TECHNICAL NOTE 73348

Carbohydrate determinations by HPAE-PAD using a PdH reference electrode

Authors: Manali Aggrawal and Jeffrey Rohrer
Thermo Fisher Scientific, Sunnyvale, CA

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Goal

To demonstrate the performance of a palladium hydrogen (PdH) reference electrode in comparison to a silver/silver chloride reference electrode (Ag/AgCl) for two carbohydrate applications: honey sugars and glycoprotein monosaccharides

Introduction

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for carbohydrate determinations. It offers the advantages of fast analysis, high resolution, ease of automation, and direct injection of the sample without analyte derivatization (i.e., direct detection). HPAE-PAD has been widely used for the determination of carbohydrates (from monosaccharides to oligosaccharides) in the pharmaceutical, biotechnology, agricultural, and food industries.

Because of the weakly acidic nature of carbohydrates (pKa 12–14), they can be separated at high pH using a strong anion-exchange stationary phase. After separation, they are detected by electrochemical detection. Each complete electrochemical detector (ED) assembly consists of an amperometric detection cell and the detector electronics required to collect data. The ED cell is a miniature flow-through amperometric detection cell that includes three different electrodes: a titanium cell body (the counter electrode), a working electrode, and a reference electrode (RE). Amperometric detection is typically performed at a solid anode (working electrode), such as gold, platinum, silver, or glassy carbon, either under constant applied potential or with a series of potentials where the analyte is detected at one or more potentials. Reference electrodes are used to assure application of the proper working electrode potential. A reference electrode should have constant electrochemical potential. Reference potential shifting can lead to unusually high background response from the working electrode, reduced signal response, or a combination of both effects.

The most common REs are saturated calomel (SCE), silver/silver chloride (Ag/AgCl), and palladium-hydrogen (PdH). Both SCE and Ag/AgCl are aqueous electrodes based around a saturated aqueous solution, while PdH is a solid-state RE. Solid-state REs are known for providing a more stable reference potential and offer some other advantages compared to conventional REs, for example, longer lifetime, less maintenance, and ease-of-use. We recently introduced a PdH RE for HPAE-PAD carbohydrate determinations. In this work, we demonstrate two popular carbohydrate applications (honey sugars and glycoprotein monosaccharides) using a PdH RE and compare the results to using an Ag/AgCl RE. Our preliminary data indicate that the PdH RE provides comparable results to the standard Ag/AgCl RE for these two applications that use hydroxide eluents.

Experimental

Equipment

- High-pressure 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 074925) with cooling tray 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)
- Sterile assembled microcentrifuge tubes with screw cap, 1.5 mL (Sarstedt® P/N 72.692.005)

*The technique in this technical note can be adapted for any Thermo Scientific™ Dionex™ IC system that supports electrochemical detection.

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Trehalose, Fluka (P/N 90208)
- D-Glucose, Sigma-Aldrich (P/N G8270)
- D-Fructose, Sigma-Aldrich (P/N F2543)
- Sucrose, Sigma-Aldrich (P/N S-9378)
- Isomaltose 98%, Sigma-Aldrich (P/N I7253-100MG)
- Melezitose hydrate, Sigma-Aldrich (P/N M5375)
- Kozibiose, Sigma-Aldrich (P/N K476-1MG)
- Raffinose pentahydrate, Sigma-Aldrich (P/N R0250-25G)
- Gentiobiose, Sigma-Aldrich (P/N G3000)
- 1-Kestose, Sigma-Aldrich (P/N 72555)
- Turanose, Sigma-Aldrich (P/N T2754)
- Palatinose, Sigma-Aldrich (P/N P2007)
- Erlose, Sigma-Aldrich (P/N E1895-50MG)
- Maltose monohydrate, Sigma-Aldrich (P/N M5885)
- Bovine serum fetuin (Sigma P/N F2379)
- Thermo Scientific™ Pierce™ trifluoroacetic acid (TFA), sequencing grade (TFA purity: ≥99.5%) (P/N 28904)
- Thermo Scientific™ Pierce™ hydrochloric acid (c(HCl) = 6 M) (P/N 24308)
- Thermo Scientific™ Dionex™ MonoStandard™ Mix of Six carbohydrate standard (P/N 043162)

Preparation of solutions and reagents

Eluent solutions

Generate the potassium hydroxide (KOH) eluent online by pumping high-quality degassed DI water through the Dionex EGC 500 KOH cartridge. The Chromeleon CDS tracks the amount of KOH used and calculates the remaining lifetime of the cartridge.

The authors strongly recommend eluents prepared by an eluent generator for concentrations of hydroxide <5 mM. However, eluents can be prepared manually, if needed. If eluents must be prepared manually, use a 50% (w/w) NaOH solution in place of KOH and prepare according to the general instructions for hydroxide eluents in Thermo Scientific Technical Note 71.[1]

Standard solutions

Stock standard solutions: Prepare individual stock standard solutions (100 mg/L / 1000 mg/L) of trehalose, glucose, fructose, sucrose, isomaltose, melezitose, raffinose, gentiobiose, turanose, 1-kestose, maltose and erlose, by dissolving 0.01 g / 0.1 g of individual sugar in 100 mL DI water. Maintain the stock solution at -20 °C until needed.

Working standards: Using the individual stock standards, prepare mixed sugar standards by diluting individual stock standard solutions into a 100 mL volumetric flask with DI water. Five calibration standards (concentrations listed in the table below) are prepared similarly by diluting the individual stock standards in DI water.

Methods

TFA hydrolysis: Prepare TFA hydrolysates of bovine fetuin by combining 20 µL of 3 mg/mL protein solution, 150 µL DI water, and 30 µL TFA in a 1.5 mL microcentrifuge tube. Heat the solutions for 4 h at 100 °C and then dry at room temperature for ~3 h in a Thermo Scientific™ Savant™ SpeedVac™ concentrator equipped with an acid trap. Reconstitute each vial with 300 µL of DI water. Vortex for 30 s and centrifuge for 5 min. Inject 2.5 µL of the supernatant (0.5 µg protein per injection) into the ion chromatography system.

HCl hydrolysis: Combine 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin solution in a 1.5 mL microcentrifuge tube. Heat the solutions for 4 h at 100 °C and then dry at room temperature for ~3 h in a Thermo Scientific™ Savant™ SpeedVac™ concentrator equipped with an acid trap. Reconstitute each vial with 300 µL of DI water. Vortex for 30 s and centrifuge for 5 min. Inject 2.5 µL of the supernatant (0.5 µg protein per injection) into the ion chromatography 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. When using the Dionex ICS-5000+ EG Eluent Generator module for electrochemical applications, install the vacuum degas conversion kit (P/N 063353). This degasser will remove gases generated

Table 1. Working standards

Sugar	Stock solution (mg/L)	Standard 1 (mg/L)	Standard 2 (mg/L)	Standard 3 (mg/L)	Standard 4 (mg/L)	Standard 5 (mg/L)
Trehalose	100	0.4	1.00	2.00	5.00	10.0
Glucose	1000	10	20	40	80	160
Fructose	1000	18.75	37.5	75	150	300
Sucrose	100	0.40	2.00	4.00	8.00	20.0
Isomaltose	100	1.00	2.50	5.00	10.0	20.0
Melezitose	100	0.50	1.00	2.00	4.00	10.0
Raffinose	100	0.40	0.80	1.60	3.20	6.40
Gentiobiose	100	0.20	0.40	0.80	2.00	4.00
1-Kestose	100	0.50	1.00	2.00	4.00	10.0
Turanose	1000	3.75	7.50	15.0	30.0	60.0
Maltose	1000	2.50	5.00	10.0	20.0	40.0
Erlose	100	1.00	2.50	5.00	10.0	20.0

by the EG and help maintain a stable baseline. This kit is not necessary when preparing eluents manually. Always keep the eluent water blanketed under 34–55 kPa (5–8 psi) of nitrogen to reduce carbonate contamination and opportunistic microorganisms.

Prepare the electrochemical cell by rinsing the cell body, the well of the RE, and the inlet tube thoroughly with DI water and dry with an absorbent tissue. Caution: Do not touch the working electrode gold surface with any paper products as this can contaminate the working electrode. Assemble the cell following the Dionex ICS 5000+ operator's manual[2] and Dionex ED User's Compendium for Electrochemical Detection[3] by first installing the working electrode gasket flat against the cell body. Avoid any wrinkles in the gasket, as this will cause a poor fit and subsequent leaks and poor detection. Install the working electrode with the metal face down over the gasket. Install the yoke block by squeezing the tabs and sliding it on the cell body. Align the yoke block parallel to the cell body and rotate the yoke block knob clockwise until you hear three "clicks". Install the cell into the ED module and connect the yellow cable to the yellow port.

Ag/AgCl reference electrode installation

Before installing the Ag/AgCl RE, calibrate it by first installing the RE blue cable into the black port. Then, immerse the RE in pH-7 buffer to at least mid-level of the electrode. Select the "pH Calibration" button on the ED Panel and follow the instructions to calibrate the electrode including using pH-10 buffer. After calibration is complete, rinse the buffer solution from the electrode with DI water, and gently, but firmly, screw the RE clockwise into the RE port of the electrochemical cell until the RE is finger tight. For best results, replace the RE after six months of use. While running the ED cell, air bubbles may be trapped in the cell, which can cause spikes in the baseline. Make sure there is adequate tubing connected to the cell outlet to produce enough backpressure to prevent air bubbles from becoming trapped in the cell. The backpressure limit for the ED cell is 690 kPa (100 psi). Do not exceed this limit. Six feet of black (0.01" i.d.) PEEK tubing at the cell outlet should generate 30–40 psi backpressure, which can prevent bubble formation.

PdH reference electrode installation

As shown in Figure 1, the first step to installing the PdH RE is, while wearing lab gloves, to add an O-ring to the electrode, then align the two PdH electrode knobs to register with the two alignment grooves of the cell body (Step 2), and insert the PdH electrode into the RE well (Step 3). Rotate the PdH reference electrode clockwise into the RE port of the electrochemical cell until the RE is finger tight (Step 4). If necessary, tighten the bolt an extra ~1/8 turn with a wrench (Step 5). Warning: Avoid overtightening, which could damage the electrode.

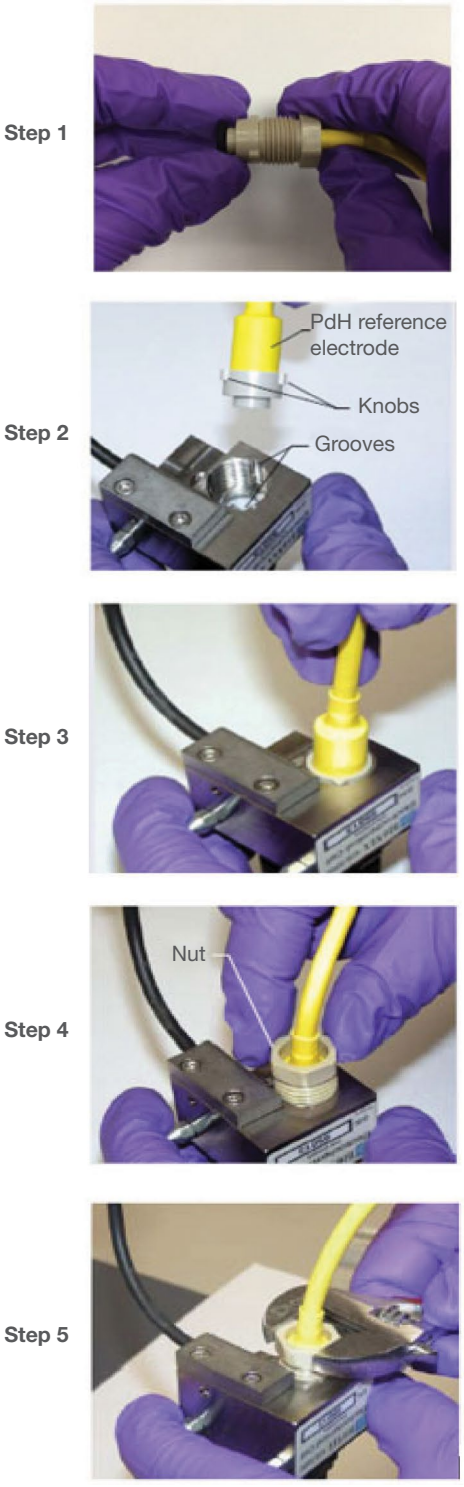


Figure 1. Stepwise installation of a PdH reference electrode

Initialization procedure of a new PdH RE

Set the pump proportioning at “100” A” (A: water), the flow rate to the recommended value for the column, (i.e., 0.25 mL/min for the 2 × 100 mm Thermo Scientific™ Dionex™ CarboPac™ PA20-4µm column) and turn on the pump flow. On the Eluent Generator panel enter “100 mM” in the Target Concentration field. This applies the current to the EGC. Turn on the CR-TC power control. Verify that eluent is exiting the cell. On the ED panel, set the ED Reference Electrode type to “PdH”. This turns on the PdH power control. (Attention: Do not turn on the ED cell. Otherwise, it can damage the working electrode due to the use of an incorrect RE potential from the unconditioned PdH RE.) Condition the electrode for 2 h at these settings. After conditioning, select the preprogrammed waveform from the dropdown menu (Figure 2) and turn on the ED cell.

Creating an instrument method for using a PdH reference electrode

The potential of the PdH RE is pH-dependent. When the PdH RE is selected, you will be asked to input the eluent type (acid or base), the concentration of the eluent, and the detection temperature (Figure 3). From those inputs, Chromeleon CDS calculates the offset potential of the PdH RE versus the Ag/AgCl RE. After the selection of a pre-programmed waveform in Chromeleon CDS, the software creates a waveform for use with the PdH RE by adjusting the offset potential (Figure 4). No pH readout is available.

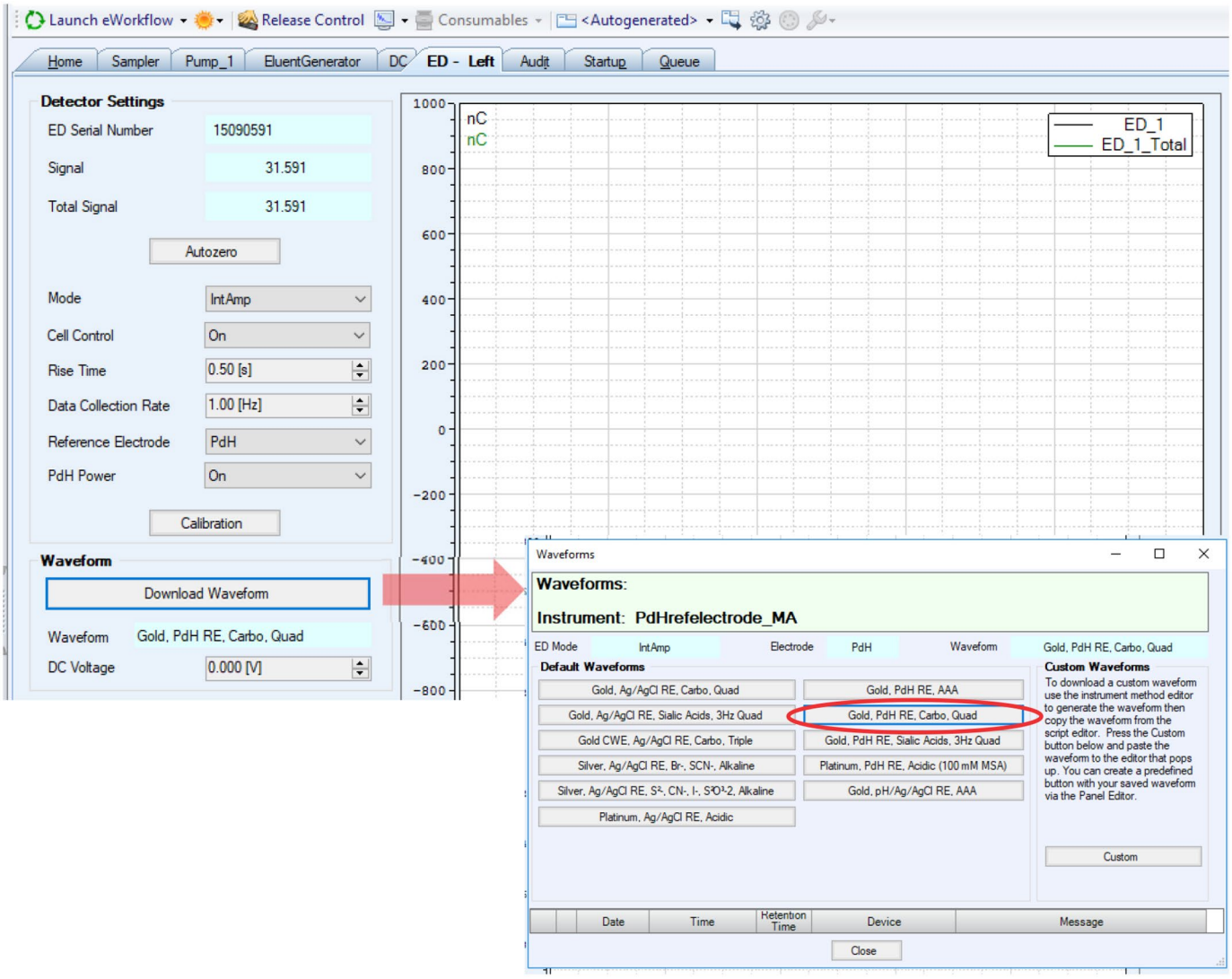


Figure 2. Picking a waveform from the ED panel

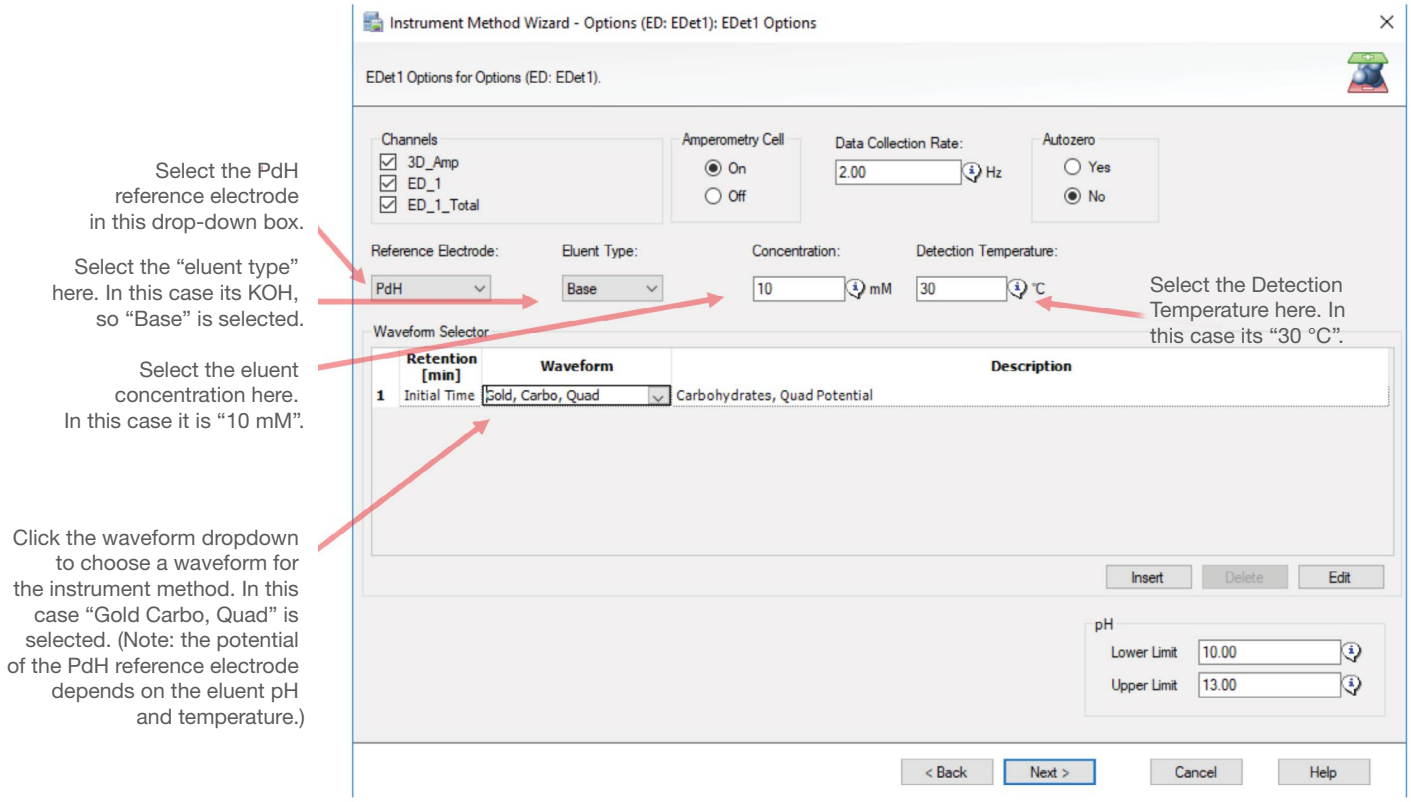
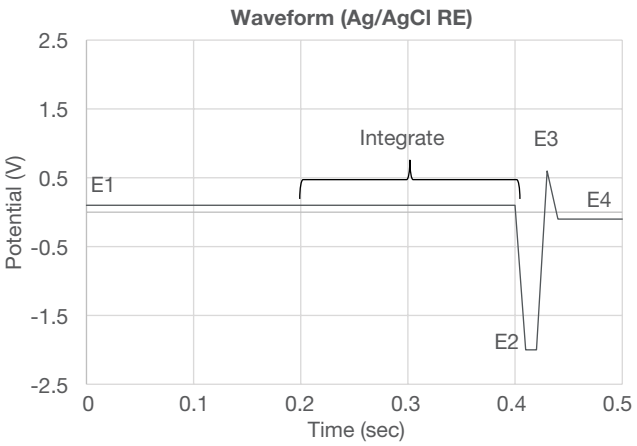
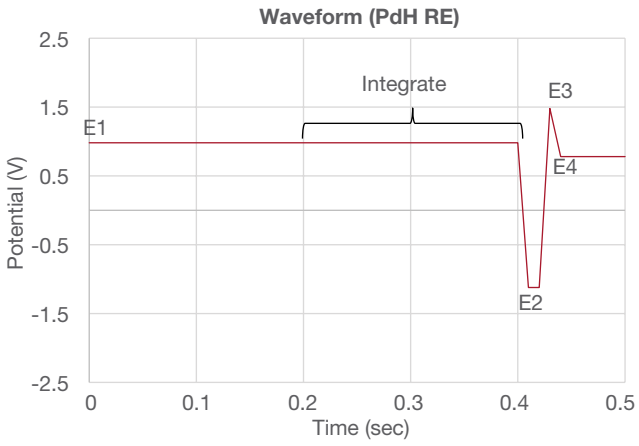


Figure 3. Creating an instrument method for using a PdH reference electrode



Time (s)	Voltage (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



Eluent concentration:	12 mM	29 mM	12 mM	29 mM	
Detection temperature:	20 °C	20 °C	30 °C	30 °C	
		Waveform Voltage (V)			
Time (s)					Integration
0	0.96	0.98	0.98	1.01	Off
0.2	0.96	0.98	0.98	1.01	On
0.4	0.96	0.98	0.98	1.01	Off
0.41	-1.14	-1.12	-1.12	-1.09	Off
0.42	-1.14	-1.12	-1.12	-1.09	Off
0.43	1.46	1.48	1.48	1.51	Off
0.44	0.76	0.78	0.78	0.81	Off
0.5	0.76	0.78	0.78	0.81	Off

Figure 4. Carbohydrate waveform(s) for Ag/AgCl and PdH reference electrodes

Short-term storage of a PdH reference electrode

If keeping the PdH power "ON", reduce the flow rate to 0.1 mL/min. (Attention: The flow needs to be maintained. Otherwise, the PdH RE may be damaged.) If turning the PdH power "OFF", stop the flow or reduce it to 0.1 mL/min for future quick start-up and prevention of possible contamination in the liquid line.

Long term storage of a PdH reference electrode

If the PdH RE is not going to be used within a known short time (e.g., one or two days), then it should be removed from the ED cell. The removal procedure is the reverse of the installation procedure. After the PdH RE is removed, rinse the eluent contact area of the PdH RE with DI water and blow it dry with a flow of nitrogen if possible. Finally, cap and store it safely.

Results and discussion

Discussion of all of the potential HPAE-PAD carbohydrate applications is beyond the scope of this document; however, two typical carbohydrate applications will be discussed to illustrate the performance of the PdH RE.

Application I: Honey sugars

As described in Application Note 1158 (AN1158)[4], a Dionex CarboPac PA210-Fast-4µm column (150 × 4 mm) in series with a Dionex CarboPac PA210 guard column (50 × 4 mm) was used to separate honey sugars. This column set provides fast, high-resolution separations for most mono- through tetrasaccharides in a variety of food and beverage samples.

Figure 5 displays chromatograms of the CarboPac PA210-Fast-4µm column QAR standard run using the Ag/AgCl and PdH reference electrodes. The background signal with both reference electrodes is ~35 nC. The noise is ~35 pC/min peak-to-peak for both reference electrodes. The peak area response is slightly higher (Table 3) with the PdH RE in comparison to using the Ag/AgCl RE.

Table 2. Conditions for the honey sugars application

Parameter	Value
System	ICS-5000+ HPIC System
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm Guard, 2 × 30 mm (P/N 088955) Dionex CarboPac PA210-Fast-4µm Analytical, 2 × 150 mm (P/N 088954)
Column temp.	30 °C
Compartment temp.	20 °C
Eluent source	EGC 500 KOH
Eluent	0–25 min: 29 mM KOH 25–30 min: 100 mM KOH 30–45 min: 29 mM KOH
Flow rate	0.2 mL/min
Injection volume	2.5 µL
Inject mode	Push full
Loop overfill factor	5
Detection	Pulsed amperometry, Carbohydrate Certified Disposable Gold Working Electrode
Reference electrode	PdH or Ag/AgCl
Waveform (For Ag/AgCl):	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 (For PdH)	Time (s) / Potential (V) / Integration 0.00 / +0.98 0.20 / +0.98 / Begin 0.40 / +0.98 / End 0.41 / –1.12 0.42 / –1.12 0.43 / +1.48 0.44 / +0.78 0.50 / +0.78
System backpressure	~3700 psi
Background	30–35 nC (same for both reference electrodes)
Noise	~35 pC /min peak-to-peak (for both reference electrodes)
Run time	45 min

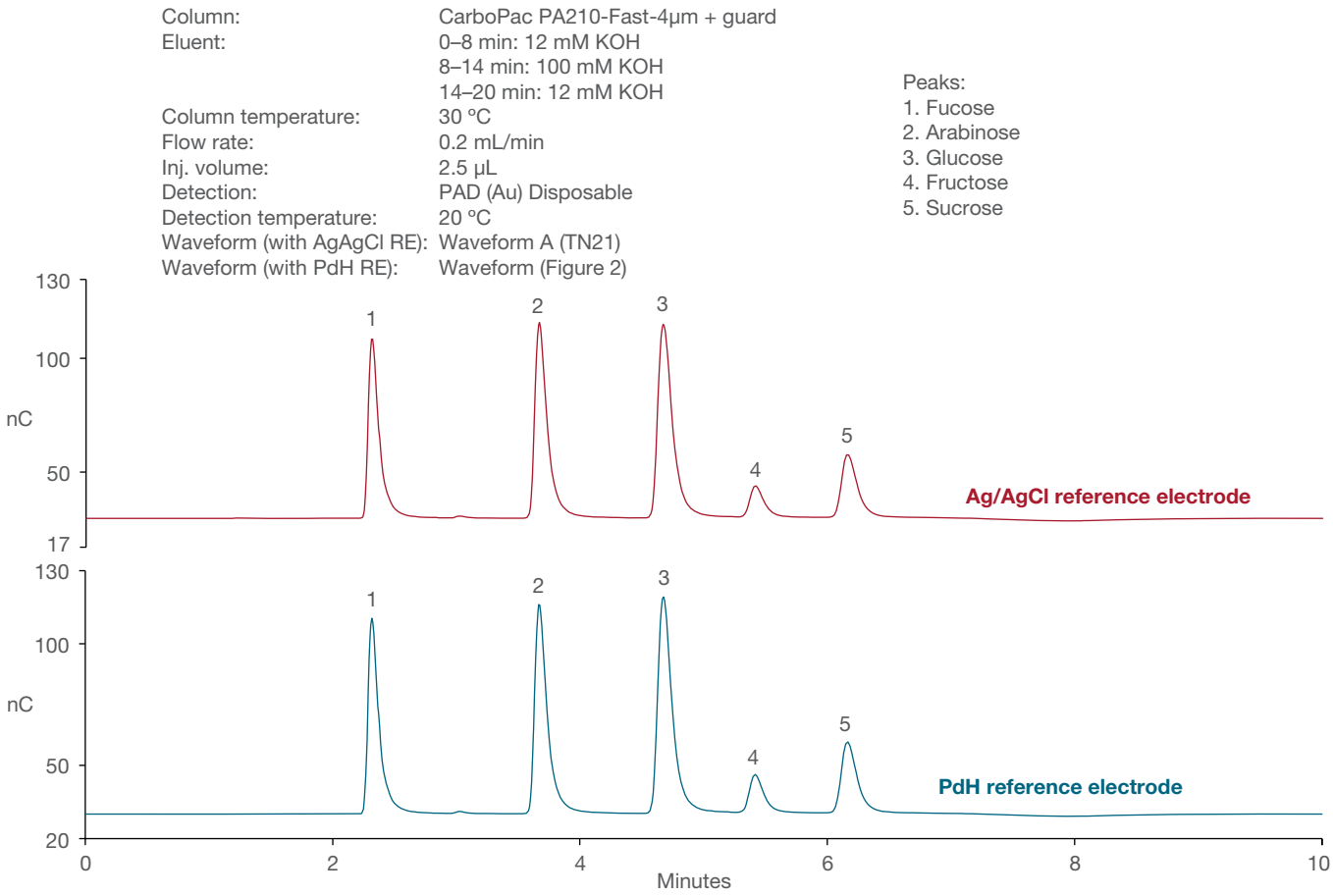


Figure 5. Chromatograms of the column's QAR standard using PdH and Ag/AgCl reference electrodes

Table 3. Electrochemical response of five sugars run using PdH and Ag/AgCl reference electrodes

Peak no.	Peak name	Peak area (nC*min)		% Diff
		PdH	Ag/AgCl	
1	Fucose	8.683	8.539	1.65
2	Arabinose	10.85	10.63	2.06
3	Glucose	12.88	12.79	0.66
4	Fructose	2.109	2.105	0.22
5	Sucrose	4.693	4.625	1.43

Then we ran the 14 honey sugar mixed standard using both RE (Figure 6). The peak area response with the PdH RE is higher for all 14 sugars ranging between 1.25% and 4.81% (Table 4). Compared to AN1158,[4] all the sugars exhibited a 5–10% earlier shift in retention time. This small change could be due to the column packing difference between column lots. All the sugars are resolved as in AN1158 except the kestose/turanose pair. Here this pair is unresolved but they are separated from palatinose, which partially coelutes with turanose in AN1158. To achieve a separation where kestose is resolved from the palatinose/turanose pair while maintaining the resolution of the other sugars, we reduced the eluent concentration to 29 mM KOH and used these conditions for the remainder of the work (Figure 7).

Table 4. Electrochemical response of 13 honey sugars run using PdH and Ag/AgCl reference electrodes

Peak name	Peak area (nC*min)		% Diff
	PdH	Ag/AgCl	
Trehalose	5.77	5.66	1.85
Glucose	30.55	29.08	4.81
Fructose	16.17	15.46	4.39
Sucrose	5.74	5.67	1.29
Isomaltose	3.72	3.58	3.91
Malezitose	4.44	4.26	4.09
Kozibiose	2.78	2.73	1.82
Raffinose	1.88	1.83	2.75
Gentiobiose	14.62	14.29	2.23
Kestose	3.30	3.16	4.44
Turanose	4.89	4.83	1.25
Maltose	9.13	8.75	4.21
Erlose	3.42	3.35	2.12

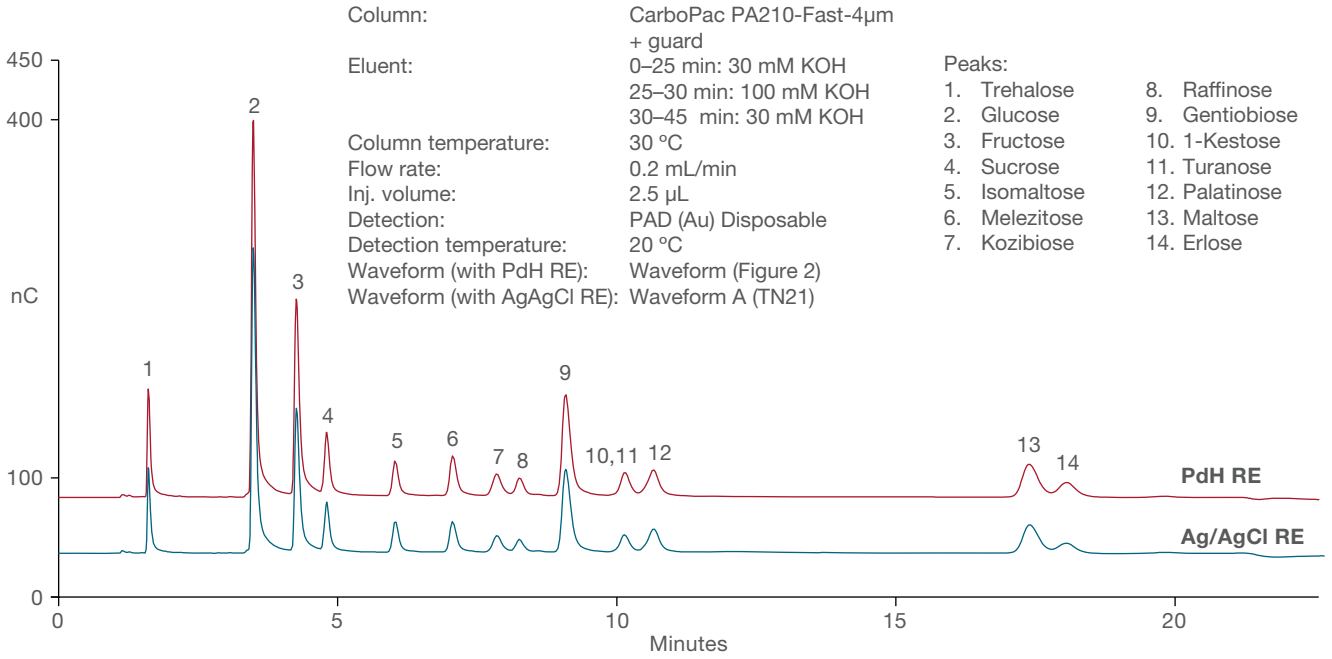


Figure 6. Comparison of the 14 honey sugar mixed standard run using PdH and Ag/AgCl reference electrodes

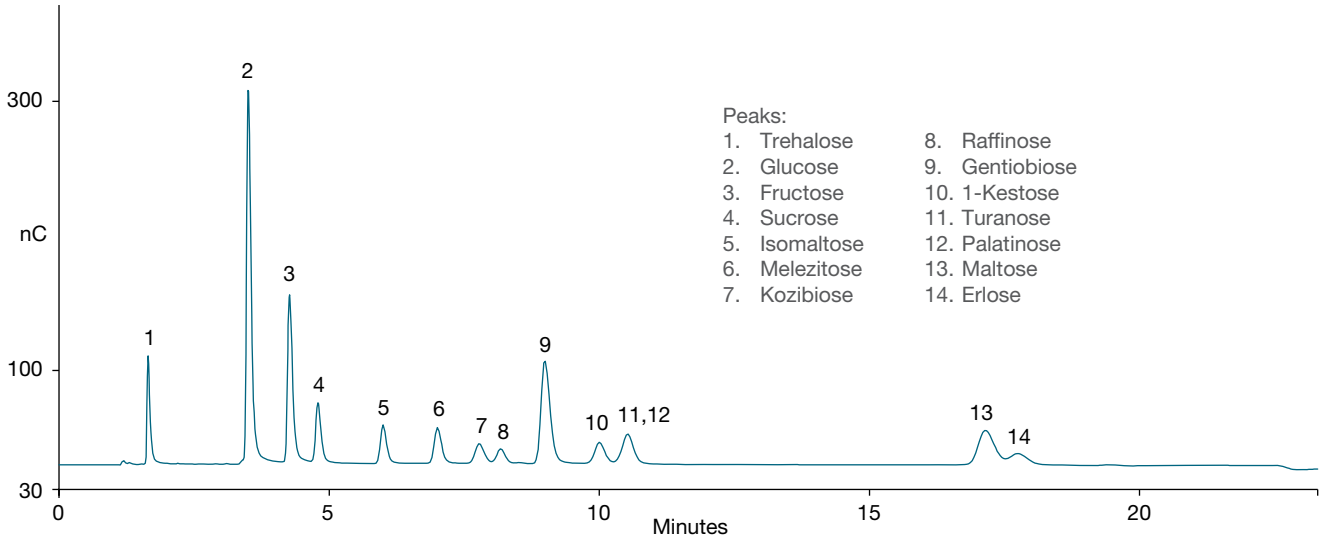
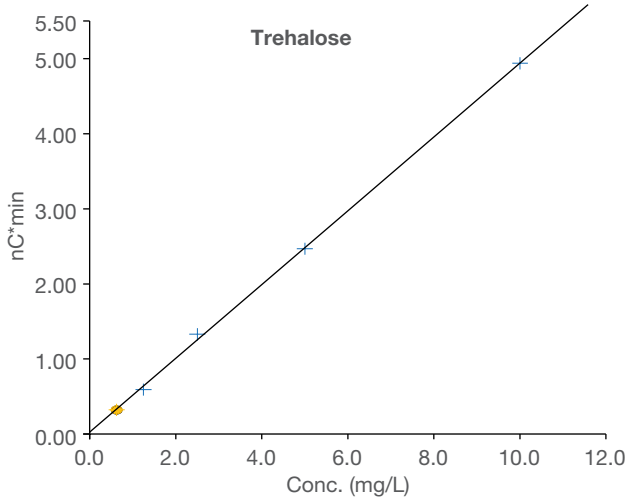


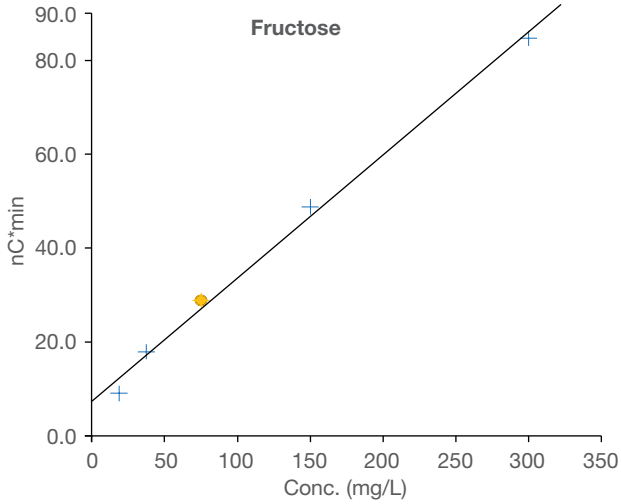
Figure 7. Chromatograms of a 14 honey sugar mixed standard run at 29 mM KOH using a PdH reference electrode

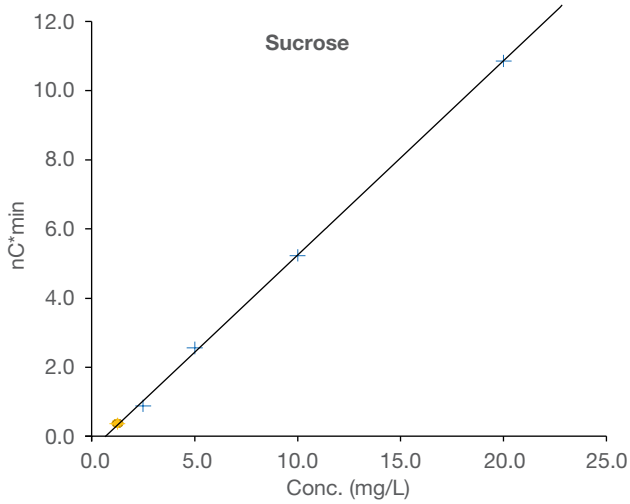
Linearity and precision

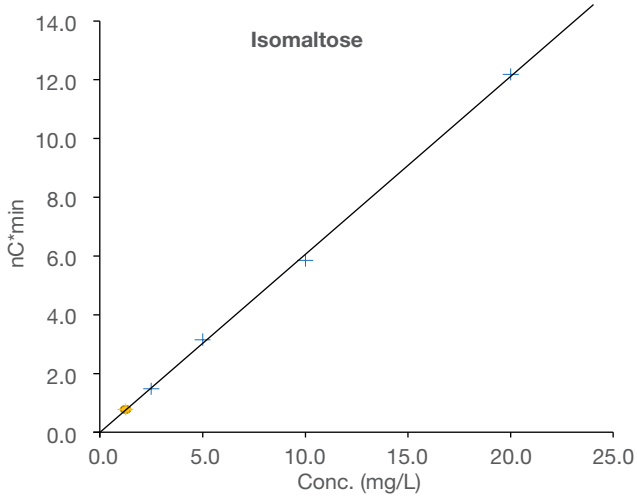
Quantification was performed with 12 sugar standards: two monosaccharides (glucose and fructose), six disaccharides (trehalose, isomaltose, sucrose, gentiobiose, turanose, and maltose), and four trisaccharides (melezitose, raffinose, 1-kestose, and erlose). The calibration curves for these 12 sugars are shown in Figure 8. The coefficient of determination (r^2) is greater than 0.999 for all sugars except glucose and fructose. This is due to the larger calibration range and thus high concentrations of glucose and fructose, which saturate the detector response above 100–150 mg/L. A linear curve fit was used for all sugars except glucose and fructose, which were fit using a second-order polynomial curve fit. The calibration curves for all the sugars fit like those reported in AN1158 that used a Ag/AgCl reference electrode (Table 5).

The method precision was determined by preparing and injecting a honey sugar standard mix at a single concentration with three replicates on three days to give nine total injections. Table 6 lists peak area precision for all 13 sugars using PdH and Ag/AgCl reference electrodes. For both reference electrodes, the peak area RSD values were less than 2%.









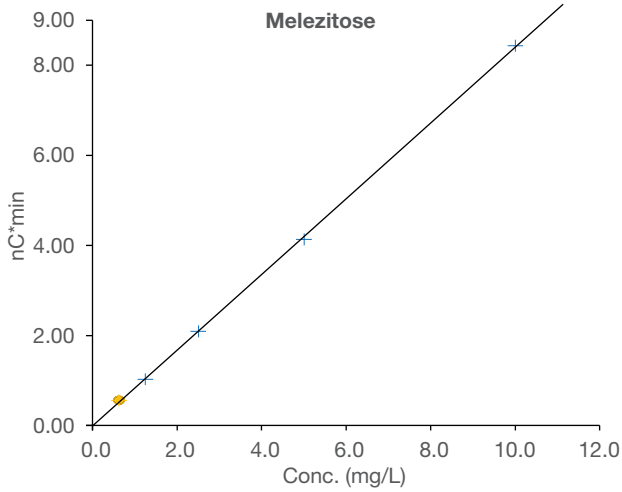
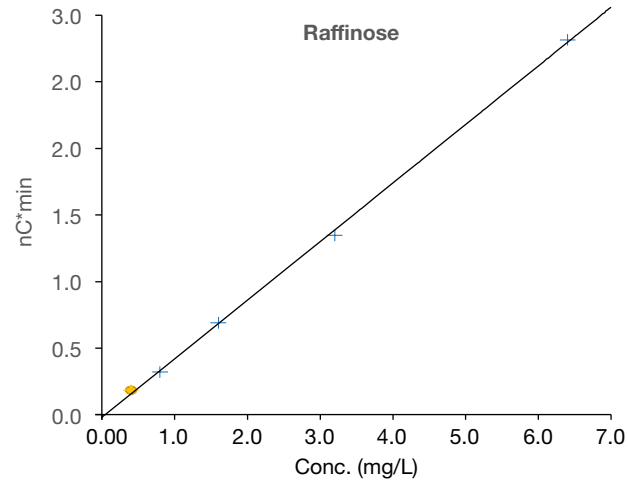
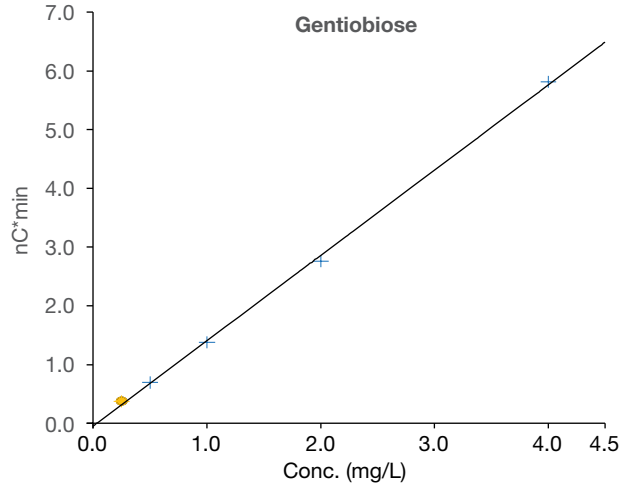
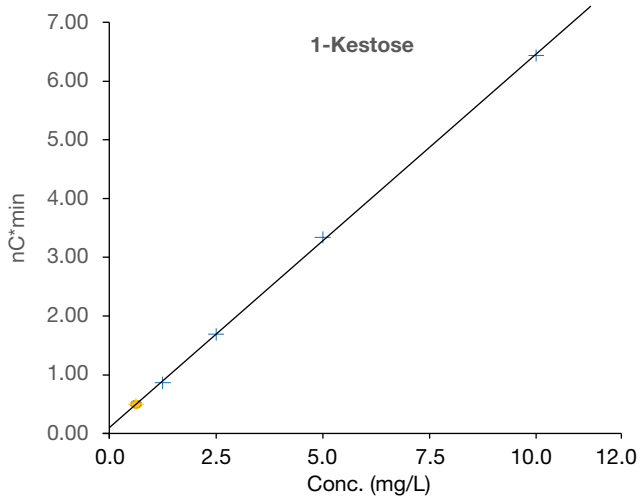
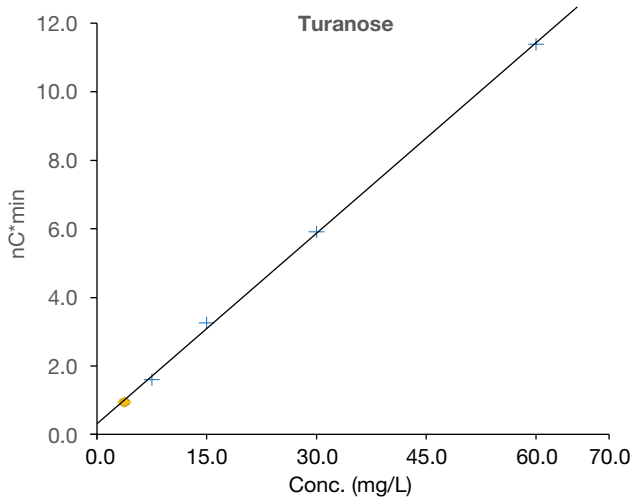


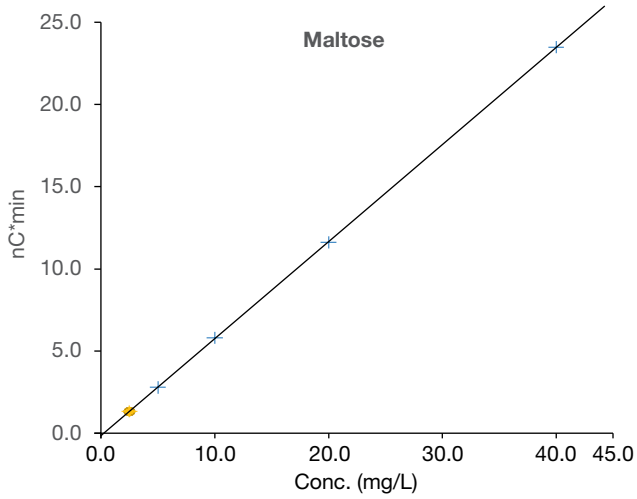
Figure 8 (part 1). Calibration curves of honey sugars











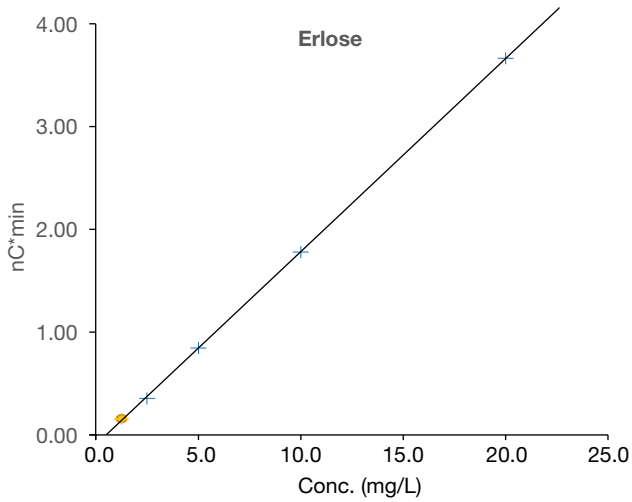


Figure 8 (part 2). Calibration curves of honey sugars

Table 5. Calibration data for 12 honey sugars (n=3)

Sugar	Calibration range (mg/L)	Levels	Coefficient of determination (using PdH)	Coefficient of determination (AN1158, using Ag/AgCl)
Trehalose	0.4–10	5	0.9999	0.9991
Glucose	10–160	5	0.9936	0.9961
Fructose	20–300	5	0.9955	0.9984
Sucrose	0.4–20	5	0.9995	0.9992
Isomaltose	1–20	5	0.9999	0.9999
Melezitose	0.5–10	5	0.9997	0.9997
Raffinose	0.4–6.4	5	0.9996	0.9997
Gentiobiose	0.2–4	5	0.9997	0.9997
Kestose	0.5–10	5	0.9995	0.9997
Turanose	3.75–60	5	09998	0.9997
Maltose	2.5–40	5	0.9994	0.9992
Erlose	1–20	5	0.9991	0.9998

Table 6. Precision data for 13 honey sugars (n=9)

Sugar	Peak area RSD	
	PdH	Ag/AgCl
Trehalose	1.90	1.37
Glucose	1.96	1.40
Fructose	2.02	1.62
Sucrose	1.32	1.06
Isomaltose	1.00	1.93
Melezitose	1.47	1.44
Kozibiose	1.72	1.55
Raffinose	1.97	0.48
Gentiobiose	1.03	1.38
Kestose	1.76	1.05
Turanose	1.39	1.12
Maltose	1.07	1.20
Erlose	1.81	1.28

Sample analysis

Previously in AN1158, twelve commercial honey samples were tested to evaluate the HPAE-PAD method. Here we chose three honey samples to evaluate the performance of a PdH RE for this application. Figure 9 displays the chromatograms of the three honey samples along with the honey sugar standard mix. For all three honey samples, reducing sugars, fructose and glucose, were found to be the major constituents, and their amounts were within the limits established by the Codex Alimentarius Committee on Sugars (2001).[5] The combined concentration of fructose and glucose, as well as their ratio, are useful indicators for the classification of monofloral honeys. For all three honey samples the fructose content (F) varied between 35.4 and 41.9 g/100 g, and glucose content (G) was within a range of 28.1 to 35.2 g/100 g.

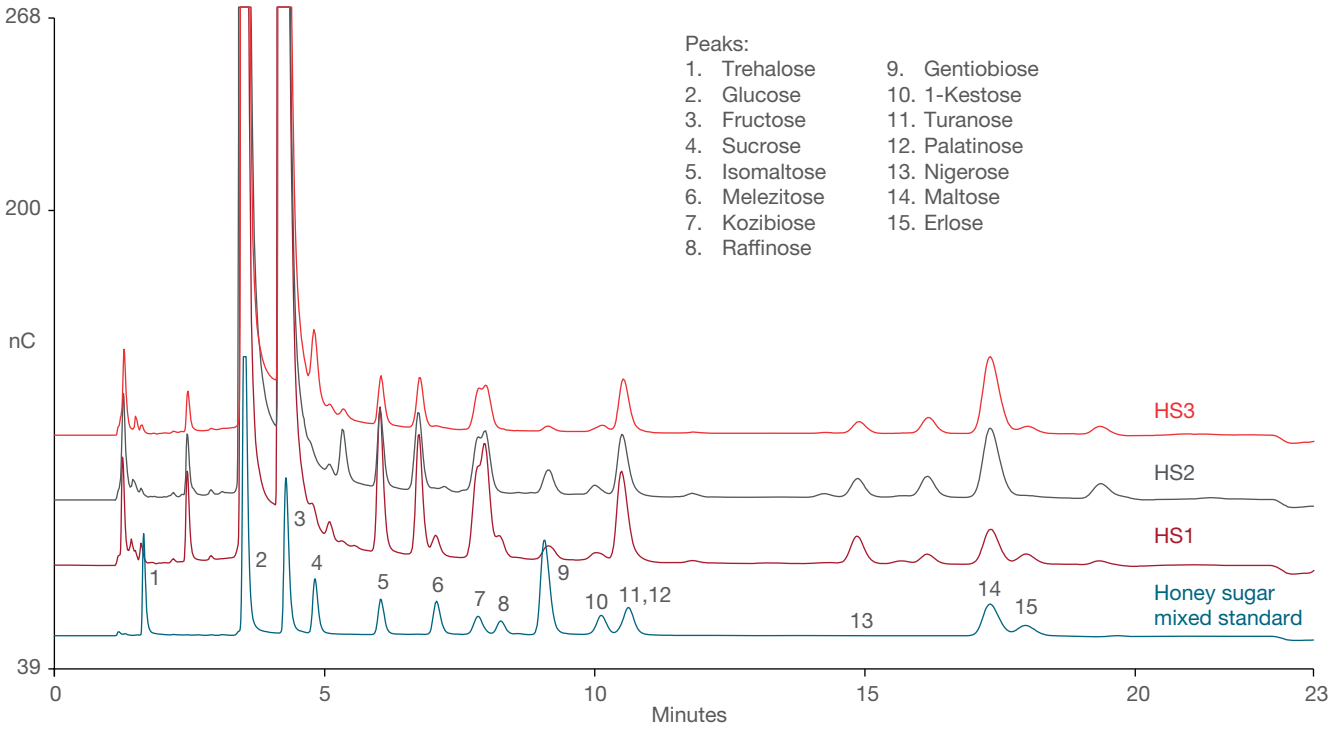


Figure 9. Chromatograms of three honey samples run @ 30 °C using a PdH reference electrode

Sum (F+G) ranged from 63.5 to 76.1 g/100 g. The sum of fructose and glucose for the honey samples used in this study all exceeded the limit required by the Codex, i.e., 60 g/100 g. Table 7 lists the carbohydrate content in three honey samples. In all three honey samples, the fructose concentration is highest, and the ratio of fructose to glucose (F/G) is greater than 1. As described in AN1158, the disaccharide and trisaccharide profile of honey depends upon the sugars and the enzymes present in the bee and nectar.[6] Among disaccharides (Table 7), turanose was the main component followed by maltose, isomaltose, and sucrose. The sucrose content of honey sample HS3 was found to be 0.34 g/100 g. For HS1 and HS2, the sucrose content was very low or not detected. The international norm established by the Codex Alimentarius Commission requires that good quality honey should not contain more than 5 g sucrose/100 g honey.[5] For trisaccharides, four sugars were analyzed and quantified: melezitose, raffinose, 1-kestose, and erlose. Of the three honey samples, HS1 had a higher percentage of trisaccharides. Kestose was found in all three honey samples, while melezitose and raffinose were only found in HS1. It has been reported that honeydew honey contains higher amounts of trisaccharides, such as melezitose and raffinose, and oligosaccharides compared to blossom honey.[7] The sugar profiling of all three honey samples was compared with that run using a Ag/AgCl RE. As listed in Table 8, the amount of sugars (major sugars) calculated in three honey samples was comparable for both reference electrodes with a difference of <1.8%.

Table 7. Carbohydrate content (g/100 g) in honey samples

	HS1 (Manuka honey)	HS2 (Wildflower honey)	HS3 (Multifloral honey)
Monosaccharides (g/100g)			
Glucose	28.14	32.93	35.26
Fructose	35.38	41.88	40.83
F+G (>60g/100g)	63.52	73.81	76.09
F/G ratio (>1)	1.26	1.27	1.16
Disaccharides (g/100g)			
Trehalose	0.04	<0.01	0.04
Sucrose	<0.02	0.02	0.34
Isomaltose	1.20	0.60	0.38
Gentiobiose	0.02	0.03	<0.02
Turanose	2.10	1.95	1.62
Maltose	0.69	1.45	1.59
Trisaccharides (g/100g)			
Melezitose	0.113	Not found	Not found
Raffinose	0.40	Not found	Not found
Kestose	0.07	<0.01	0.08
Erlose	0.63	Not found	0.6

Application II: Glycoprotein monosaccharides

Determination of the monosaccharide composition of a glycoprotein pharmaceutical is a key quality control assay for glycoprotein-based therapeutics. As described previously in AN72580,[8] separation of monosaccharides was achieved using a Dionex CarboPac PA20-Fast-4µm column (2 × 100 mm). Figure 10 displays the chromatogram of the Dionex MonoStandard mix containing fucose, galactosamine, glucosamine, galactose, glucose, and mannose, each at 10 µM concentration (25 pmol). Table 9 lists the chromatographic conditions for the QAR standard. The same conditions were used for the sample analysis. In the first 8 min, all six monosaccharides are eluted using 10 mM KOH. After 8 min, a column clean-up/regeneration step is initiated using 100 mM KOH. This step removes many strongly retained analytes from the column.

Table 8. Carbohydrate content (g/100 g) in honey samples

	HS1			HS2			HS3		
Sugar	Ag/AgCl	PdH	% difference	Ag/AgCl	PdH	% difference	Ag/AgCl	PdH	% difference
Glucose	301	299	-0.65	337	339	0.77	350	353	0.85
Fructose	375	369	-1.37	420	418	-0.50	411	411	0.08
Sucrose	0.00	0.00	na	2.05	2.11	3.03	3.72	3.75	0.67
Isomaltose	11.1	11.0	-0.57	6.41	6.43	0.24	3.75	3.73	-0.45
Maltose	7.19	7.25	0.89	14.9	15.2	1.77	16.1	15.9	-0.88

Table 9. Chromatographic conditions (glycoprotein monosaccharides)

Parameter	Value
System	ICS-5000+ HPIC System
Columns	Dionex CarboPac PA20-Fast-4µm, 2 × 100 mm column (P/N 302749) Dionex CarboPac PA20-Fast-4µm, 2 × 30 mm guard column (P/N 302750)
Column temp.	30 °C
Compartment temp.	20 °C
Flow rate:	0.25 mL/min
Eluent source	Dionex EGC 500 KOH (P/N 075778)
Eluent	0–8 min: 10 mM KOH 8.01–14 min: 100 mM KOH 14.01–20 min: 10 mM KOH
Flow rate	0.25 mL/min
Injection volume	2.5 µL (full loop)
Inject mode	Push full
Loop overfill factor	5
Detection	Pulsed amperometry, Carbohydrate Certified Disposable Gold Working Electrode, Ag/AgCl reference
Reference electrodes	1. Ag/AgCl, 2. PdH
Waveform (PdH reference electrode) @ 10 mM KOH and @ 20 °C detection temp.	Time (s) / Potential (V) / Integration 0.00 / +0.96 0.20 / +0.96 / Begin 0.40 / +0.96 / End 0.41 / –1.14 0.42 / –1.14 0.43 / +1.46 0.44 / 0.76 0.50 / 0.76
Waveform (Ag/AgCl reference electrode)	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
System backpressure	~3750 psi
Background	30–32 nC (for both reference electrodes)
Noise	~30 pC/min peak-to-peak (for both reference electrodes)
Run time	20 min

The top chromatogram in Figure 10 was run using a Ag/AgCl RE and the bottom one using a PdH RE. The background signal for both reference electrodes is about ~31 nC. Except for fucose, peak area response was slightly higher for the chromatogram run using a PdH RE compared to a Ag/AgCl RE (Table 10).

Linearity and precision

The linearity of monosaccharide response was studied by generating calibration curves for all six monosaccharides using a monosaccharide standard mix containing 1.56 to 300 µM of each of the six monosaccharides (Figure 11). A linear curve fit was used for four of the six monosaccharides, and a quadratic curve fit was used for galactosamine and glucosamine. The method precision was determined at three concentrations of the mix of six-monosaccharide standard with three replicates of each standard concentration to give nine total injections. Table 11 shows peak area precision for all three concentration levels tested with RSD values below 3%. Tables 11 and 12 show that precision and linearity results are comparable to those reported in AN72580, which used a Ag/AgCl RE.

Sample analysis

To demonstrate the performance of the PdH RE for this application, we chose commercially available bovine fetuin glycoprotein and subjected it to two sets of hydrolysis conditions—using HCl, which is best for the amino sugars galactosamine and glucosamine, and using TFA, which is best for neutral sugars like mannose, glucose, and galactose. Figure 12 shows typical injections of bovine fetuin TFA as well as HCl hydrolysates. As expected, the neutral monosaccharides are observed at higher concentrations in the TFA hydrolysate, and the amino sugars at a higher concentration in the HCl hydrolysate. Table 13 lists the amount of each monosaccharide present in fetuin glycoprotein. The amounts calculated here are comparable to those reported in AN72580,[8] which used the Ag/AgCl RE, with the understanding that acid hydrolysis also contributes to the variation in results.

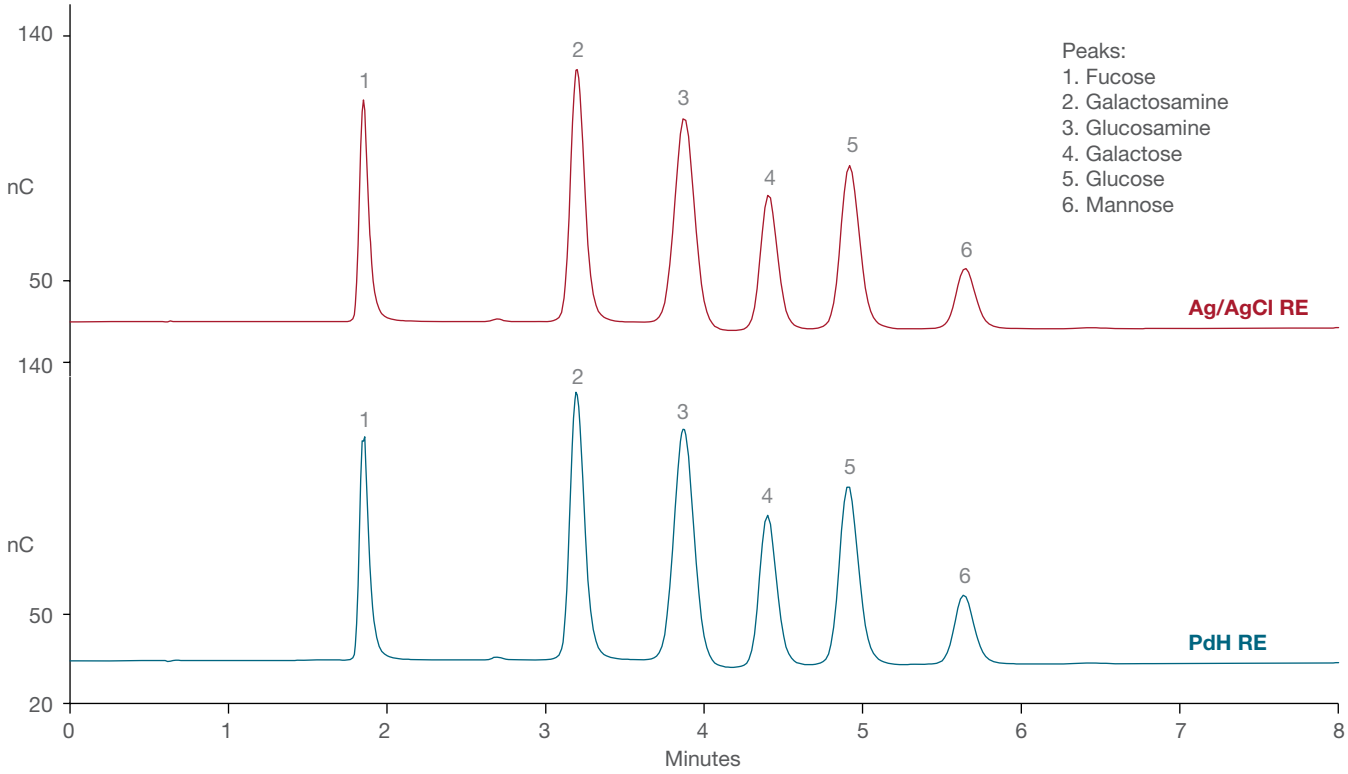


Figure 10. Chromatogram of the column's QAR standard using Ag/AgCl and PdH reference electrodes

Table 10. Electrochemical response of six glycoprotein monosaccharides run using PdH and Ag/AgCl reference electrodes

Peak no.	Peak name	Peak area (nC*min)		% Diff
		Ag/AgCl	PdH	
1	Fucose	5.82	5.72	-1.75
2	Galactosamine	9.80	9.83	0.25
3	Glucosamine	11.52	11.94	3.48
4	Galactose	6.45	6.65	3.07
5	Glucose	8.67	8.68	0.13
6	Mannose	3.25	3.42	4.94

Table 11. Precision data for six monosaccharides

Peak area RSDs (using PdH RE)						
Std conc.	Fucose	Galactosamine	Glucosamine	Galactose	Glucose	Mannose
1.56	1.43	2.90	2.09	0.77	1.57	2.41
12.5	2.76	0.79	0.53	2.07	0.77	0.48
50	0.52	0.08	2.66	1.31	0.30	0.34
Peak area RSDs (AN72580, using Ag/AgCl RE)						
Std conc.	Fucose	Galactosamine	Glucosamine	Galactose	Glucose	Mannose
1.56	4.53	1.09	2.86	1.71	1.26	1.71
12.5	2.2	1.99	1.54	1.55	1.48	2.31
50	0.9	1.3	1.36	1.75	2.06	2.43

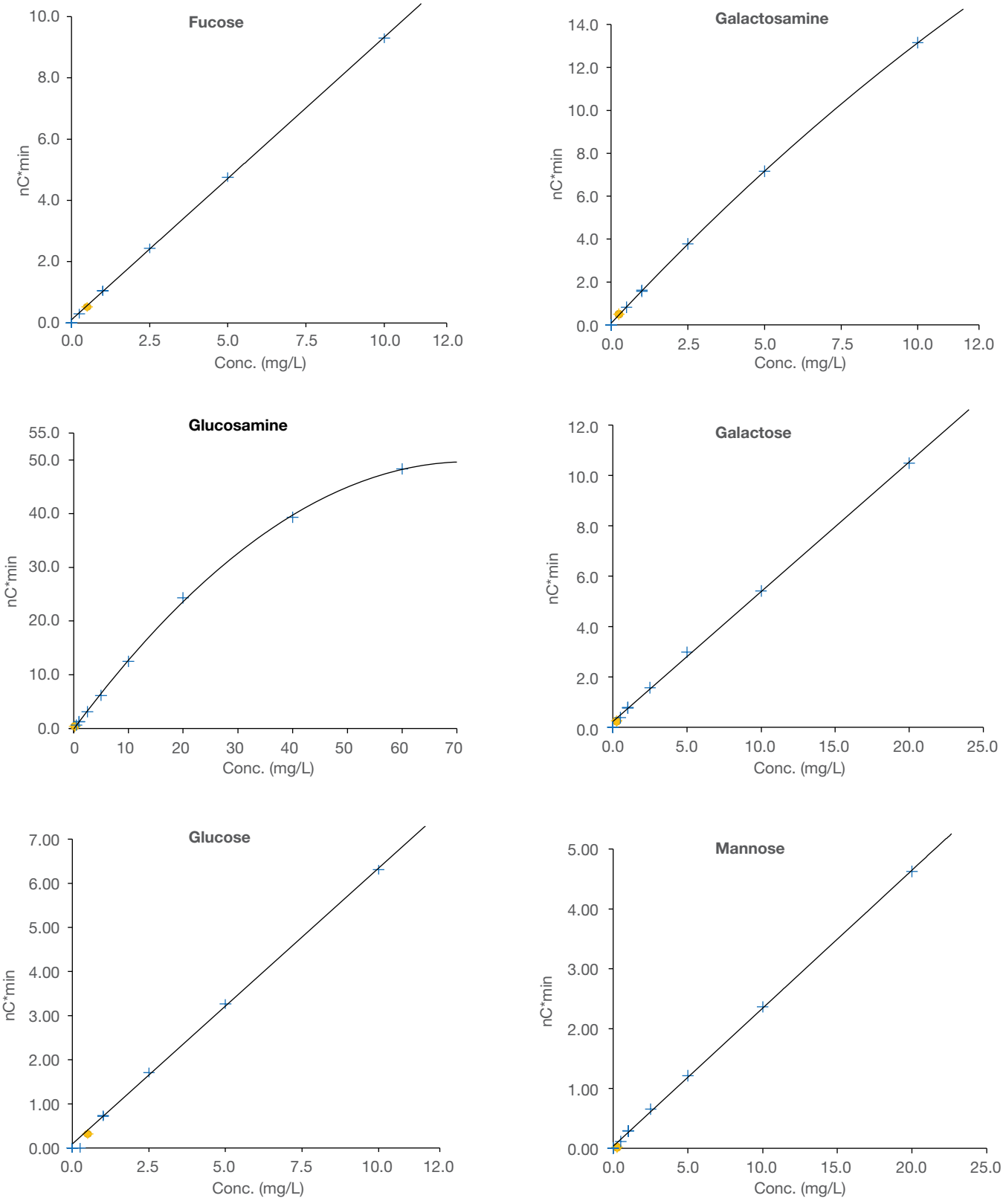


Figure 11. Calibration curves of six glycoprotein monosaccharides

Table 12. Calibration data for six monosaccharides (n=3)

Peak no.	Peak name	Calibration range	Levels	Coefficient of determination (using PdH)	Coefficient of determination (AN72580, using Ag/AgCl)
1	Fucose	1.56–50	6	0.998	0.995
2	Galactosamine	1.56–50	6	>0.9999	1.000
3	Glucosamine	1.56–300	9	>0.9999	1.000
4	Galactose	1.56–100	7	0.998	0.995
5	Glucose	1.56–50	6	0.993	0.993
6	Mannose	1.56–100	7	0.997	0.994

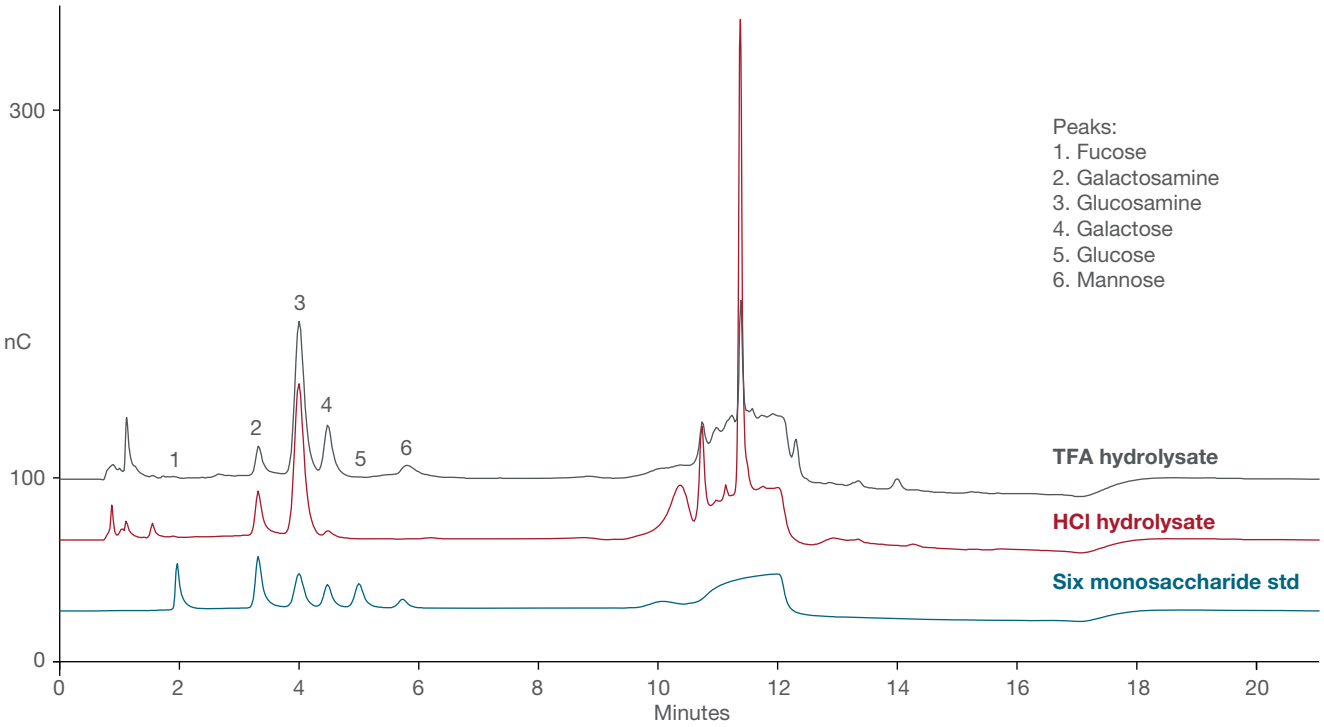


Figure 12. Chromatogram of bovine fetuin TFA and HCl hydroysates using PdH reference electrode

Table 13. Amount of monosaccharide present in fetuin glycoprotein

Sample	Monosaccharide	Amount present (µM)	
		This work (using PdH RE)	AN72580 (using Ag/AgCl RE)
HCl extract	Galactosamine	9.15	8.73
	Glucosamine	47.9	53.7
TFA extract	Galactose	29.3	28.1
	Mannose	20	18

Short-term and long-term stability of a PdH reference electrode

To evaluate the short-term stability of electrochemical response using the PdH RE, 50 consecutive injections of five sugar standards and a six-monosaccharide standard were made and peak area was plotted against the number of injections (Figures 13 and 14) for fifty consecutive injections.

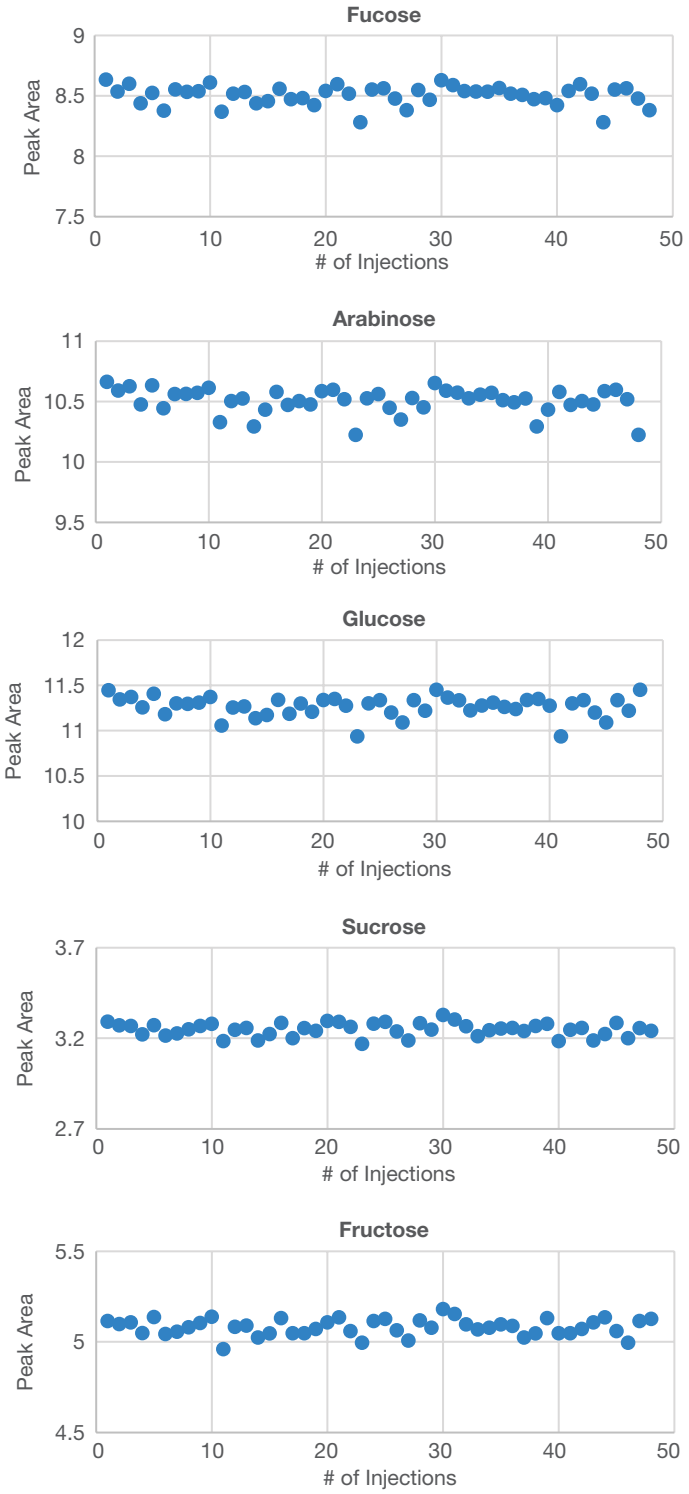


Figure 13. Electrochemical response stability of five sugars over 50 consecutive injections (using a PdH RE)

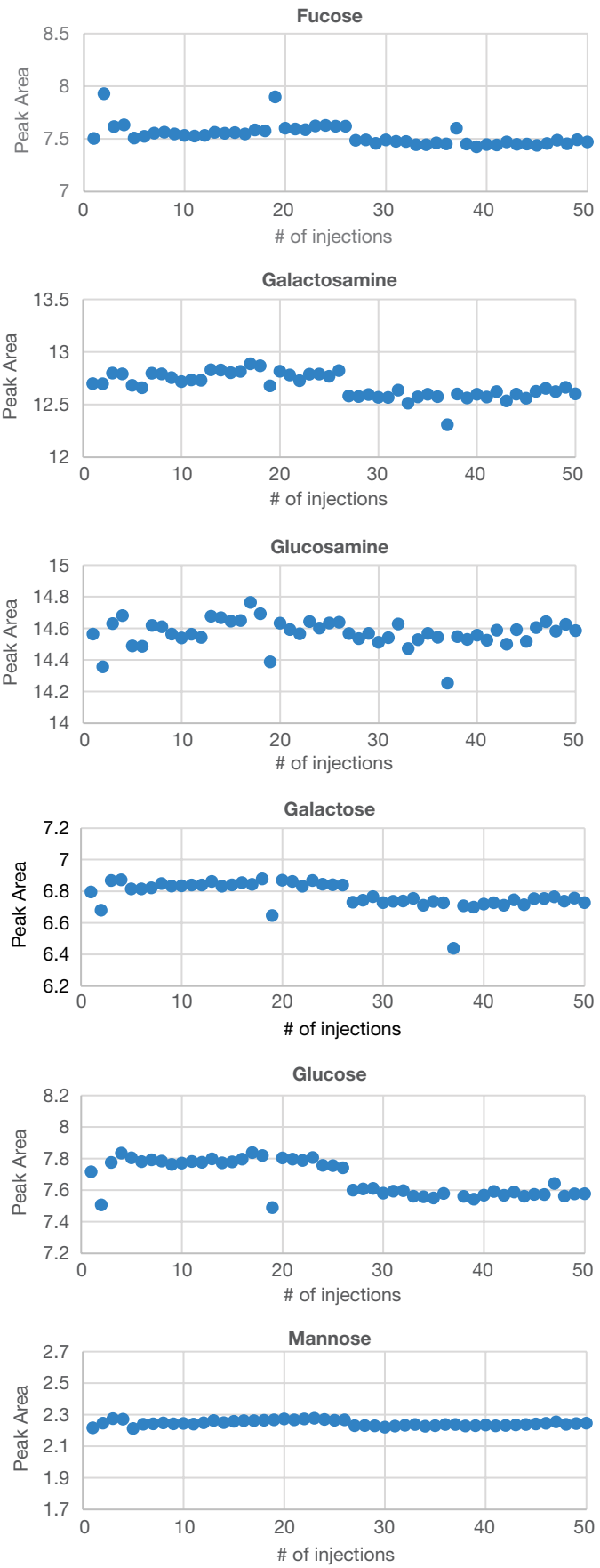


Figure 14. Electrochemical response stability of six monosaccharides over 50 consecutive injections (using a PdH RE)

We also evaluated the long-term stability for a PdH RE by comparing the concentrations of sugars calculated in honey sample 1 run twice, with the second analysis six months later. Figure 15 displays the chromatograms from that analysis. During the six-month period, approximately 1500 injections were made and at least four disposable electrodes were used. The retention time of peaks shifted earlier ~2–6%, which could be due to the loss of column capacity (same column used for the 1500 injections). The concentration of sugars in the honey sample, which was also six months older, from the two dates varied from -0.38 to 11.1% (Table 14). All the sugars except gentiobiose and raffinose, differed by <5%, which indicates good stability of the electrochemical response over the time period of six months.

Table 14. Amount of sugars in honey sample 1, run using the same PdH reference electrode 6 months apart

Peak name	Amount (mg/L)		% Difference
	Run on 06.04.2019	Run on 11.19.2019	
Trehalose	0.353	0.370	4.60
Glucose*	284	298	4.70
Fructose*	355	370	4.05
Sucrose	0.00	0.00	na
Isomaltose	11.7	11.4	-2.63
Melezitose	1.68	1.67	-0.38
Raffinose	3.52	3.17	-11.1
Gentiobiose	0.248	0.278	10.6
Kestose	0.72	0.74	2.89
Turanose	20.1	21.1	4.71
Maltose	7.51	7.54	0.35
Erlose	5.34	5.71	6.48

* Amounts of glucose and fructose were calculated using 3 times diluted sample

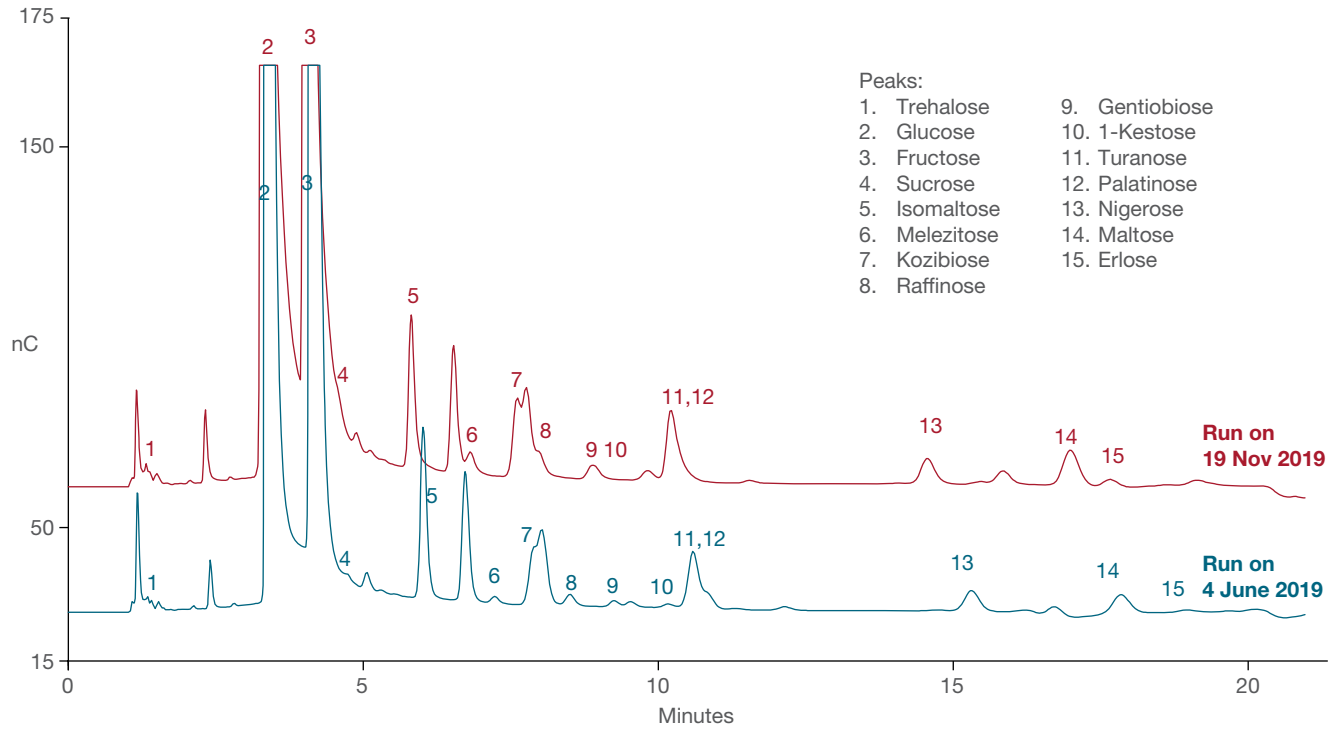


Figure 15. Chromatogram of honey sample 1, run using the same PdH reference electrode 6 months apart

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Conclusion

In this technical note, we demonstrated the performance of a PdH RE using two typical HPAE-PAD applications and compared the performance to the same applications run previously with a Ag/AgCl rRE. The signal response was found to be similar (slightly higher) with a PdH RE, for both applications. The response linearity and peak area reproducibility were found to be comparable to the previous data generated using a Ag/AgCl RE.

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