	5
Detection	Pulsed amperometry, Carbohydrate Certified Disposable Gold Working Electrode, 2 mil (50.8 µm) gasket
Reference electrode	PdH or Ag/AgCl
Waveform (For Ag/AgCl RE)	Time (s) / Potential (V) / Integration 0.00 / +0.1 0.20 / +0.1 / Begin 0.40 / +0.1 / End 0.41 / –2.0 0.42 / –2.0 0.43 / +0.6 0.44 / –0.1 0.50 / –0.1
Waveform (PdH RE @ 100 mM NaOH and @ 20 °C detection temp.)	Time (s) / Potential (V) / Integration 0.00 / +1.01 0.20 / +1.01 / Begin 0.40 / +1.01 / End 0.41 / –1.09 0.42 / –1.09 0.43 / +1.51 0.44 / +0.81 0.50 / +0.81
System backpressure	~2900 psi (100 psi = 0.689 MPa)
Background	20–30 nC (with both PdH and Ag/AgCl REs)
Noise	~35 pC/min peak-to-peak (with both PdHs and Ag/AgCl REs)
Run time	25 min

Results and discussion

Separation

The goal for this project was to establish conditions that would resolve the early eluting peaks glucose, fructose, allulose, and sucrose as well as elute sucralose a short time later, well resolved from the other sugars. Sucralose is retained longer on the Dionex CarboPac PA20 column, likely due to the three electronegative chlorine atoms in the molecule, increasing the dissociation of alcohol-groups due to a minus-I effect. Using 100 mM sodium hydroxide ensures sucralose ionization and good detection sensitivity. Acetate is a stronger eluent than hydroxide. Hence it was

used to control retention. We experimented with isocratic separations, using a fixed concentration of sodium hydroxide with variable sodium acetate concentrations. Low concentrations of sodium acetate in the eluent resulted in long sucralose retention times (>25 min using <20 mM sodium acetate), and poor peak response (<75 mM) due to peak broadening. Higher concentration (>75 mM sodium acetate) resulted in poor resolution of early eluting peaks: glucose-fructose and fructose-allulose pairs. Thus, we switched to an acetate gradient with fixed concentration of sodium hydroxide. After trying several gradient separations, one was selected (Table 3). The method starts with 100 mM sodium hydroxide, then applies an acetate gradient with the same concentration of sodium hydroxide and finishes with a short column wash and a return to starting conditions. Figure 1 displays a chromatogram of the six sugar standards analyzed using the conditions listed in Table 3. Figure 2 displays a comparison of the chromatograms of the six sugar standards analyed using either an Ag/AgCl or PdH RE. The background signal with both the REs is ~25 nC. The noise is ~35 pC /min peak-to-peak for both the REs. The peak area response is slightly higher (Table 4) with the PdH RE in comparison to using the Ag/AgCl RE, consistent with the data reported in TN73348.[6] Figure 3 displays the electrochemical response stability of six sugars over 50 consecutive injections using a PdH RE.

Column:	Dionex CarboPac PA20 + guard
Eluent A:	100 mM NaOH
Eluent B:	1 M NaOAc/100 mM NaOH
Gradient:	0–3 min: 100 mM NaOH 3–10 min: 0–300 mM NaOAc/100 mM NaOH 10–15 min: 300 mM NaOAc/100 mM NaOH 15–20 min: 100 mM NaOH
Column temp.:	30 °C
Flow rate:	0.5 mL/min
Inj. volume:	2.5 µL
Detection:	PAD (Au) Disposable Waveform A (TN21)
Peaks:	1. Erythritol 2. Glucose 3. Fructose 4. Allulose 5. Sucrose 6. Sucralose

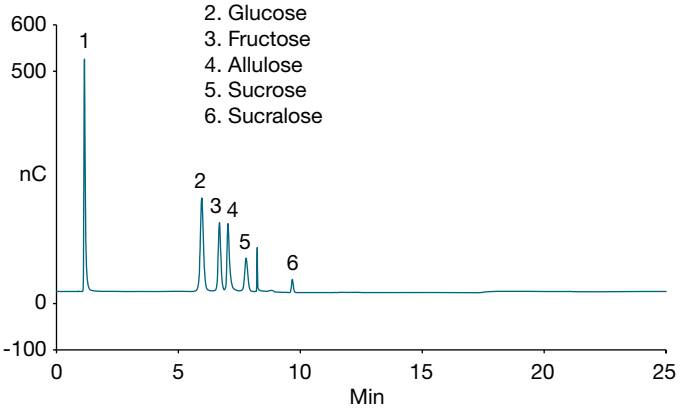

Figure 1. Chromatogram of the 10 mg/L six-sugar mixed standard

Table 4. Electrochemical response of six sugars run using PdH and Ag/AgCl REs

Peak	Analyte	PdH	Ag/AgCl	% Difference
		Peak area (nC·min)		
1	Erythritol	33.9511	31.8556	6.17
2	Glucose	30.7487	29.9224	2.69
3	Fructose	18.0802	16.9036	6.51
4	Allulose	17.8038	17.7248	0.44
5	Sucrose	11.0432	10.0364	9.12
6	Sucralose	2.4205	2.4157	0.20

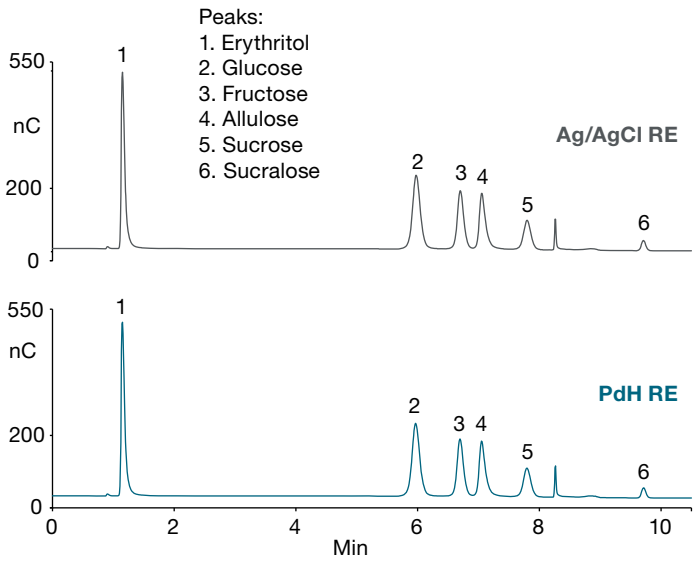

Figure 2. Chromatogram of the 10 mg/L six-sugar mixed standard using Ag/AgCl and PdH REs

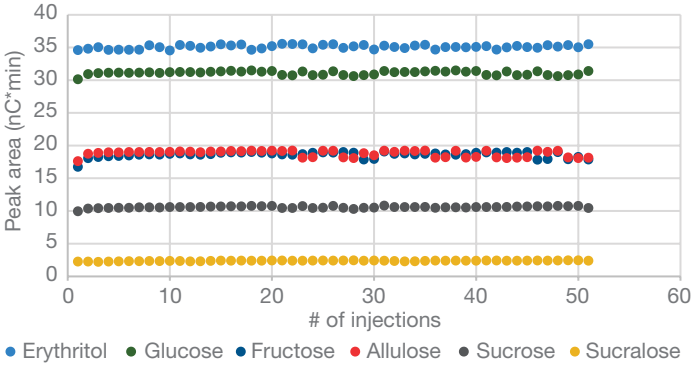

Figure 3. Electrochemical response stability of six sugars over 50 consecutive injections (using a PdH RE)

Sample analysis

For this study, we purchased the five commercial low sugar/sugar-free products listed in Table 2 and analyzed them using HPAE-PAD. Figure 4 displays the chromatograms of the three food samples, two protein bars, and one cookie baking mix. All three samples were diluted as appropriate. Protein bar I (S#1) was diluted 50× and found to contain erythritol, allulose, and sucrose. Protein bar II (S#2) and cookie baking mix (S#3) were diluted 200×. For both protein bar samples (S#1 and S#2), the label indicates the presence of sucralose, but we did not detect a sucralose peak in these samples. To confirm that it was not lost during sample preparation steps, sucralose standard was spiked in the sample, followed by sample preparation. See the section titled "Accuracy and precision" for more details on spiking experiments.

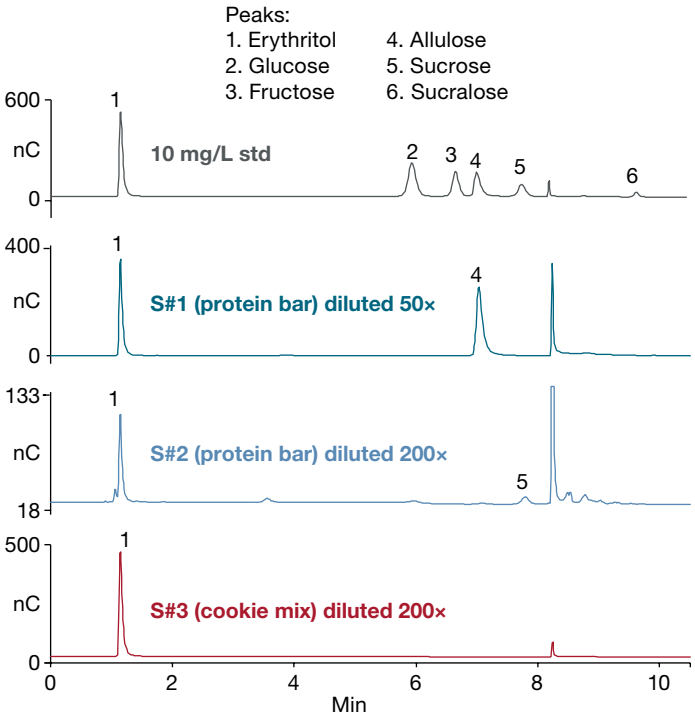

Figure 4. Chromatograms of samples S#1,S#2, S#3 and a 10 mg/L mixed sugar standard

Figure 5 displays the chromatograms of two beverage samples (S#4 and S#5). Consistent with the label, S#4 contains erythritol, and S#5 contains sucrose and sucralose. Table 5 lists the amounts of sugars determined in the five samples. Three samples were chosen to be reanalyzed using a PdH RE. Table 6 shows that the amounts of the analytes determined in the three sugar-free samples were comparable for both REs with a difference of <5%.

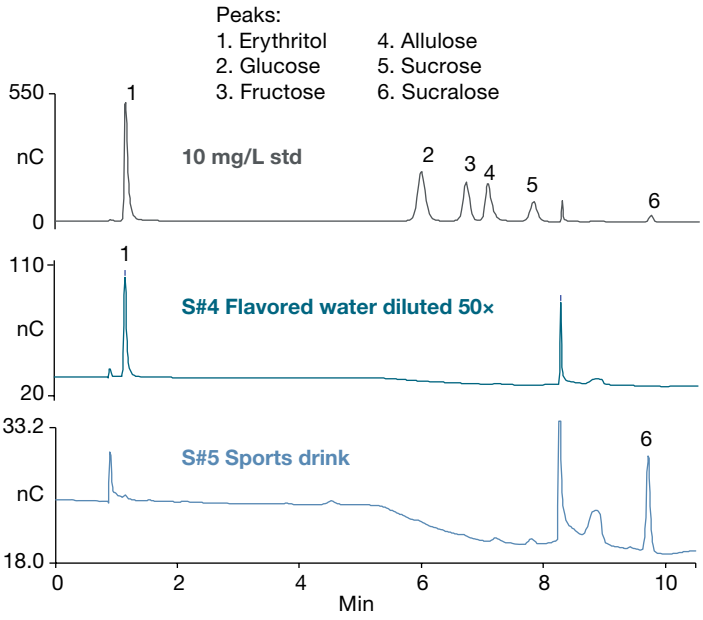

Figure 5. Chromatograms of samples S#4, S#5, and a 10 mg/L mixed sugar standard

Table 5. Sugar content (mg/g) in sugar-free products

Sample	Amount (mg/g)			
	Erythritol	Allulose	Sucrose	Sucralose
1. Protein bar I	63.5	158	6.54	not detected
2. Protein bar II	27.9	n.a	20.0	not detected
3. Keto cookie baking mix	314	n.a	22.8	n.a
4. Flavored water	4.70	n.a	n.a	n.a
5. Sports drink	n.a	n.a	n.a	0.069

Table 6. Sugar content (mg/g) in sugar-free products

	Sample #1			Sample #3			Sample #5		
	PdH	Ag/AgCl	% Difference	PdH	Ag/AgCl	% Difference	PdH	Ag/AgCl	% Difference
Erythritol	66.9	63.5	5.08	328	314	4.27	Not present		
Allulose	161	158	1.86	Not present			Not present		
Sucrose	6.83	6.54	4.25	22.2	22.8	-2.70	Not present		
Sucralose	Not detected			Not present			0.06	0.07	-4.78

Calibration

Calibration was performed for four sugar standards: erythritol, allulose, sucrose, and sucralose. The calibration curves for these four standards are shown in Figure 6. A linear curve fit was used for all sugars except erythritol, which was fit using a second-order polynomial curve. This choice was necessary as erythritol concentrations above 20 mg/L saturate the detector response. The coefficient of determination (r^2) is greater than 0.999 for all sugars. Table 7 lists the calibration results for all four sugars and using both REs.

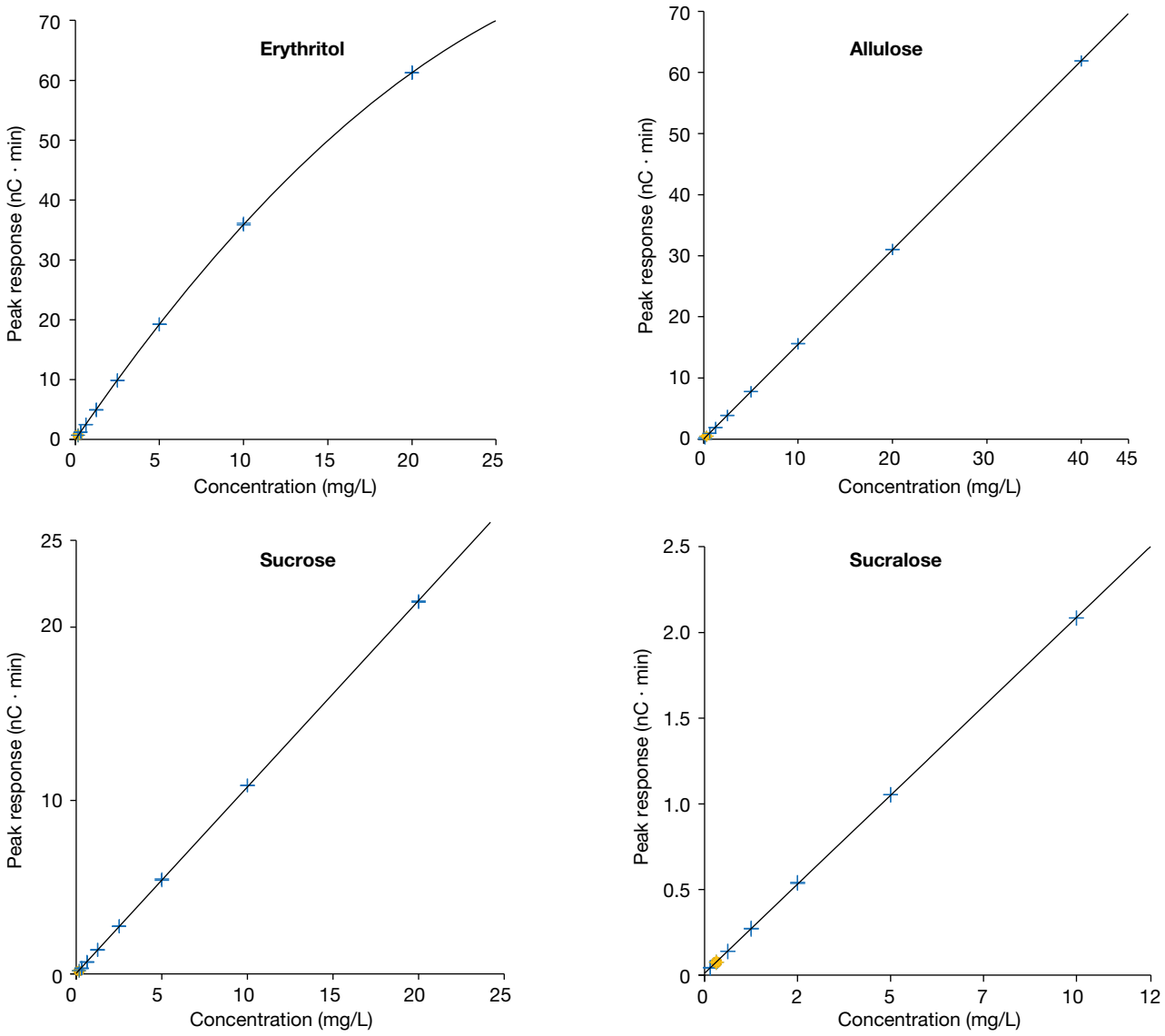

Figure 6. Calibration curves of four sugars

Table 7. Calibration data for four sugars

Sugar	Calibration range (mg/L)	Levels	Fit	Coefficient of determination (using PdH)	Coefficient of determination (using Ag/AgCl)
Erythritol	0.156-20.0	8	Quadratic	0.9998	0.9998
Allulose	0.156-40.0	9	Linear	0.9997	0.9994
Sucrose	0.156-20.0	8	Linear	0.9998	0.9999
Sucralose	0.156-10.0	7	Linear	0.9995	0.9993

Accuracy and precision

Method accuracy was evaluated by measuring recoveries of four sugar standards spiked into low sugar/sugar-free products. For spiking experiments, three samples (Samples #1, #3, and #5) were chosen and spiked with a four-sugar standard mix at two concentration levels. For the S#1 spiking experiment, Figure 7 shows a negative peak in the spiked sample at sucralose RT, and the intensity of the negative signal is proportional to the concentration of sucralose. When a 50× sample dilution was used, however, a sucralose peak was clearly observed (Figure 8) at both spike levels with an 84% recovery. Figures 9 and 10 show the chromatograms of unspiked and spiked S#3 and S#5, respectively. The recovery percentages were calculated using the formula below:

$$\text{Recovery \%} = \frac{C \text{ spiked sample} - C \text{ unspiked sample}}{C \text{ analyte added}} \times 100$$

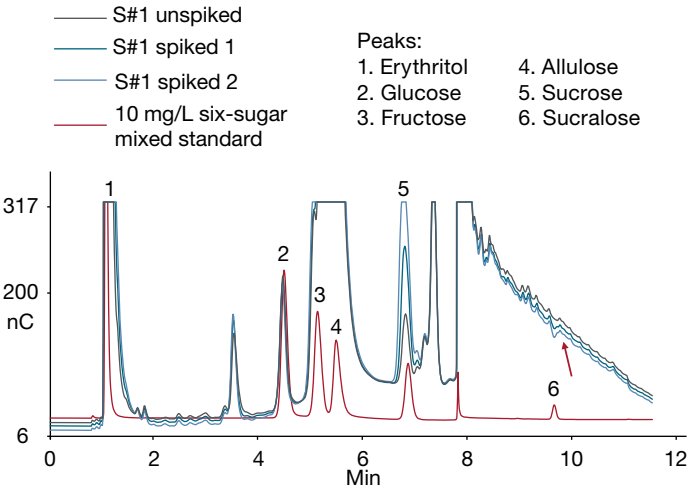

Figure 7. Chromatograms of S#1 and S#1 spiked at two levels

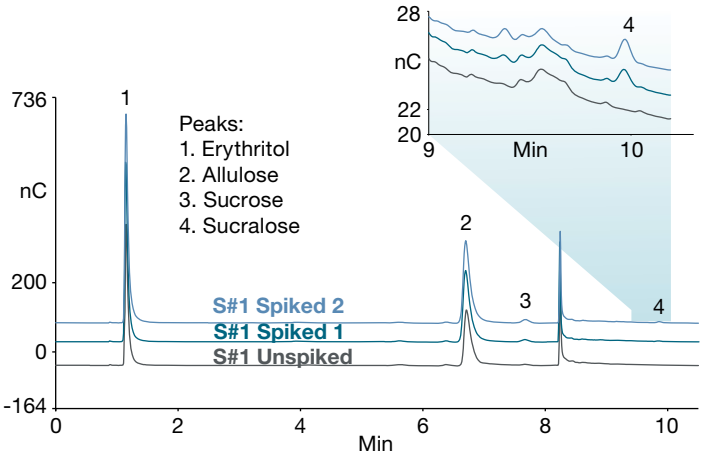

Figure 8. Chromatograms of S#1 (diluted 50×) spiked at two levels

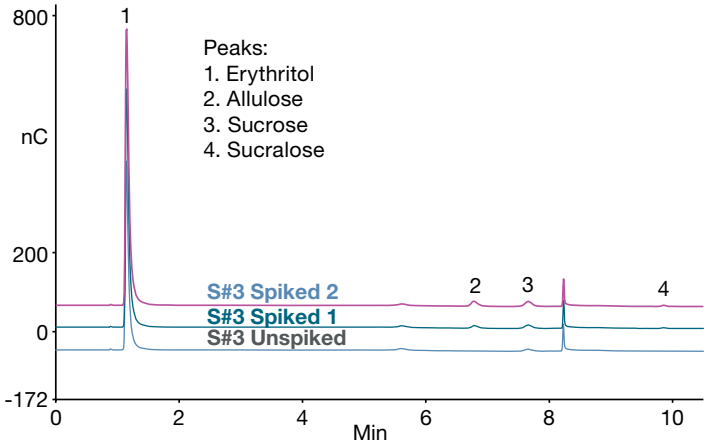

Figure 9. Chromatograms of S#3 (diluted 200×) and S#3 (diluted 200×) spiked at two levels

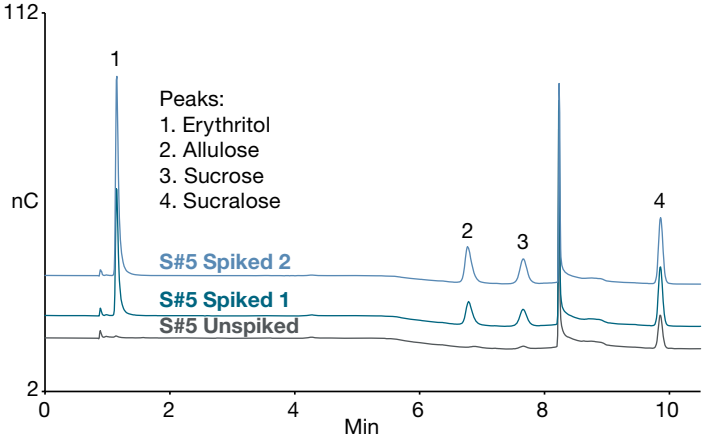

Figure 10. Chromatograms of S#5 and S#5 spiked at two levels

Table 8 lists the recovery results. For the three samples spiked, the recoveries were 83–116%.

The method precision was determined at three concentrations of the four-sugar standard with three replicates of each standard concentration to give nine total injections. Table 9 shows retention time and peak area precision for all three concentration levels tested with RSD values below 0.5% and 5%, respectively. We obtained similar values with the PdH RE using the three samples in Table 6 (Table 10).

Table 8. Spike recovery results

	Sample #1 (protein bar, diluted 50 times)						
	Found (mg/L)	Spiked 1 (mg/L)	Recovered (mg/L)	Recovery %	Spiked 2 (mg/L)	Recovered (mg/L)	Recovery %
Erythritol	6.50	3.00	8.98	82.7	6.00	11.5	83.4
Allulose	16.0	5.00	20.2	84.0	10.0	24.3	82.8
Sucrose	0.178	0.50	0.610	86.7	1.00	1.02	84.4
Sucralose	0.00	0.50	0.448	89.5	1.00	0.968	96.8
	Sample #3 (cookie baking mix, diluted 200 times)						
	Found (mg/L)	Spiked 1 (mg/L)	Recovered (mg/L)	Recovery %	Spiked 2 (mg/L)	Recovered (mg/L)	Recovery %
Erythritol	6.50	3.00	8.98	82.7	6.00	11.5	83.4
Allulose	16.0	5.00	20.2	84.0	10.0	24.3	82.8
Sucrose	0.178	0.50	0.610	86.7	1.00	1.02	84.4
Sucralose	0.00	0.50	0.448	89.5	1.00	0.968	96.8
	Sample #5 (sports drink)						
	Found (mg/L)	Spiked 1 (mg/L)	Recovered (mg/L)	Recovery %	Spiked 2 (mg/L)	Recovered (mg/L)	Recovery %
Erythritol	0.00	0.50	0.563	113	1.00	1.12	112
Allulose	0.00	0.50	0.515	103	1.00	1.08	108
Sucrose	0.00	0.50	0.582	116	1.00	1.12	112
Sucralose	3.32	3.00	6.01	89.5	6.00	8.50	86.3

Table 9. Precision (RSD, n=9) of peak area and retention time of four sugars

Standard concentration (mg/L)	Erythritol		Allulose		Sucrose		Sucralose	
	RT	Peak area	RT	Peak area	RT	Peak area	RT	Peak area
0.156	0.48	1.17	0.23	4.47	0.04	2.33	0.20	4.07
2.50	0.37	1.06	0.46	3.56	0.04	1.83	0.21	4.80
40.0	0.48	1.75	0.45	0.88	0.23	0.34	0.17	4.54

Table 10: Precision (RSD, n=9) of peak area and retention time of four sugars (samples)

Sample	Erythritol		Allulose		Sucrose		Sucralose	
	RT	Peak area	RT	Peak area	RT	Peak area	RT	Peak area
S#1 (50×)	0.42	1.23	0.37	2.46	0.26	3.50	Not detected	
S#3 (200×)	0.37	1.06	n.a.		0.04	1.83	n.a.	
S#5	n.a.		n.a.		n.a.		0.17	4.54

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Conclusion

An HPAE-PAD method was successfully developed and validated for the determination of four low- or non-caloric sweeteners in five commercial sugar-free or low-sugar products using a Dionex CarboPac PA20 column. This column allows the separation of the four sweeteners in sugar-free samples with minimum sample preparation and an overall cycle time of 25 min. The method showed satisfactory precision and accuracy, with a recovery range of 83–116% for the three samples tested.

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