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Technical Note 110

Carbohydrate Determination by HPAE-PAD with Disposable Au on PTFE Working Electrodes

INTRODUCTION

Many samples, ranging from food,[1] biological products,[2] biofuel feedstocks,[3] and fermentation media,[4] are analyzed for carbohydrate content. As with the sample matrix, determination methods can vary widely, with analyte detection frequently being a challenge. Carbohydrates do not have a strong chromophore; therefore nonspecific detection methods such as refractive index (RI) or low UV wavelengths are used. These detection methods greatly increase the potential for chromatographic interferences and have limited sensitivity. To compensate for this lack of detection sensitivity and specificity, numerous labeling techniques exist. However, these methods are indirect and have varying degrees of labeling specificity, relying on a reactive property of the analyte, which may be shared by other compounds. Additionally, these methods are labor intensive and rely on expensive reagents.

Electrochemical detection offers specificity by directly detecting analytes based on their oxidation potentials with no need for complex or time-consuming sample preparation. For carbohydrates, this can be accomplished by detecting the analytes based on oxidation at high pH.[5] However, if a single potential is applied to the electrode, oxidation products gradually poison the electrode surface, causing a loss of analyte signal. To remove oxidation products, and therefore prevent signal loss, the electrode surface can be cleaned by a series of potentials that are applied for fixed time periods after the detection potential, as in pulsed amperometric detection (PAD).

In PAD applications, the electrode is automatically cleaned and prepared for detection by each cycle of the programmed series of potentials (i.e., the waveform), thereby minimizing electrode fouling by oxidation products of the analyte and other sample compounds, and thus maintaining consistent response.[6] This detection technique is commonly combined with high-performance anion-exchange (HPAE) chromatography to detect carbohydrates that are charged at high pH. This method allows selective separation based on anion-exchange, as well as specific detection based on the oxidation potential of carbohydrates.

High-performance anion-exchange chromatography with pulsed amperometric detection was originally developed with working electrodes embedded in a polymer block. These electrodes require periodic polishing to remove strongly bound compounds, which reduce electrode response. This cleaning step takes time and requires a re-equilibration period after the polished electrode is installed. The introduction of disposable working electrodes simplifies this process by eliminating electrode polishing. Disposable electrodes are easily installed and when the electrode response declines, they are replaced.

Here, gold on polytetrafluoroethylene (Au on PTFE) disposable working electrodes have been evaluated in a variety of carbohydrate applications to determine lifetime, background, and noise characteristics. The electrodes were evaluated using diverse analytes such as aminoglycoside antibiotics, mono- and disaccharides, oligosaccharides, sialic acids, and—although it is a carbohydrate hydrolysis product and not a carbohydrate—hydroxymethylfurfural. The chromatographic conditions for these analytes varied widely, providing a collection of evaluation data that describes many common HPAE-PAD carbohydrate applications. These electrodes provided consistent performance for four weeks with <15% loss in response for the applications evaluated, electrode-to-electrode response consistency, and simple installation without the need to polish.

EQUIPMENT

Thermo Scientific Dionex ICS-3000 or ICS-5000 Reagent-Free™ Ion Chromatography (RFIC™) system including:
- SP Single Pump or DP Dual Pump module
- DC Detector/Chromatography module
- AS Autosampler
- EG Eluent Generator

Electrochemical Detector (ED) (P/N 061719) with Dionex ICS-3000/5000 Analytical EG Vacuum Degas Conversion Kit (P/N 063353)

Electrochemical Cell (P/N 061757)

Disposable Gold Working Electrode, Au on PTFE,* (P/N 066480)

Reference Electrode (P/N 061879)

Thermo Scientific Dionex Chromeleon™ 7 Chromatography Workstation

Polypropylene injection vials with caps, 0.3 mL (P/N 055428)

Polypropylene injection vials with caps, 1.5 mL (P/N 061696)

Thermo Scientific Nalgene™ 1000 mL, 0.2 µm nylon filter units (Fisher Scientific P/N 09-740-46)

Polypropylene microcentrifuge screw cap tubes, 1.5 mL (Sarstedt P/N 72.692.005)

*Unless otherwise noted, 2 mil gaskets supplied with Au on PTFE disposable working electrodes were installed.

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 (P/N 059326)

Additional reagents for specific applications are listed elsewhere.[7–12]

DETECTION CONDITIONS

Carbohydrate 4-Potential Waveform for the ED

Time (s)	*Potential (V)*	*Gain Region**	*Ramp**	*Integration*
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000, but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference). See Dionex Technical Note 21 for more information.[6]

Chromatography

Applications spanning a variety of typical elution conditions were performed with the Au on PTFE disposable working electrodes. The chromatographic conditions are listed in Tables 1 and 2. Additional information about individual applications can be found in the respective documents listed in the References section; elaboration is not provided here.

Table 1. Chromatographic Conditions for Hydroxide Eluent Applications				
Application Analyte	Hydroxymethylfurfural (HMF)	Aminoglycoside antibiotics	Carbohydrates in coffee	Carbohydrates in biofuel feedstocks
Columns	Dionex CarboPac PA1 Analytical, 4 × 250 mm and Dionex CarboPac PA1 Guard, 4 × 50 mm	Dionex CarboPac MA1 Analytical, 4 × 250 mm and Dionex CarboPac MA1 Guard, 4 × 50 mm	Dionex CarboPac PA1 Analytical, 4 × 250 mm and Dionex CarboPac PA1 Guard, 4 × 50 mm	Dionex CarboPac SA10 Analytical, 4 × 150 mm and Dionex CarboPac SA10 Guard, 4 × 50 mm
Eluent	50 mM KOH	115 mM NaOH	DI water with 300 mM NaOH post column	1 mM NaOH
Eluent Source	EGC II KOH with CR-ATC	Manual preparation	Manual, post column hydroxide	EGC II KOH with CR-ATC
Gradient	Isocratic	Isocratic	Isocratic	Isocratic
Flow Rate	1.0 mL/min	0.5 mL/min	0.6 mL/min	1.5 mL/min
Injection Volume	10 µL (full loop)	20 µL (full loop)	10 µL (full loop)	0.4 µL (full loop)
Temperature	30 °C	30 °C	25 °C column, 30 °C detector	45 °C column, 30 °C detector
Detection	PAD	PAD	PAD	PAD, 15 mil gasket
Backpressure	2400 psi	1500 psi	2700 psi	2200 psi
Working Electrode	Au on PTFE Disposable	Au on PTFE Disposable	Au on PTFE Disposable	Au on PTFE Disposable
Background	30 nC	35 nC	22 nC	42 nC
Noise	30 pC	30 pC	90 pC	50 pC
Reference	Application Note 270[7]	Application Note 267[8]	Application Note 280[9]	Application Note 282[10]

Table 2. Chromatographic Conditions for Hydroxide-Acetate Eluent Applications		
Application Analyte	Oligosaccharides in proteins	Sialic acids
Columns	Dionex CarboPac PA200 Analytical, 3 × 250 mm and Dionex CarboPac PA200 Guard, 3 × 50 mm	Dionex CarboPac PA20 Analytical, 3 × 150 mm and Dionex CarboPac PA20 Guard, 3 × 30 mm
Eluents	100 mM NaOH, 1 M sodium acetate	100 mM NaOH, 1 M sodium acetate
Eluent Source	Manual preparation	Manual preparation
Gradient	20–150 mM acetate in 100 mM NaOH	70–300 mM acetate in 100 mM NaOH
Flow Rate	0.5 mL/min	0.5 mL/min
Injection Volume	20 or 50 µL (full loop)	10 µL (full loop)
Temperature	30 °C	30 °C
Detection	PAD	PAD
Backpressure	3200 psi	3000 psi
Working Electrode	Au on PTFE Disposable	Au on PTFE Disposable
Background	20 nC	20 nC
Noise	30 pC	40 pC
Reference	Application Update 176[11]	Application Update 180[12]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Generation Applications Using an RFIC System

Generate potassium hydroxide (KOH) eluent on-line by pumping high-quality degassed DI water through the EGC II KOH cartridge. Chromeleon software will track the amount of KOH used and calculate the remaining lifetime.

The authors strongly recommend eluents prepared by an eluent generator for concentrations of hydroxide <100 mM; however, eluents can be prepared manually if needed. If eluents must be prepared manually, use a 50 % (w/w) NaOH solution or a KOH solution of similar purity and prepare according to the general instructions for hydroxide eluents in Dionex Technical Note 71.[13]

Manual Eluents (Hydroxide Concentrations of ≥100 mM)

Use high-quality DI water containing as little dissolved carbon dioxide as possible. Biological contamination must be absent. Obtain source water using a water purification system consisting of filters manufactured without electrochemically active substances such as glycerol. Filter through a 0.2 µm nylon membrane vacuum filter unit to remove particulates, then sonicate to reduce dissolved gases.

Take care to minimize carbonate contamination, which will cause a loss of chromatographic efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and must not be used. A commercially prepared 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the recommended source for sodium hydroxide.

Maintain all manual eluents under 34 to 55 kPa (5 to 8 psi) of nitrogen at all times after preparation to reduce diffusion of atmospheric carbon dioxide. Within as little as 15 minutes, enough carbon dioxide can dissolve from the air to negatively impact chromatography.[13]

Sodium Hydroxide, 100 mM

Add 5.2 mL of 50% (w/w) NaOH to 994.8 mL of degassed DI water.

Sodium Hydroxide, 115 mM

Dilute 5.9 mL of a 50% (w/w) sodium hydroxide into 994.1 mL of degassed DI water.

Sodium Hydroxide, 300 mM

Add 15.6 mL of 50% (w/w) NaOH to 984.4 mL of degassed DI water.

Sodium Acetate, 1 M, in 100 mM Sodium Hydroxide

Prepare 1 L of 1 M sodium acetate in 100 mM sodium hydroxide by dissolving 82.0 g of anhydrous sodium acetate in ~800 mL of DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% (w/w) NaOH, and fill the flask with degassed DI water.

Sodium Hydroxide, 1 M

Dilute 51.5 mL of a 50% (w/w) sodium hydroxide into 948.5 mL of degassed DI water.

SYSTEM PREPARATION AND CONFIGURATION

RFIC Systems

Methods that use eluent generation require installation of the Dionex ICS-3000/5000 Analytical EG Vacuum Degas Conversion Kit (P/N 063353) to allow sufficient removal of the hydrogen gas formed with the potassium hydroxide eluent. If there is a leak in the installed conversion kit, baseline instability will result. Ensure that all connections and fittings for the vacuum degas are vacuum tight before using the system.

Postcolumn Applications

Applications requiring the postcolumn addition of NaOH solution will require installation of a knitted reaction coil reactor after the column but before the detector. Install a PEEK™ mixing tee (P/N 048227) after the column and use the second pump of the DP to deliver the postcolumn solution to the tee. Direct the third port on the tee to the reaction coil, followed by the electrochemical detector cell. Increased pump noise (and therefore detector noise) is expected when delivering postcolumn reagents in this manner.

Precautions

System Preparation and Maintenance

Before running applications, prepare the system as described in section 2.5 of the Thermo Scientific Dionex CarboPac™ SA 10 manual with a 1 h 2 M NaOH wash to thoroughly clean all tubing and system parts.[14] If metal contamination is suspected, an EDTA wash (as described in section 5.6 of the same manual) is also recommended. When using manually prepared eluents, particularly those that contain sodium acetate, take care to keep the system metal free and sterile. Ensure that all acetate eluents contain some hydroxide, preferably >50 mM, to prevent biological contamination. Once the system is in operation with manual eluents, continue running the pump as consistently as possible to avoid any precipitation in the pump components. If the pump must be shut down for a long period of time, first remove the column and flush the pump with DI water to remove as much of the basic eluent as possible. This step will improve the life of the pump and minimize downtime.

When installing a new column, fully equilibrate it to eluent conditions before restoring the fluidic connections to the electrochemical cell.

Reference Electrode

Keep a spare pH-Ag/AgCl reference electrode at all times. For best results, replace the reference electrode every six months, depending on how frequently it is used and the hydroxide eluent concentration. As a result of exposure to alkaline solutions—such as flowing sodium hydroxide—the 3 M KCl electrolyte inside the reference electrode gradually becomes alkaline and the silver chloride layer on the Ag wire in the electrode dissolves or converts to a mixture of silver oxide and silver hydroxide. When this happens, the reference potential shifts and becomes increasingly unstable.

Reference potential shifting can lead to unusually high background response from the working electrode, reduced signal response, or a combination of both effects. If the reference electrode fails while the electrochemical detection cell is on, replace the disposable working electrode as well as the reference electrode. A large shift in reference potential can damage the disposable working electrode.

If the reference electrode is allowed to dry out, it will be irreversibly damaged. Store the reference electrode in saturated KCl when not in use.

If the pH reading is not consistent with the hydroxide eluent strength, replace the reference electrode. For most applications, the pH reading will fall within the range of 12–13.

Other Considerations

If a leak occurs at the working electrode in the electrochemical cell, thoroughly clean the cell and replace the gasket and the working electrode before running samples. Reduced response will be observed if the working electrode used while a cell was leaking is reinstalled after correcting the leak.

Waveforms other than those specifically recommended for use with disposable electrodes may significantly shorten the electrode lifetime.

RESULTS AND DISCUSSION

Discussion of each of the potential applications is beyond the scope of this document; however, a few select analytes will be discussed to illustrate the performance of the Au on PTFE disposable working electrode. These electrodes provide the advantage of increased lifetime compared to previous disposable Au on polyester working electrodes (P/N 060139). Disposable Au working electrodes on a polyester substrate are limited to 100 mM NaOH or less in eluent strength and have lifetimes of two weeks for carbohydrate applications at flow rates between 0.5 and 1.5 mL/min. Frequently, after two weeks of use, the electrode fails by delaminating from the substrate, resulting in a sudden loss of response.

To resolve this issue, Au on PTFE disposable working electrodes were developed. These disposable working electrodes do not delaminate from the substrate and response consistency is improved, with gradual response reduction as the electrode ages but no sudden loss of response. Additionally, these working electrodes are stable at higher hydroxide concentrations (>100 mM) than the Au on polyester disposable working electrodes and can be used for applications that previously required a conventional Au working electrode.

Applications Using Hydroxide Eluents

High-performance anion-exchange chromatography with pulsed amperometric detection with hydroxide eluents is a technique commonly used for mono- and disaccharide separations. Figure 1, for example, illustrates the elution of a mixed standard of commonly determined carbohydrates present in coffee, as well as the separation of free and total carbohydrates from soluble coffee. This application uses weak hydroxide elution conditions along with 300 mM NaOH delivered postcolumn to improve sensitivity. Under these conditions, peak area response measured by injecting 0.050 mg/mL galactose—interspersed within a sequence of samples and calibration standards—gradually decreases over the electrode lifetime with an 11% loss over one month, as shown in Figure 2.

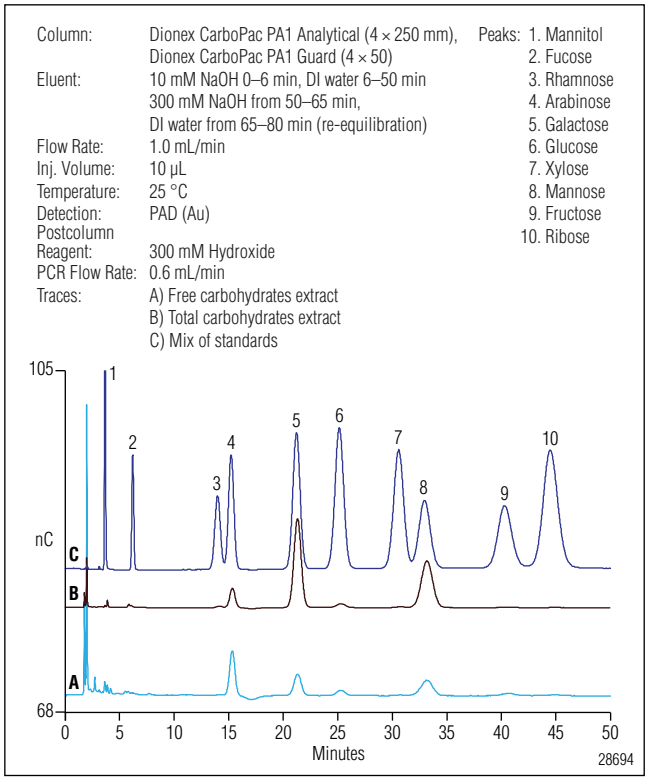

Figure 1. Separation of free carbohydrates extract from instant coffee (A), total carbohydrates extract from instant coffee (B), and mixed carbohydrate standards (C) using a modified AOAC method 995.13.[9]

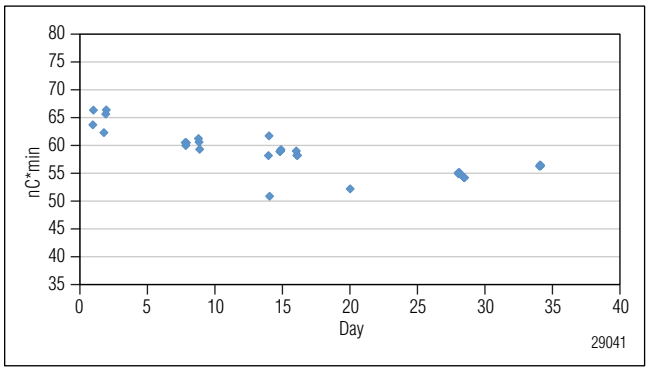

Figure 2. Galactose peak area response on a single Au on PTFE disposable working electrode over four weeks. Standards were interspersed with samples and additional calibration standards (periods of time with no data points displayed). See Figure 1 for chromatographic conditions.

For comparison, 1 mM NaOH from eluent generation without postcolumn base was also investigated using a 62 mil gasket designed for samples with high concentrations of carbohydrates. In this example, the peak area response to 0.05 mg/mL galactose was equal within the replicate error of 10 serial injections, with an average peak area of 0.24 ± 0.02 nC*min and precision, as RSD, of 5.9 over four weeks. Furthermore, multiple sequential injections over a four-day period had a precision (RSD) of 1.98, as shown in Figure 3. Electrode-to-electrode peak area response was within 8% during these experiments.

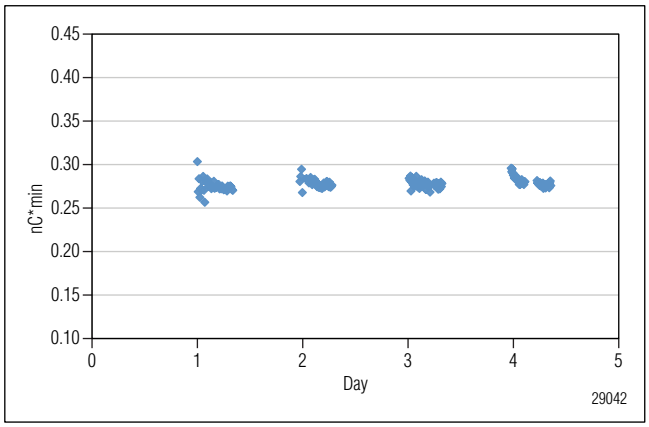

Figure 3. Stability of galactose peak area response over four days when eluted with 1 mM NaOH and using a 62 mil gasket in the ED cell. Standards are interspersed with samples (periods of time with no data points displayed). For chromatographic conditions, see Reference 10.

Stronger base elution conditions (such as the 50 mM hydroxide used for HMF determination)[7] had peak area responses within an RSD of 1.7 across five days. The aminoglycoside antibiotics kanamycin and amikacin—eluted with 115 mM hydroxide[8]—had peak area response precision within 7.2 and 5.0 (RSD), respectively, over four weeks of sample analysis.

This performance illustrates the reproducible response possible with the Au on PTFE disposable working electrodes.

Typical Performance Characteristics

Background and noise observed during applications that use hydroxide eluents are summarized in Table 1. The typical background signal for a newly installed electrode ranges from 22–64 nC, depending on the column used and application elution conditions. As the electrode ages, the background will increase 10–20 nC. Biological contamination can also increase the background; therefore, monitoring the background can be useful when evaluating system performance both in terms of system cleanliness and electrode age.

Postcolumn addition applications show greater noise than those without. This is expected, due to the additional noise that is generated from a second pump delivering the postcolumn base. Applications with postcolumn addition had baseline noise ranging from 90–110 pC. Applications without postcolumn base had noise ranging from 20–70 pC.

Equilibration Period

When a new electrode is installed, the background and noise observed will equilibrate within 1 h using 2 mil gaskets. However, RFIC applications at low (1 mM) hydroxide concentrations and a 15 mil gasket may show a gradual increase in response over time.

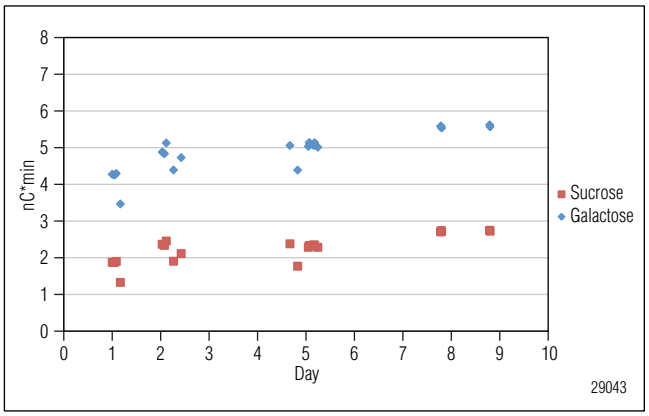

Figure 4. Galactose (0.5 mg/mL) and sucrose (0.5 mg/mL) peak area responses using an Au on PTFE disposable working electrode and a 15 mil gasket. Standards are interspersed with samples and additional standards (periods of time with no data points displayed). See Reference 10 for chromatographic conditions.

For example, when injecting high concentrations of galactose (0.5 mg/mL), the peak area response (measured in the intervals between injections of samples and calibration standards) increases 23% over 10 days of injections under these conditions (Figure 4). This change in response was not observed during other application conditions. Daily calibration or a reduced set of daily check standards is recommended to confirm calibration is valid before samples are injected.

Response Linearity for Selected Applications

Table 3 lists the linearity for a variety of applications using the Au on PTFE electrode. The linearity, as measured by the coefficient of determination (r^2), is generally >0.99. The linear range may be reduced for high concentrations of carbohydrates, such as in biomass hydrolysates. Use of the 62 mil gasket is recommended for such samples with high concentrations of carbohydrates that cannot be highly diluted.

Table 3. Analytical Data from Hydroxide Eluent Applications

Application Analyte	HMF	Kanamycin Sulfate	Amikacin	Galactose in biofuel feedstocks
Calibration Range	0.1–50 µg/mL or 0.5–1000 µg/mL	2–16 µg/L	4–40 µg/L	0.4–2 mg/mL
Coeff. of Determination (r^2)	0.9998 or 0.9965	0.9993	0.9991	0.9887
Reference	Application Note 270	Application Note 267	Application Note 267	Application Note 282

System Idle Recommendations

For best consistency in system performance and minimal startup time, keep the system at the chromatographic conditions for the application. Reducing the flow rate to extend eluent lifetime during system idle conditions, such as over a weekend, did not impact electrode response for the applications discussed here (i.e., those with hydroxide eluents). For extended system shutdown, turn off the cell, remove the Ag/AgCl reference electrode from the cell, and store the electrode in a saturated KCl storage solution. Disconnect the column from the cell and plug the column. For manual eluent applications, if the system will be idle for more than one week, flush the pump with DI water for several hours to remove residual hydroxide, thereby extending the pump seal lifetime.

Applications Using Sodium Acetate Eluents

Figure 5 shows the separation of fetuin oligosaccharides on the Dionex CarboPac PA200 column detected using both a conventional working electrode and the Au on PTFE working electrode. As shown, the response and signal-to-noise ratio (S/N) characteristics of both electrodes are similar, with slightly improved response for the disposable electrode. The S/N for the trisialylated oligosaccharide that is labeled as peak 1 in Figure 5 is 645 for a conventional working electrode and 882 for the disposable Au on PTFE working electrode.

Typical Performance Characteristics

Background signal and noise observed during applications that use acetate with hydroxide eluents are summarized in Table 2. The typical background for a newly installed electrode was 20 nC; however, this may vary based on the application conditions. As the electrode ages, the background signal will gradually increase by 10–20 nC. Noise for these applications ranged from 30–80 pC.

The background signal observed when first installing a disposable Au on PTFE working electrode will equilibrate within 30 min for applications using sodium acetate in hydroxide eluents. However, when tested with 75 pmol injections of sialic acid standards, peak area response stabilized within 6–12 h when the electrode was installed with the 2 mil gasket supplied with the electrodes.

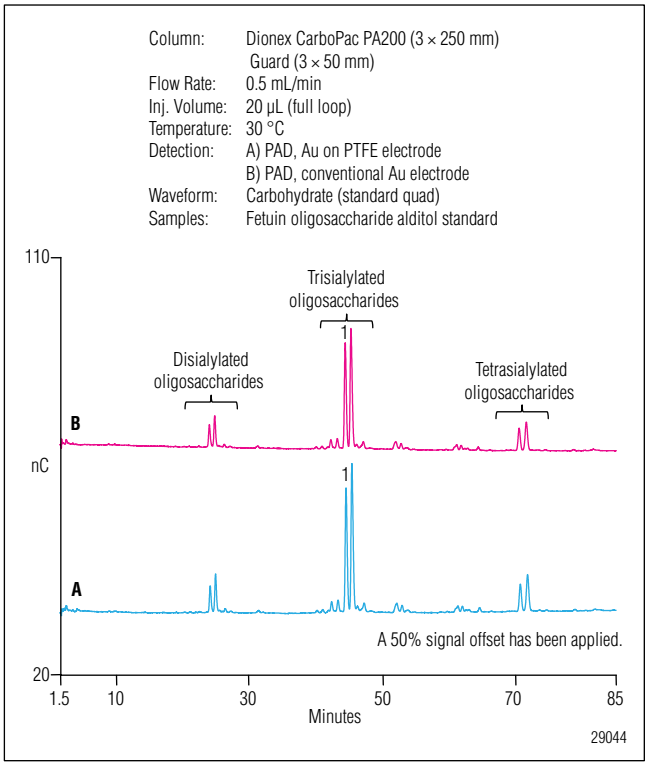

Figure 5. Comparison of oligosaccharide peak area response with an Au on PTFE working electrode (A) and a conventional Au working electrode (B).

For convenience, it may be simplest to install the electrode at the end of a day and begin a sequence the following morning. Under these conditions, the response will be consistent. After this initial equilibration, the response should be stable with a decrease in response of 5.8% over two weeks of standard injections (Figure 6). Three electrodes were installed over the course of three months. The electrode peak area responses for 11 pmol injections of Neu5Ac after equilibration were within a relative standard deviation (RSD) of 3.1%. For the most accurate results, daily calibration or check standards are recommended.

Sialic acid peak area response using the Au on PTFE working electrode is linear with respect to concentration. Calibration coefficients of determination (r^2) are >0.999 between 1.0–100 pmol Neu5Ac and 0.39–7.8 pmol Neu5Gc.[12] These results are equivalent to previous results on a conventional gold working electrode.[15]

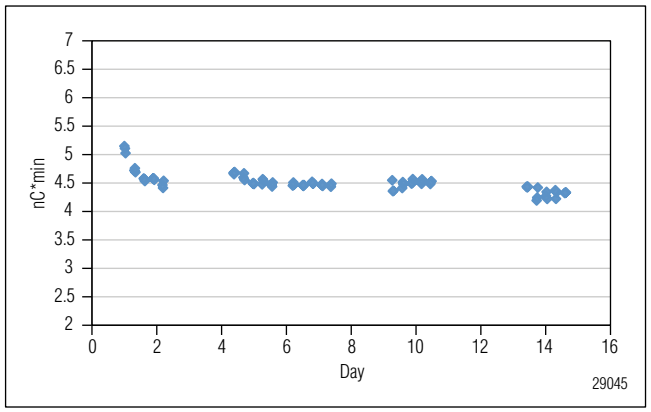

Figure 6. Equilibration and stabilization of 75 pmol Neu5Ac peak area response over two weeks. With exclusion of the initial 12 h of injections, peak area response declines 5.8% based on triplicate injections on day 2 compared to day 15.

System Idle Recommendations

For applications that use an acetate gradient, do not decrease the flow rate to conserve eluent while not running samples. For best performance, maintain the system at chromatographic conditions for the application. For gradient applications, no electrode response differences were observed between maintaining the idle system at initial gradient conditions compared to final gradient conditions. Working electrode response can fall by 15–60%, regardless of the age of the electrode, if the system is maintained for long periods (i.e., over a weekend or longer) at flow rates of 0.35 mL/min, compared to maintaining the system at an application flow rate of 0.5 mL/min. For this reason, it is strongly recommended that the system be maintained at the flow rate and eluent conditions of the application. Working electrode response was found to be consistent under these conditions.

CONCLUSION

Here, disposable Au on PTFE working electrodes were evaluated with multiple application conditions, providing a collection of data that describes many common analyses. With the recommendations described here, these electrodes provide consistent performance for four weeks with <15% loss in response for the applications evaluated and excellent electrode-to-electrode response consistency. Furthermore, these electrodes offer convenience with simple installation and no need for electrode polishing.

SUPPLIERS

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