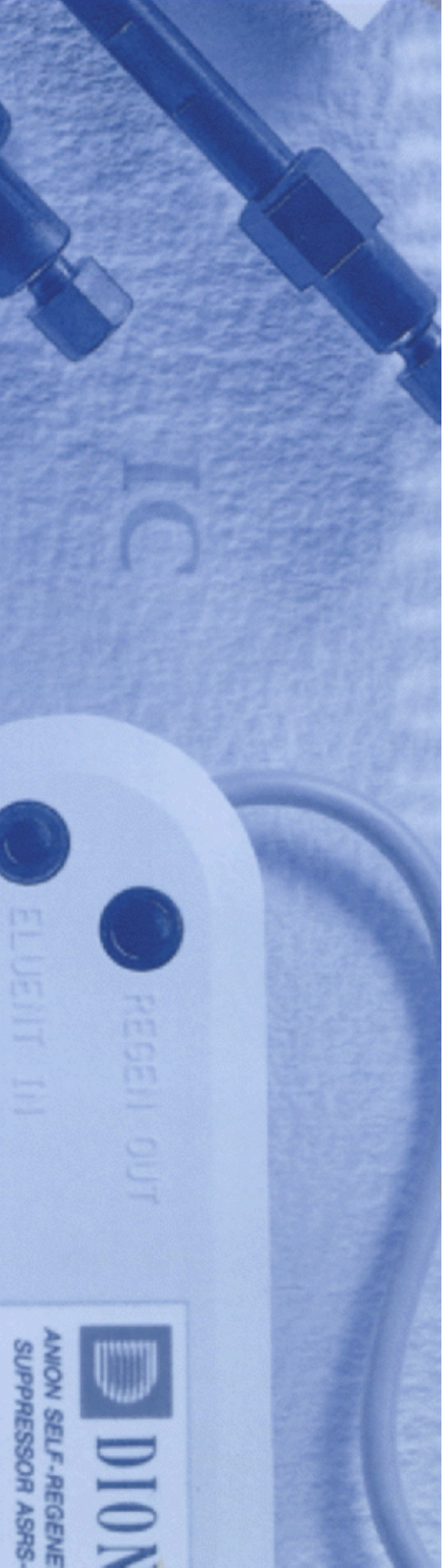

PRODUCT MANUAL

for the
CARBOPAC PA200 Guard Column
(3 x 50 mm, P/N 062895)

CARBOPAC PA200 Analytical Column
(3 x 250 mm, P/N 062896)

QUICKSTART STEPS AND LINKS
Click blue text below to get started.

1. See Section 4, “Before You Start” and Section 5, “Preparation of Eluents and Standards.” Note general operating conditions, precautions and chemical purity requirements. Make the required solutions and eluents.

2. See Section 8, "Example Applications" for example applications.

3. See Appendix A, “Quality Assurance Reports”. Run the Production Test Chromatogram as a system check.

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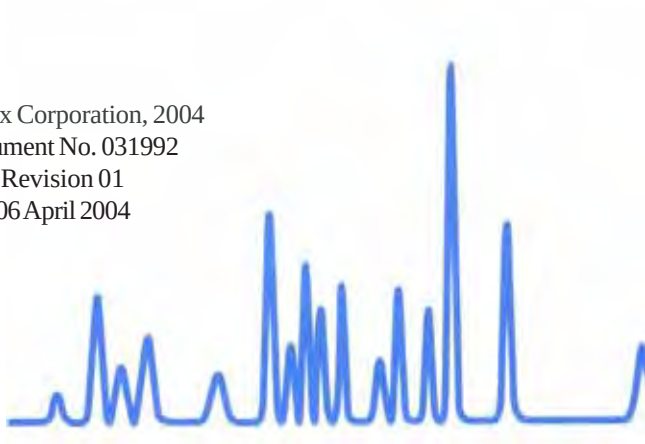

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SECTION 1 - INTRODUCTION

1.1 CarboPac PA200

The CarboPac PA200 column is the latest addition to the CarboPac family of columns for carbohydrate separations. This column has been specially developed to provide high resolution separations of charged and neutral oligosaccharides and is the recommended column for these applications. The CarboPac PA200 columns are packed with a hydrophobic, polymeric, pellicular anion exchange resin stable over the range of pH 0–14. This unique pH-stability of the packing material allows the use of eluent compositions that are conducive to anodic oxidation of carbohydrates at gold electrodes.

Resin Characteristics:

Particle Size:	5.5 µm
Pore Size:	microporous (< 10 Å)
Cross-linking:	55%
Ion exchange capacity:	35 µeq per column
Latex Characteristics:	
Functional Group:	quaternary ammonium ion
Latex Diameter:	43 nm
Latex Cross-linking:	6%

Typical Operating Parameters:

pH range:	0–14
Temperature Limit:	4–60 °C
Pressure Limit:	3500 psi
Organic Solvent Limit:	100% compatible
Typical eluents:	High purity water (18.2 megohm-cm), sodium hydroxide, sodium acetate

1.2 Disposable Gold Working Electrodes

Carbohydrates separated by high pH anion exchange chromatography are detected by pulsed amperometric detection and the signal is reported in coulombs (C). Amperometric detection is used to measure the current or charge resulting from oxidation or reduction of analyte molecules at the surface of a working electrode. During oxidation reactions electrons are transferred from molecules of electroactive analytes, such as carbohydrates, to the working electrode in the amperometry cell. Detection is sensitive and highly selective for electroactive species, since many potentially interfering species cannot be oxidized or reduced, and are not detected. When a single potential is applied to the working electrode, the detection method is d.c. amperometry. Pulsed amperometry and integrated amperometry employ a repeating sequence of potentials. Pulsed amperometric detection at a gold working electrode is a reproducible and sensitive method for all carbohydrates of molecular weight up to ten thousand.

Although carbohydrates can be oxidized at a gold working electrode, the products of the oxidation reaction poison the surface of the electrode, inhibiting further analyte oxidation. By repeatedly pulsing between high positive and negative potentials, a stable and active electrode surface can be maintained. However, the gold working electrode is very slowly consumed during this process and will eventually need to be replaced. Occasionally the electrode may be ‘poisoned’ by other contaminants, resulting in a significantly reduced response. When this occurs, the active surface can be renewed by polishing the electrode. However, this can be a tedious and time-consuming process.

The Dionex disposable gold electrodes (P/N 060139 for 6, P/N 060216 for 4 packages of 6) make electrode reconditioning by polishing and other methods superfluous. They are less expensive and can thus be replaced more often than the conventional electrodes. The more frequent replacement of working electrodes renders electrochemical detection more predictable and reproducible. The disposable electrodes also make easier any troubleshooting of electrochemical detection problems. The gold hydroxide (AuOH) catalyzed mode of oxidation of carbohydrates differs from the gold oxide catalyzed oxidation of amino acids at higher potentials. Although both gold electrodes can be mounted in the same ED50 detection cell, and thus in principle it is feasible to convert a gold electrode from one mode of detection to another, in practice this may require an extensive period of time and is thus not recommended. The Au electrodes for carbohydrate analysis have been tested for and are guaranteed to work for carbohydrate analyses.

NOTE

Dionex Technical Note 21 Waveform A MUST BE USED with disposable electrodes. Waveform B and Waveform C CANNOT BE USED with Disposable Electrodes. Waveforms B and C will strip the gold surface of the disposable electrode within 24 hours

1.3 BioLC Carbohydrate System (Without Columns)

System Part Numbers and Description

The following system components are recommended for carbohydrate analysis.

Basic Gradient System	Standard System
BioLC gradient pump, with degas	BioLC gradient pump, with degas
Chromatography oven with injection valve and regulator assembly	Autosampler with thermal compartment
Electrochemical detector without cells	Electrochemical detector without cells
Electrochemical cell, Au for Chromatography oven	Electrochemical cell, Au for Autosampler
EO1 eluent organizers	EO1 Eluent organizers

1.4 Electrochemical Gold Cells

Part Number	Product Description
055290	Electrochemical Cell, Au, for ED50 with AS50 autosampler
052556	Electrochemical Cell, Au, for ED50 with LC25 chromatography oven
044108	Electrochemical Cell, Au, for ED50 with LC30 chromatography oven
044112	Working Electrode, Au, for ED50 electrochemical detector
044198	pH-Ag/AgCl reference electrode

Please note that in this manual, ED50 can stand for ED40/ED50/ED50A

1.5 Guard Column Use

A guard column is usually placed before the analytical column to prevent sample contaminants from eluting onto the analytical column. The addition of the guard to the analytical also increases the column capacity by about 20%, which translates into an increase of about 20% in the retention times for isocratic runs. If a guard is added to a system running a gradient method that was initially developed for just an analytical column, the gradient schedules should be increased by about 20%, to ensure similar resolution between the eluting peaks.

1.6 CarboPac PA200 Anion Exchange Columns

Part Number	Product Description
062896	CarboPac PA200 Analytical Column, 3-mm
062895	CarboPac PA200 Guard Column, 3-mm

Always remember that assistance is available for any problem that may be encountered during the shipment or operation of Dionex instrumentation and columns through the Dionex North America Technical Call Center at 1-800-DIONEX-0 (1-800-346-6390) or through any of the Dionex offices listed in section 10, "Dionex Worldwide Offices."

SECTION 2 – OPERATION AND SYSTEM REQUIREMENTS

2.1 System Requirements

The carbohydrate separations with the CarboPac PA200 columns are optimized for use with Dionex 2-mm systems, whether they are the 2-mm Dionex DX 500, DX 600 or BioLC. The pump should be configured for microbore pumping (microbore pump heads, pump heads volume 25 µL). All of these systems are metal-free.

For carbohydrate analysis with microbore pumpheads, the active mixer in the pump's priming block must be bypassed and the gradient mixer GM-4 is used between the pump and the injector. Tubing anywhere between the injection valve and detector should be < 0.005 in i.d. PEEK tubing. Minimize the length of all liquid lines, but especially that of the tubing between the column and the detector cell. The use of larger diameter and/or longer tubing may decrease peak resolution.

Each of the possible configurations offers multiple sampling option; however, a consistently reproducible quantitation and an absence of disturbing artifacts are achieved best using the "full loop" mode and in conjunction with a 25 µL loop (P/N 042857). Good reproducibility of retention times requires the use of temperature controlled modules from Dionex and applications of the exact settings described in the following sections of this manual.

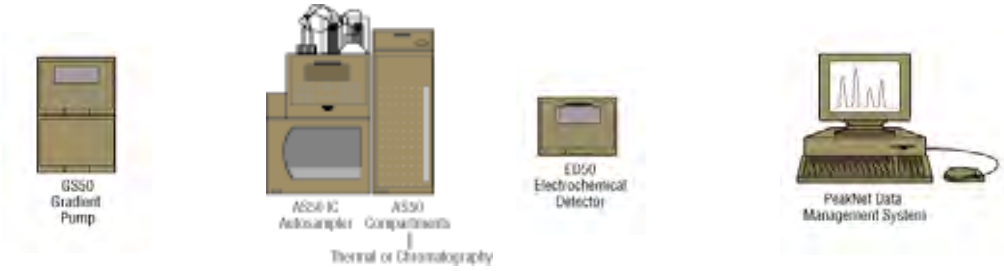


Figure 1 Carbohydrate System Configuration

2.2 System Operation Requirements

The Dionex Carbohydrate systems should be configured to comply with the following key requirements:

1. Mobile phase components are kept under helium or nitrogen at all times
2. Online degassing of eluents
3. Accurate and precise flow rates at 0.5 mL/min
4. Ag/AgCl reference electrode
5. Programmable PAD waveforms with frequencies of 1 Hz or higher
6. Minimized contribution to the background signal by contaminants from the system and reagents
7. Column oven for constant temperature control of the guard column, separation column and detection cell.
8. The heat exchange coil in the AS50 thermal compartment must be 0.005" i.d. PEEK tubing (Dionex P/N 052311)

2.3 CarboPac PA200 Column Operational Parameters

pH range:	pH = 0–14
Temperature limit:	60 °C
Pressure limit:	3500 psi
Organic Solvent Limit:	100% Acetonitrile, methanol, acetone, if required for cleaning
Typical Eluents:	High purity water (18.2 megohm-cm), sodium hydroxide, sodium acetate

SECTION 3 – PURITY REQUIREMENTS FOR CHEMICALS

Obtaining reliable, reproducible and accurate results requires eluents that are free from impurities and prepared only from the chemicals recommended below. Dionex cannot guarantee proper column performance when alternate suppliers of chemicals or lower purity water are utilized.

3.1 Deionized Water

The deionized water used to prepare eluents should be Type I reagent grade water with a specific resistance of 18.2 megohm-cm. The water should be free from ionized impurities, organics, microorganisms and particulate matter larger than 0.2 µm. The availability of UV treatment as a part of the water purification unit is recommended. Follow the manufacturer's instructions regarding the replacement of ion exchange and adsorbent cartridges. All filters used for water purification must be free from electrochemically active surfactants. Expanding their period of use beyond the recommended time may lead to bacterial contamination and as a result, a laborious cleanup may be required. Use of contaminated water for eluents can lead to high background signals and gradient artifacts.

3.2 Sodium Hydroxide

Use 50% w/w sodium hydroxide (Certified Grade, Fischer Scientific P/N UN 1824) for preparation.

3.3 Sodium Acetate

Dionex highly recommends the use of Dionex Sodium Acetate Reagent (P/N 059326) for carbohydrate analysis. However, anhydrous sodium acetate from Fluka Biochemika (MicroSelect, P/N 71183) is also adequate. Dionex cannot guarantee proper detection performance when different grades or alternate suppliers of sodium acetate are utilized.

SECTION 4 – BEFORE YOU START

4.1 The Most Important Rules

ALWAYS	use 50% NaOH solution rather than NaOH pellets to make eluents
ALWAYS	use dedicated glassware and disposable glass or plastic ware for volume adjustments.
ALWAYS	keep your NaOH eluent blanketed with helium or nitrogen. Prepare new NaOH eluent if left unblanketed for more than 30 minutes.
ALWAYS	pull at least 40 mL of new eluent through the lines when changing eluent or adding fresh eluent. This will ensure that your fresh eluent is primed through the lines up to the pump heads.
ALWAYS	use 25 µL loop size; larger loops will cause loss of resolution.
NEVER	go to the next step of the installation if the previous step has failed.
NEVER	start an installation with any of the check list items below missing
NEVER	use 'communal' filtration units or filters made of unknown or unsuitable (cellulose derivatives, polysulfone) materials.
NEVER	use MeOH or other organic solvents as rinse fluid in the autosampler. Use only water, replaced daily.
NEVER	run above 60 °C or 3500 psi.

4.2 Initial Check List

These items MUST be available in your lab. The absence of any of these may compromise your analysis.

__ Laboratory water unit delivering 18.2 megohm-cm water at the installation site.

__ Vacuum pump available for use with the vacuum filtration units.

__ Inert gas cylinder (helium or nitrogen) with a regulator valve (ca. 0–200 psi at the low pressure side) and the appropriate size adaptors plus tubing.

__ Sterile-packed 10 mL and 25 mL disposable pipets and suitable pipeting bulbs or pumps.

__ Disposable, plastic (PE) large-size (at least 20 mL) syringe for priming the pump.

__ Plastic eluent bottles.

SECTION 5 – PREPARATION OF ELUENTS AND STANDARDS

NOTE

Always sanitize the entire analyzer with 2M NaOH prior to initial start-up (see Section 6) and after idle periods.

Obtaining reliable, consistent and accurate results requires eluents that are free from ionic and electrochemically active impurities. Chemicals and deionized water used to prepare eluents must be of the highest purity available. Maintaining low trace impurities and low particle levels in eluents also help to protect your ion exchange columns and system components. Dionex cannot guarantee proper column performance when the quality of the chemicals, solvents and water used to prepare eluents is substandard.

5.1 Eluent E1: Deionized Water

Vacuum degas the water by placing the eluent reservoir in a sonicator and drawing a vacuum on the filled reservoir with a vacuum pump. Vacuum degas the reservoir for 5–10 minutes while sonicating. Cap each bottle and minimize the length of time the bottle is opened to the atmosphere. Online degassing is supported through the use of the GP40, GP50 and GS50 gradient pumping systems and the IS20 and IS25 isocratic pumping systems.

5.2 Eluent E2: 200 mMSodium Hydroxide

The first step in the preparation of sodium hydroxide eluent is to degas an aliquot (typically 900 mL) of the deionized water, as described above. To make 0.2 M NaOH, add 16 g (10.4 mL) of 50% (w/w) NaOH to the degassed deionized water by removing the NaOH aliquot from the middle of the stock solution where sodium carbonate is least likely to have formed. Do not pipet from the bottom where sodium carbonate precipitate may have fallen, and always discard the bottle of 50% sodium hydroxide when it reaches about 2/3 empty. Place the tip of the pipet containing the aliquot of NaOH about 1 inch below the surface of the water and dispense the NaOH. If done properly, without stirring, most of the concentrated sodium hydroxide will stay at 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. Remember to replace the cap to the 50% hydroxide bottle immediately as well. Mix the contents of the tightly sealed container holding the 1M hydroxide.

NOTE

DO NOT prepare NaOH eluents from sodium hydroxide pellets! The pellets are coated with a layer of carbonate.

Always degas and store NaOH eluents in plastic eluent bottles blanketed with helium or nitrogen to avoid carbon dioxide contamination from the air. Carbonate in the eluent can significantly reduce retention times for carbohydrates.

The eluents can be prepared by either weight or by volume. Using a volumetric pipet is more effective in preventing contamination than the weight method, but is less precise. For applications requiring less than or equal to 100 mM hydroxide, an on-line eluent generator may be used. Thus the preparation of caustic eluents may be avoided altogether. Table 1, below, lists the mass or volume, of NaOH (50% w/w) required in 1 L to make the listed concentrations. Decide which technique to use, mass or volume, and then always use the same methodology to ensure consistent chromatographic results.

The sodium hydroxide eluents used with the CarboPac PA200 column will readily absorb carbon dioxide, producing sodium carbonate. The presence of variable amounts of carbonate will lead to inconsistent retention times, therefore always degas the water prior to use, discard the 50% sodium hydroxide once it is 2/3 empty and keep the prepared eluent blanketed under an inert gas.

Table 1
Mass or Volume of NaOH Required to Make 1 L of Common Eluents

Eluent concentration	NaOH (g)	NaOH (mL)
0.1 M	8.0	5.2
0.2 M	16.0	10.5
0.3 M	24.0	15.7
0.4 M	32.0	20.9
0.5 M	40.0	26.1
0.6 M	48.0	31.4
0.7 M	56.0	36.6
0.8 M	64.0	41.8
0.9 M	72.0	47.1
1.0 M	80.0	52.3

5.3 Eluent E3: 100 mM Sodium Hydroxide

To make 0.1 M NaOH, add 8.0 g (5.2 mL) of 50% (w/w) NaOH to the degassed deionized water by removing the NaOH aliquot from the middle of the stock solution where sodium carbonate is least likely to have formed. Do not pipet from the bottom where sodium carbonate precipitate may have fallen, and always discard the bottle of 50% sodium hydroxide when it reaches about 2/3 empty. Place the tip of the pipet containing the aliquot of NaOH about 1 inch (2.54 cm) below the surface of the water and dispense the NaOH. If done properly, without stirring, most of the concentrated sodium hydroxide will stay at 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. Remember to replace the cap to the 50% hydroxide bottle immediately as well. Mix the contents of the tightly sealed container holding the 0.1 M hydroxide.

5.4 Eluent E4: 100 mM Sodium Hydroxide/ 1 M Sodium Acetate

To maintain baseline stability, it is important to keep the sodium hydroxide concentration constant during the sodium acetate gradient, because acetate has no buffering capacity at high pH. This is achieved by making the eluents as follows:

Eluent A: x mM NaOH
Eluent B: x mM NaOH, y mM NaOAc

To make one (1) liter of 0.1 M sodium hydroxide/ 1.0 M sodium acetate, dispense approximately 800 mL of DI water into a 1 L volumetric flask. Vacuum degas for approximately 5 minutes. Add a stir bar and begin stirring. Weigh out 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. Once 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 may take a while as the filter may clog with insoluble material from the sodium acetate. Using a plastic tip volumetric pipet, 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 about 1 inch under the surface of the acetate solution. The eluent should be kept blanketed under helium at 34 to 55 kPa (5–8 psi) at all times, and last about 1 week.

NOTE

Dionex recommends the use of dedicated glassware, pipets and filtration apparatus for exclusive use in the preparation of carbohydrate eluents.

5.5 Carbohydrate Sialylated N-linked Alditol Standard

The Dionex OligoStandard, Sialylalted N-Linked Alditols, P/N 043164 contains 25 nmol oligosaccharides purified from bovine fetuin. Dilute the standard prior to use, by adding a known volume of DI water (for example 1 mL). An injection of 25 µL will correspond to an injection of 625 pmol of the standard. Dionex recommends running this standard every time a new column is installed and subsequently anytime it becomes necessary to troubleshoot your system.

SECTION 6 – GETTING STARTED

6.1 Introduction To The Detection Methods

The carbohydrate oxidation at gold electrodes is made possible by a rapid sequence of potentials (waveform) adjusted between the working electrode (gold) and the reference electrode (Ag/AgCl). Resulting currents are measured by integration during a short time interval of the detection waveform. The standard, recommended carbohydrate waveform is shown in Table 2.

Table 2
Carbohydrate Quadruple Waveform

Time (sec)	Potential (V) vs. Ag/AgCl	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	

NOTE
Do not polish a new gold electrode prior to use. NEVER POLISH the disposable gold electrodes.

Refer to section 9 – Troubleshooting Guide of this manual for an overview of reconditioning techniques for gold working electrodes.

The reference electrode for the ED50 is a combination pH-Ag/AgCl electrode (P/N 044198). For carbohydrate analysis, this electrode is used in the Ag mode; for amino acid analyses it is used in the pH mode. Always verify the correct selection of reference electrode is made in the program file and on the ED50 module prior to turning the cell voltage on. The reference electrode selection is made in the Detail Menu Screen on the ED50 front panel. The ‘Ref’ display should read ‘Ag’.

It is advantageous to always have available at least one unused “known good” reference electrode. If stored in saturated KCl, a reference electrode can be kept for years with its reference potential virtually unchanged. In contrast, the reference electrodes mounted inside the electrochemical cell and exposed to flowing sodium hydroxide have only a limited lifetime of ca. 3 to 6 months. As a result of prolonged exposure to alkaline solutions, the 0.1 M KCl solution inside the reference electrode gradually becomes alkaline and the silver chloride layer on the Ag wire immersed into that solution either dissolves or converts to a mixture of silver oxide and silver hydroxide. As that happens, the reference potential shifts and becomes increasingly unstable. Shifting reference potential is experienced by the user either as an unusually high background or as a decrease in signal response. A combination of both effects is also possible.

CAUTION
Never leave a reference electrode inside a disconnected electrochemical cell.

A reference electrode can be irreversibly damaged by drying out. This happens most frequently by leaving the reference electrode inside a disconnected electrochemical cell. Always remove the reference cell from the electrochemical cell, when the system is not in proper use (i.e. cell inlet and outlet are not plugged or connected to a flowing eluent). After removal from the electrochemical cell, keep the reference electrode immersed in 3 M KCl solution (224 g KCl/L) at all times.

6.2 Sample Preparation

The CarboPac columns are strong anion exchangers. Thus, the normal caveats applicable to ion exchange chromatography apply to these columns. High salt concentrations in the samples should be avoided wherever possible. Special care should be taken with samples containing high concentrations of anions, which are strong eluents for the CarboPac columns (e.g. chloride, carbonate, phosphate, etc.). It is best to avoid extremes of sample pH (especially extremely acid samples). The presence of anionic detergents (e.g., SDS) in samples should be avoided entirely. Nonionic or cationic detergents may be acceptable in low concentrations. When using PED for detection, beware of high concentrations of electrochemically-active components (e.g., TRIS buffer, alcohols, and other hydroxylated compounds). Small amounts of organic solvents in the sample will not harm the column, although the organics may interfere with the chromatography or detection of the analytes of interest. If necessary, samples may be treated with reversed phase or ion exchange cartridges (such as the Dionex OnGuard cartridges) before analysis. However, because the CarboPac columns are extremely rugged, it is often worthwhile to analyze an aliquot of the sample directly, without any pre-column cleanup.

Matrix Interferent	Effect	Possible Removal
Hydroxylated compounds (e.g. Tris buffers, alcohols)	PED-active (interferes with carbohydrate detection)	Dialysis or dilution
Halides	Will bind to column, may affect retention time of analytes and interact with the gold electrode.	Dialysis, dilution, or solid-phase extraction using OnGuard Ag (silver) cartridge.
Amine-containing compounds (including proteins, peptides and free amino acids)	PED active	Solid-phase extraction using OnGuard A (anion-exchange). For inline use, the AminoTrap column is used for proteins, peptides and amino acids
Lipids	May foul column	Liquid-liquid extraction or supercritical fluid extraction.
Organic Solvents	May affect analyte retention and cause diminished electrode response	Solid phase extraction using OnGuard RP (reversed phase)
Anionic detergents (such as SDS)	Will bind irreversibly to the column	Solid phase extraction using OnGuard RP

Sample matrices in glycoprotein analysis can be greatly simplified by performing a Western blot and selectively removing the carbohydrates from the PVDF membrane-bound proteins. Please ask for Dionex **Technical Note 30**, "Monosaccharide and Oligosaccharide Analysis of Glycoproteins Electrotransferred onto Polyvinylidene Fluoride (PVDF) membranes," or retrieve it from our Web site at www.dionex.com.

SECTION 7 – INSTALLATION AND STARTUP

There are three distinct stages during an installation of a new carbohydrate system.

A. System configuration and start-up
B. Verification of system cleanliness
C. Verification of system functionality.

Make sure that each section passes before moving onto the next. If you are having problems, check the troubleshooting guide at the end of this procedure. If you are still having problems, call Dionex.

7.1 System Configuration and Startup

Configure the system with the AS50 autosampler on the left, the injection module in the middle and the pump on the right. The detector should be placed on top of the pump. Nitrogen or helium should be delivered to the eluent organizer with about 5–6 psi at each bottle. Make sure that the AS50 TC is plumbed with red (0.005" i.d., P/N 52310) tubing, NOT BLACK, and that extra care is taken to minimize dead volume (see section 6.2 below). Make all fluidic and electrical connections, but do not install the column yet. Instead install some backpressure tubing, such as a length of yellow (0.001" i.d.) tubing between the injector and detector cell inlet. Minimize the number of unions and the length of all the liquid lines. Tubing between the injection valve and the detector, on either side of the column, should be 0.005" i.d. PEEK tubing. The use of larger tubing will decrease peak resolution. Verify that the modules are communicating.

7.1.1 AS50 Thermal Compartment Modification for 2-mm Operation

NOTE
If you are using an AS50 with Thermal Compartment, read this section.

The Thermal Compartment (TC) sold with the AS50 and intended for use with carbohydrate systems is shipped pre-plumbed with black (0.010" i.d.) tubing in the L-box. This tubing in the L-box of the AS50TC is approximately 2 feet of standard bore tubing after the injection valve, but before the guard column. This tubing must be removed and replaced with the appropriate length of red (0.005" i.d.) tubing for the carbohydrate resolution to be optimal.

To confirm the tubing inside the L-box is really black, remove the top cover of the L-box (3 knurled screws) and find the “chase” or hole right near the bend, at the bottom of the assembly, where the tubing connects to the guard column(s). You will see 2 pieces of tubing near this “window,” if they are black, proceed as follows:

A. Remove the 3 Allen screws that secure the L-box to the Thermal Compartment, using a #3 metric Allen wrench.

B. Dismount the L-box from the Thermal Compartment and you will find the PEEK tubing running through a “groove channel”. The L-box has 2 groove channels, the longer one is for use with black PEEK tubing, and the shorter one is for the red PEEK tubing.

C. Remove the black tubing and attach 10–12 inches of red (0.005" i.d.). This length should be as short as possible but should be of sufficient length to bend into the thermal compartment and connect to the Rheodyne valve on one end and the guard column on the other end.

D. The groove channels have an outer diameter (o.d.) of 0.61". PEEK tubing has an o.d. of 0.62". This is so the PEEK tubing makes good contact with the metal body of the L-box in order to maximize thermal accuracy. The PEEK tubing must be fitted carefully into the groove channel so that it is seated completely. To complete this, you may need to use a flat-bladed screwdriver or similar tool to push the tubing down into the channel, be careful not to bend or otherwise distress the tubing any more than is necessary.

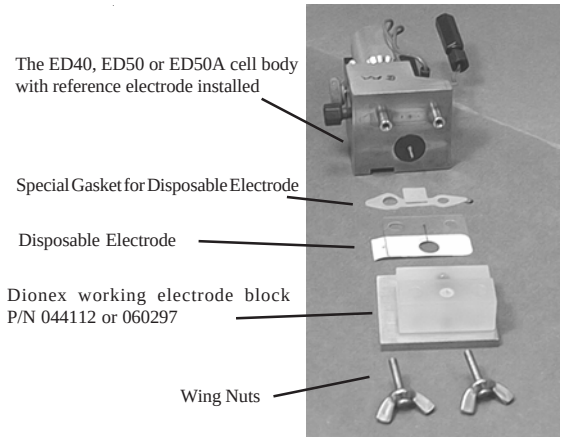
E. When completed, you should not see any tubing sticking up above or out of the groove. Remount the L-box and replace the cover, making sure that the cover is tightly secured, with no obvious gaps, again to ensure temperature stability.

F. Attach the end of the red tubing exiting the L-box to the inlet of the injection valve.

7.1.2 Installation of Disposable Electrodes

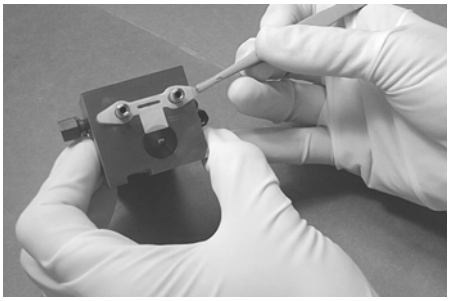
The Teflon gaskets included in each package of disposable electrodes must be used; otherwise, the disposable electrode product warranty is void. In addition, the quadruple waveform must be used for carbohydrate analysis otherwise the product warranty is void. Always wear gloves when handling electrodes. Never touch the electrode surface.

STEP 1
Check availability of all parts



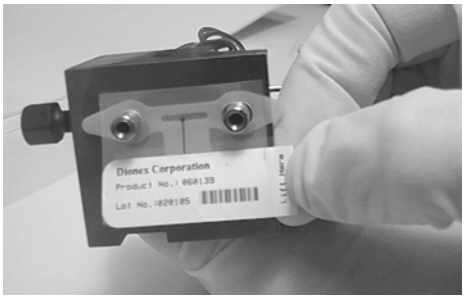
STEP 2
Install the special gasket.

Check that the gasket orientation is correct.
Avoid any wrinkles inside the sealing area of the gasket.



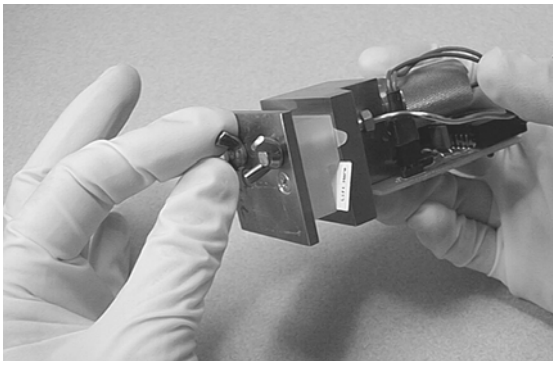
STEP 3
Install disposable electrode

Make sure the Disposable Electrode is oriented correctly.
The gold electrode must face the ED40, ED50 or ED50A cell body.



STEP 4
Place the working electrode block over the Disposable Electrode and tighten the wing nuts

Tighten the wing nuts evenly and “finger tight” only.
Do not use tools such as pliers.



7.1.3 System Rinse

NOTE
RINSE a new system with 2 M NaOH prior to use.
DO NOT install the CarboPac PA200 column before confirming that the background < 30 nC.

Prepare a solution of 2 M NaOH to rinse each bottle, by diluting 104 mL of 50% sodium hydroxide to 1 L with deionized water using the techniques described in section 4.2. Place the 2 N NaOH in a pre-rinsed bottle and place all 4 eluent lines in it. Withdraw at least 40 mL of sodium hydroxide from each line, using a syringe. Close the solvent draw-off valve and leave the pump proportioning 25/25/25/25 for 15 minutes. Make sure that all surface come into contact with the sodium hydroxide; rotate the injector valve. Repeat the process with 18.2 megohm-cm water.

7.2 Verification Of System Cleanliness

Prepare a new set of eluents as described in section 5.1, 5.2 and 5.3 and fill the eluent bottles. Set the eluent composition to 100% for each eluent line and draw out at least 40 mL of eluent from each eluent line.

7.2.1. System Background Check

This section is performed using the conditions of the test chromatogram. Make sure that

A. the cell is not yet on,
B. the pump is pumping 100 mM NaOH, 50 mM NaOAC at 0.5 mL/min,
C. a length of yellow tubing is installed between the injector and detector cell to generate ~1000 psi backpressure,
D. the columns are not yet installed.

Confirm that the pH reading on the detail screen of the detector is between 12.8 and 13.4. With the pH within this range, turn on the cell using the quadruple waveform in Table 1 (Section 6.1) and begin monitoring the background signal from the control panel for at least 30 minutes. Confirm that the baseline is < 30 nC. If the background > 30 nC or the pH is out of range, see the "Troubleshooting" section at the end of this manual.

7.2.2 Verification of Column Cleanliness

Install the CarboPac PA200 column set only after the initial system test (7.1.3. and 7.2.1) determines a background level within the specified range. A premature installation on a contaminated system will cause delays during the column equilibration.

The CarboPac PA200 is shipped in 100 mM NaOH/50 mM NaOAc. Any column that is stored long-term should be stored in the same solution. To prepare the column for standard analysis, the CarboPac PA200 must be washed for at least 20 minutes at 0.5 mL/min using 250 mM NaOAc in 100 mM NaOH. Following the wash, equilibrate the column using your starting conditions for 15–20 minutes. Next, switch your injection valve to LOAD (the loop now contains eluent equivalent to the initial conditions) and then connect the column to the cell.

Equilibrate the column set by performing two blank injections (DI water) under the test chromatogram conditions, including the column regeneration and re-equilibration steps. Should the background shift exceed 10 nC, perform the 2M NaOH wash as described in section 7.1.3.

Once the columns are equilibrated, inject a system suitability standard such as the Dionex OligoStandard, to establish the performance of the column at start-up. This chromatogram can then be referred to when troubleshooting your system. Once you obtain your expected chromatography, you are ready to proceed to running your application.

Dionex recommends that the system suitability standard be run whenever you reinstall a column after long-term storage.

SECTION 8 – APPLICATIONS

The CarboPac PA200 columns have been designed for isocratic or gradient separation using sodium hydroxide eluents up to a concentration of 1 M with sodium acetate gradients. Analyte separation is highly dependent on hydroxide concentration in HPAEC. Many separations require only an isocratic separation. However, some groups of analytes will require a step or gradient elution. Retention of carbohydrates can be varied with eluent concentration, in some cases changing the elution order as the eluent concentration increases.

Depending upon your system, you may have to make small adjustments to your gradient conditions or operating temperature to achieve resolution of all analytes.

8.1 Glycoprotein Characterization and Quality Control

Most secreted and cell surface proteins are glycosylated. They typically have multiple glycosylation sites, each site containing several structures. Therefore a protein can exist in many glycoforms. Glycans present on the protein can have a profound effect on the protein structure, and on biological function. Therefore the study of glycans is an important part of protein characterization. Biopharmaceuticals, including glycoproteins require thorough characterization and analysis to meet European, US and Japanese regulatory standards for New Drug Approval as defined by the International Conference for Harmonization (ICH) process. Even at clinical trial stage the national requirements for conduct of clinical studies necessitate clear identification, quality and purity of the investigational drug (FDA guidance documents for IND, MCA guidelines for CTX).

8.1.1 Fetuin Oligosaccharide Alditol Profiling

The high resolution of the CarboPac PA200 is exemplified in the following example. CarboPac columns separate mono-, oligo- and polysaccharides on the basis of fine structural differences in branching, linkage isomerism, anomericity and sialylation. In the fetuin oligosaccharide alditol standard shown below, peaks are separated according to branching, sialylation and linkage isomerism. The disialylated biantennary peaks are eluted before the trisialylated triantennary peaks which are eluted before the tetrasialylated tetraantennary peaks. In addition, within each grouping, the $\alpha_{2\text{-}6}$ isomer is eluted before, and well resolved from, the $\alpha_{2\text{-}3}$ isomer.

The Dionex OligoStandard, Sialylalted N-Linked Alditols, P/N 043164 contains 25 nmol oligosaccharides purified from bovine fetuin. Dilute the standard prior to use, by adding a known volume of DI water (for example 1 mL; a 25 µL injection corresponds to 625 pmol of oligosaccharide). Dionex recommends running this standard every time a new column is installed and subsequently anytime it becomes necessary to troubleshoot your system.

Columns:	CarboPac PA200 (3 x 250 mm) and CarboPac PA100 (4 x 250 mm)
Gradient:	PA200: 20–150mM NaOAc in 100 mM NaOH over 1 h PA100: 20–200 mM NaOAc in 100 mM NaOH over 1 h
Flow Rate:	 PA200: 0.5 mL/min PA100: 1mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode
Samples:	Fetuin oligosaccharide alditol stnd

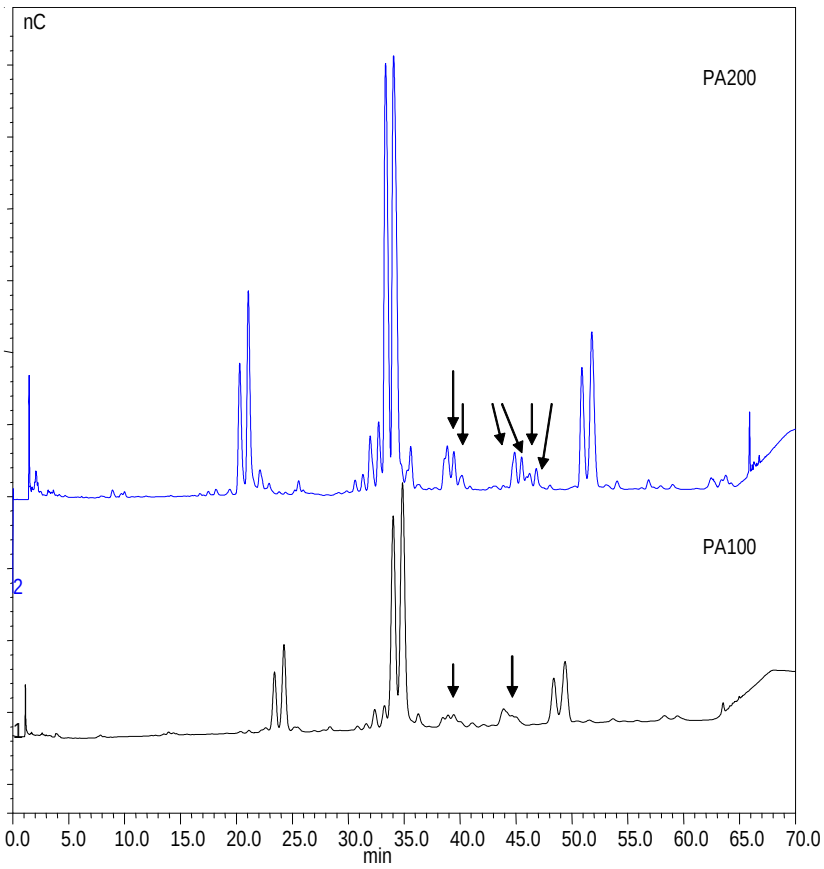


Figure 2
Fetuin Oligosaccharide Profiles: CarboPac PA200 vs. CarboPac PA100

8.1.2 Antibodies Separations

Today, monoclonal antibodies comprise the cornerstone of numerous medical and scientific procedures and thus are produced routinely for both medical and research applications. In the medical world, they are used as diagnostics, to detect cancers or infection by certain bacteria or viruses; as vaccines, to boost the body's immune response; and as therapeutics, to target foreign bacteria, viruses or cancerous cells.

Monoclonal Antibody Profile

Columns:	CarboPac PA200 (3 x 250 mm) and CarboPac PA100 (4 x 250 mm)
Gradient:	10 mM or 20 mM – 150 mM NaOAc in 100 mM NaOH over 60 min
Flow Rate:	
PA200:	0.5 mL/min
PA100:	1mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode
Sample:	48 h PNGase F digest of monoclonal antibody (100 µg)

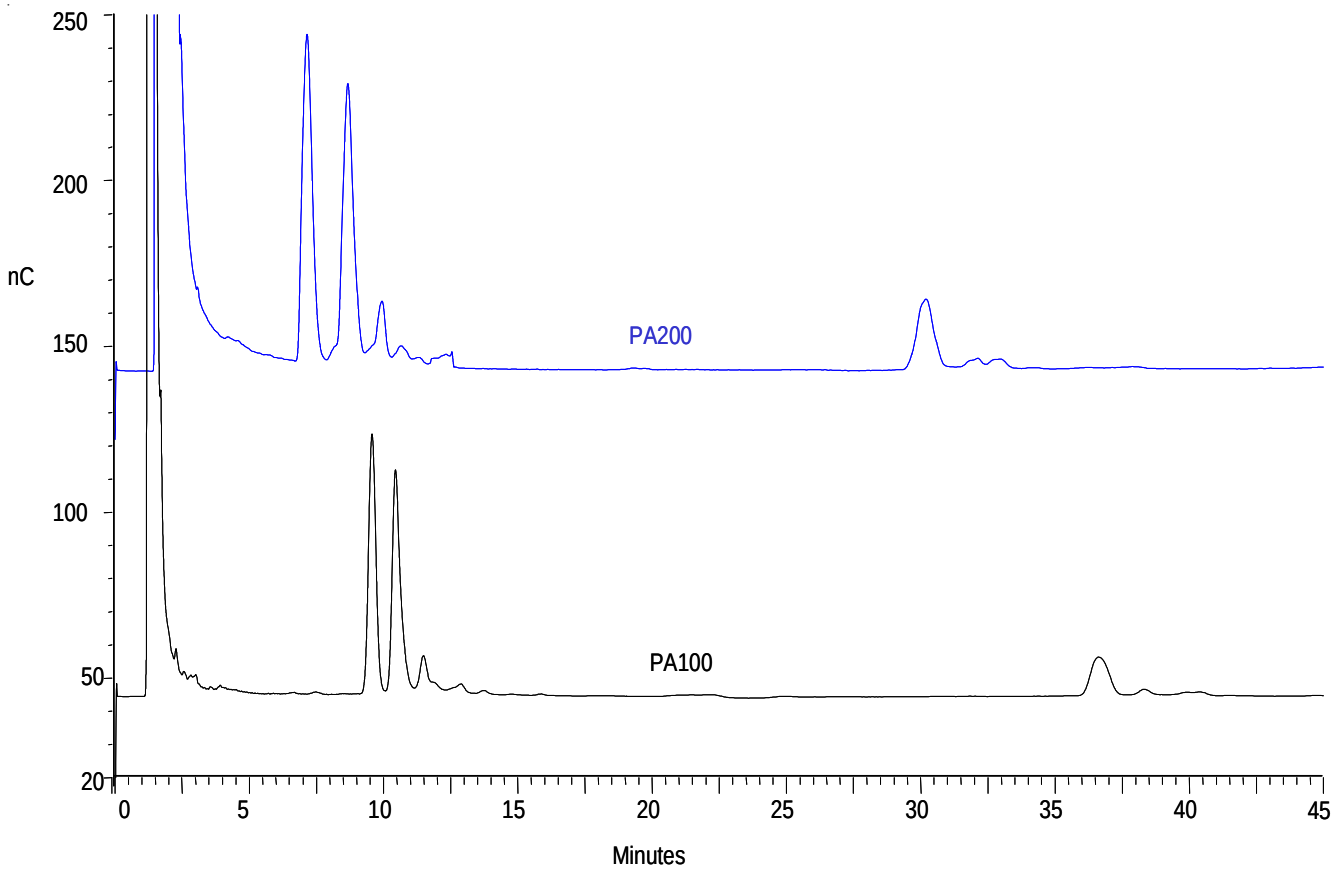


Figure 3
CarboPac PA200 vs. CarboPac PA100 Separation of Monoclonal Antibody N-linked Oligosaccharides

Polyclonal Antibody Profile

Column:	CarboPac PA200 and guard
Eluent:	Sodium acetate gradient in 100 mM sodium hydroxide
Flow Rate:	0.5 mL/min
Detection:	Pulsed amperometry, gold electrode
Sample:	PNGase-F digest of hIgG

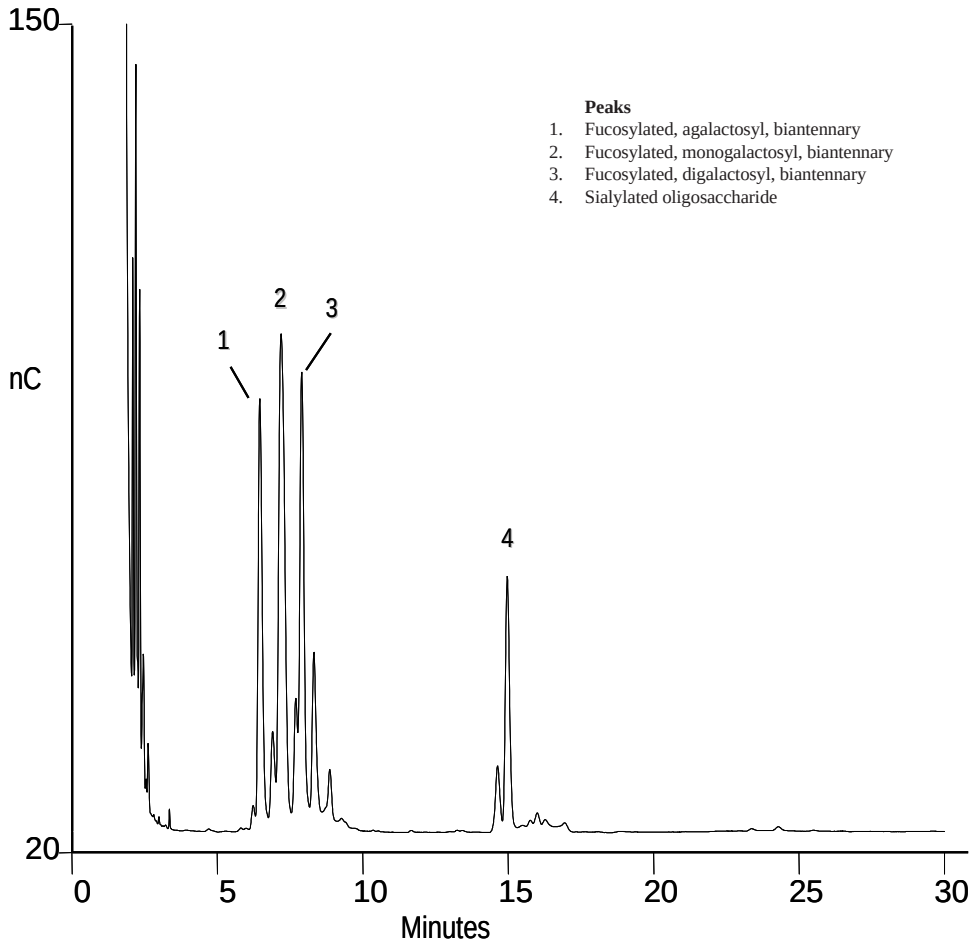


Figure 4
Polyclonal Antibody Profile

8.2 Profiling of Complex Plant Carbohydrates

8.2.1 Profiling of Inulins

Inulins and fructooligosaccharides (FOS) are increasingly being used as functional food ingredients. Chain length distribution profiles of commercial products such as those derived from inulin can be determined by using HPAE-PAD with gradient elution. By adjusting the initial gradient profile, smaller oligofructose chains can be distinguished from the inulin chains and separations upto and exceeding DP80 are possible.

Quantification of individual inulin oligomers requires a knowledge of the PAD response factors. These have been determined for the Fn and GFn oligomers from DP 2 to 8 and from DP 11 to 17, by isolation of 5–20 mg quantities of the pure oligomers using preparative scale RP-18 HPLC (see reference 9, section 8.6.2). Unfortunately, pure fractions in the range DP 6–10 could not be obtained due to coelution problems. For DP >17, the response factor appears to change very slowly with increasing DP, and relative response factors can be obtained by interpolation. Semi-preparative scale (9- and 22- mm i.d.) CarboPac PA1 and PA100 columns are available and could be used for isolation of mg quantities of all the inulin oligomers. A method that both identifies and quantifies FOS products, directly, is detailed in reference 8 (section 8.6.2).

Columns:	CarboPac PA200 (3 x 250 mm) and CarboPac PA100 (4 x 250 mm)
Gradient:	120–320 mM NaOAc in 100 mM NaOH over 40 min
Flow Rate:	
	PA200: 0.5 mL/min
	PA100: 1 mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode
Samples:	Inulin from chicory (Sigma)

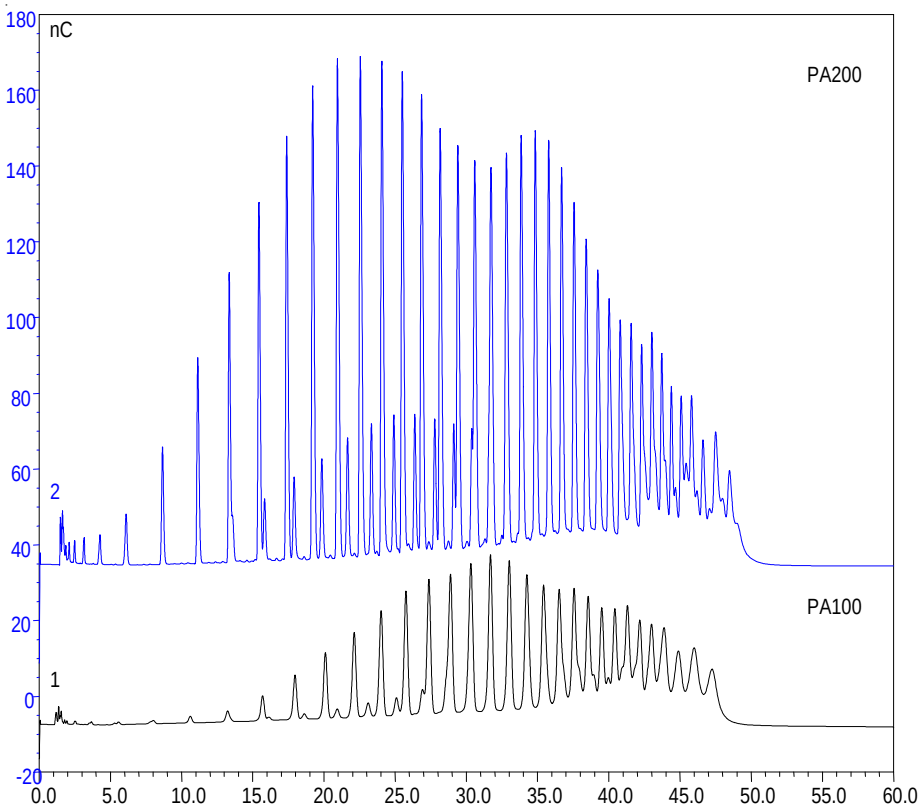


Figure 5
Inulin Profiles: CarboPac PA200 vs. CarboPac PA100

8.2.2 Amylopectins

HPAE-PAD with gradient elution has been used for structural studies on starch-derived materials such as amylopectins since the chain length distribution is an important parameter for characterizing the molecular structure. These distributions can be used as fingerprints for the amylopectin source.

Column:	CarboPac PA200 and guard
Eluent:	Sodium acetate gradient in 100 mM Sodium hydroxide 70 to 300 mM in 30 min
Flow Rate:	0.5 mL/min
Temperature:	30 °C
Injection Vol:	5 µL from 10 µL loop
Detection:	Pulsed amperometry, gold electrode
Sample:	Red Hook Amber Ale 1:50 dilution

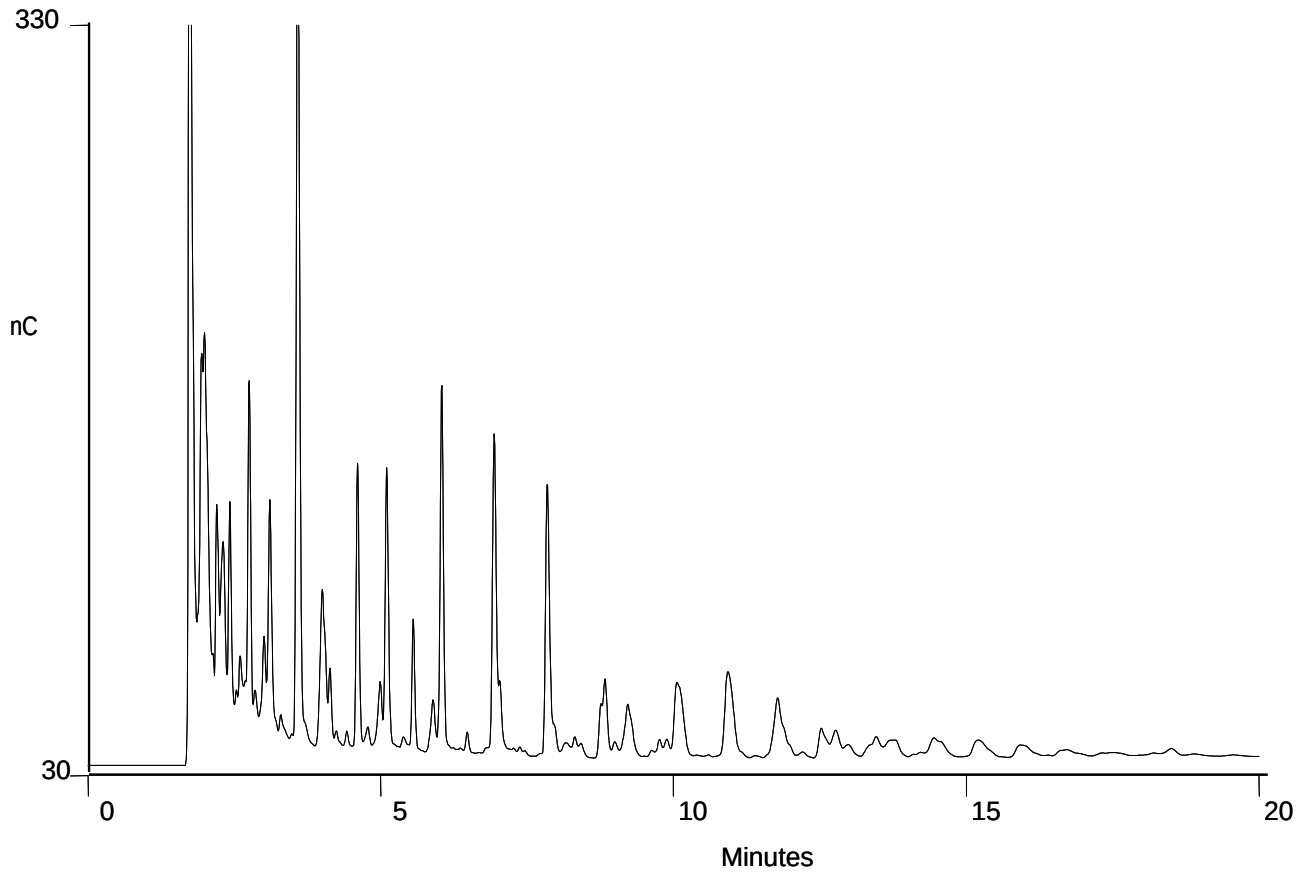


Figure 6
Amylopectins Separated on the CarboPac™ PA200 Column

8.3 Sialyl Lactose (N-Acetylneuraminyl-Lactose)

The following chromatography is used as the quality assurance test for the CarboPac PA200 column. Every CarboPac PA200 column is tested with respect to sialyl lactose peak efficiency. Chromatography of sialyl lactose on the CarboPac PA200 column exhibits greater than a twofold increase in peak efficiencies compared to chromatography of sialyl lactose on the CarboPac PA100 column.

Columns:	CarboPac PA200 (3 x 250 mm) or CarboPac PA100 (4 x 250 mm)
Gradient:	
	PA200: 100 mM NaOH, 50 mM NaOAc isocratic
	PA100: 100 mM NaOH, 100 mM NaOAc isocratic
Flow Rate:	
	PA200: 0.5 mL/min
	PA100: 1 mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode
Sample:	NanLac Std

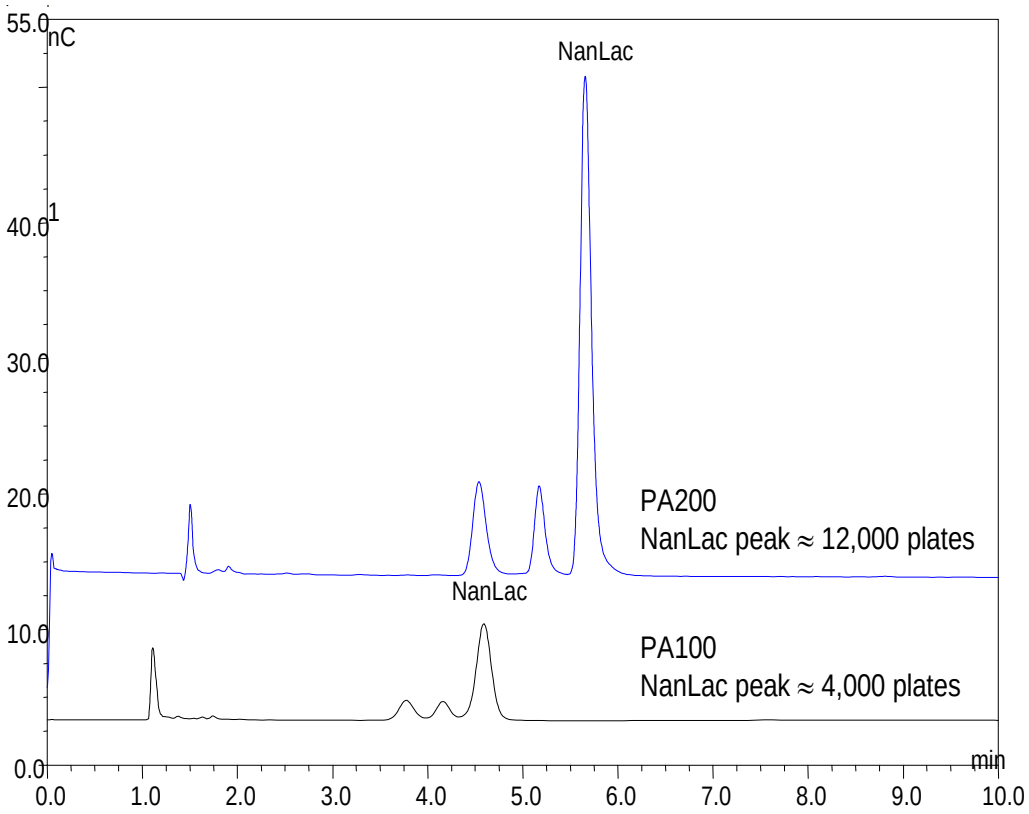


Figure 7
Chromatography of NanLac on the CarboPac PA200 exhibits > 2x the peak efficiencies compared to the CarboPac PA100

8.4 Separation of Mannose 7 D1, D3 Isomers

High mannose isomers that frequently would not be distinguishable by other techniques are well separated on both the CarboPac PA200 and PA100 columns. The separation of these isomers is better on the CarboPac PA200 column.

Columns:	CarboPac PA200 (3 x 250 mm) or CarboPac PA100 (4 x 250 mm)
Gradient:	0–200 mM NaOAc in 100 mM NaOH over 110 minutes
Flow Rate:	
	PA200: 0.5 mL/min
	PA100: 1 mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode
Sample:	Mannose 7 isomers (Dextra Labs):

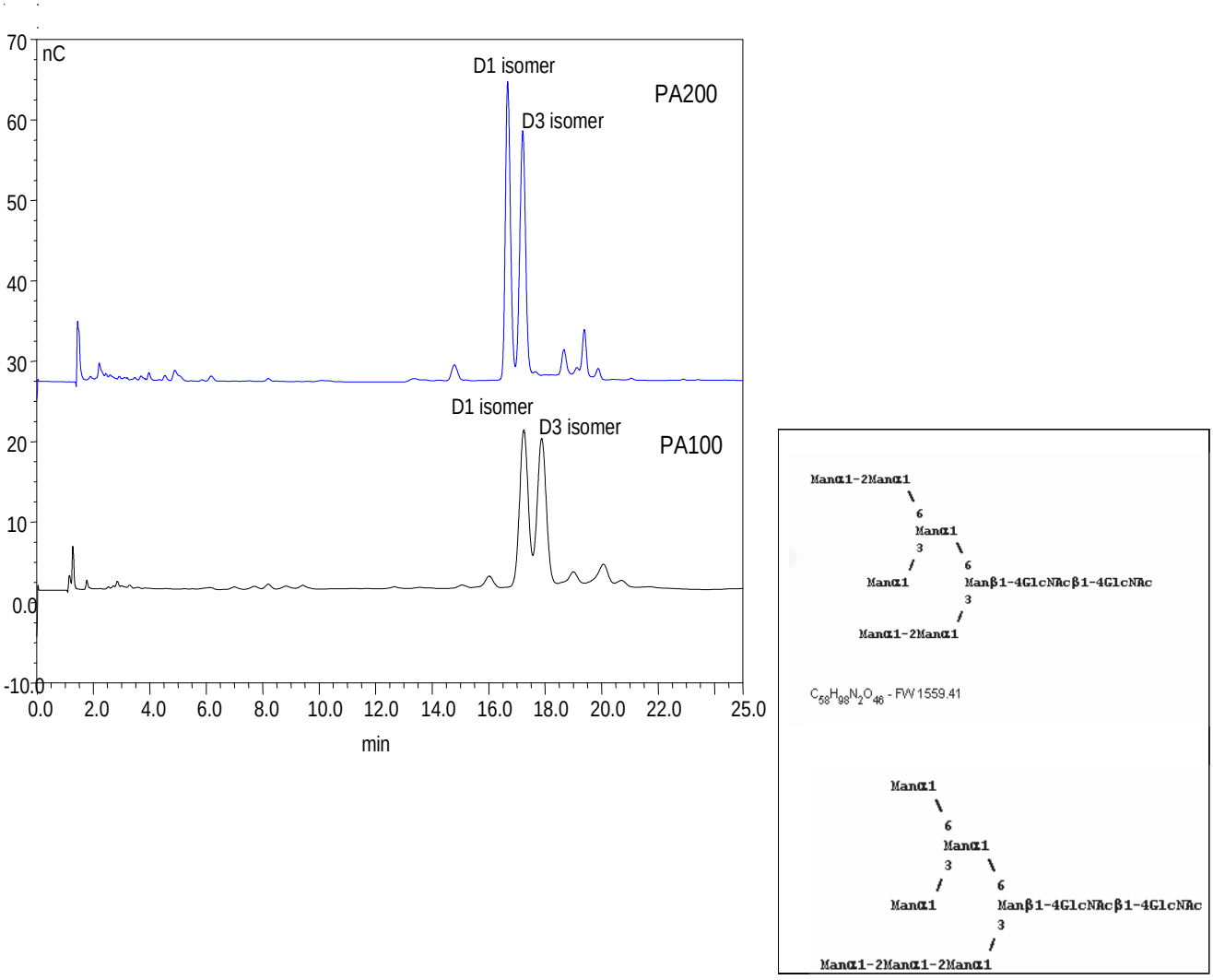


Figure 8
CarboPac PA200 vs. CarboPac PA100 Separation of Mannose 7 D1,7D3 Isomers

8.5 Separation of Neutral N-Linked Oligosaccharides

N-linked glycoproteins may exhibit micro-heterogeneity with respect to the N-linked oligosaccharides that may be present. Neutral N-linked oligosaccharides released from glycoproteins could be of the complex or high mannose variety. A comparison of chromatography of neutral and high mannose oligosaccharides on the CarboPac PA200 column compared to the CarboPac PA100 column shows that the separation window is wider for the CarboPac PA200 column. Early eluting oligosaccharides on the CarboPac PA100 column tend to elute earlier on the CarboPac PA200 column.

8.5.1 Complex Neutral Structures

Columns:	CarboPac PA200 (3 x 250 mm) or CarboPac PA100 (4 x 250 mm)
Gradient:	0–200 mM NaOAc in 100 mM NaOH over 110 minutes
Flow Rate:	
	PA200: 0.5 mL/min
	PA100: 1 mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode

Samples:

1. Asialo agalacto biantennary standard
2. Asialo galacto biantennary standard
3. Asialo galacto fuco biantennary standard
4. Asialo triantennary standard
5. Asialo tetraantennary standard

(All standards are from Dextra Labs)

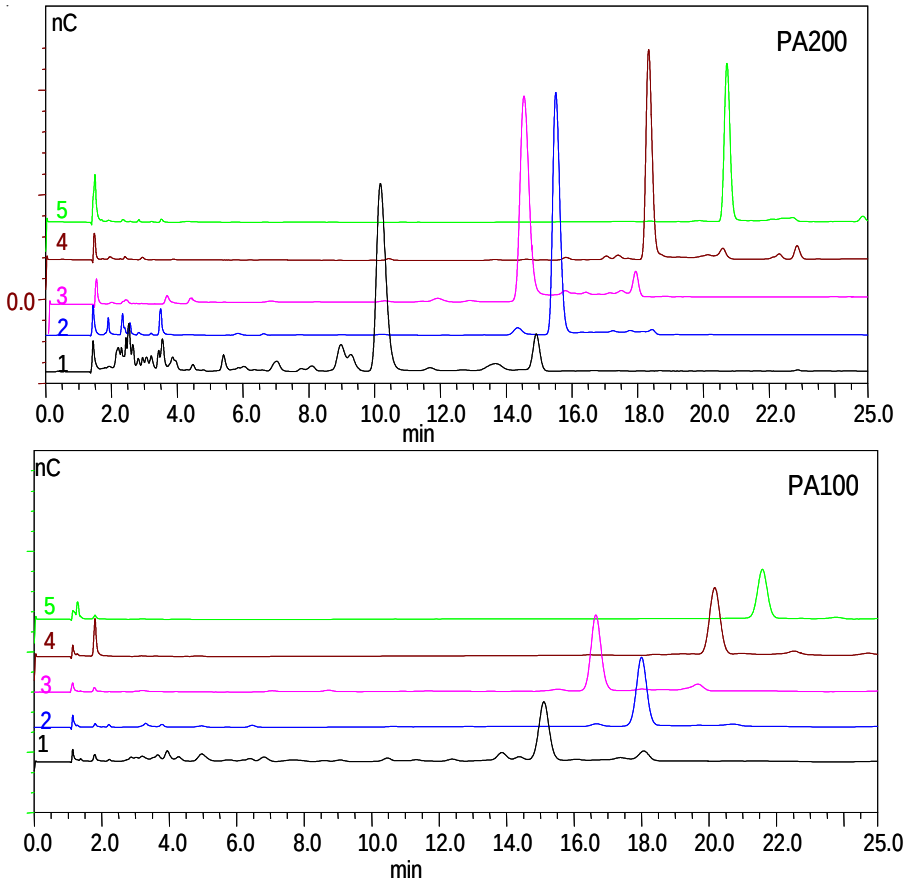


Figure 9
CarboPac PA200 vs. CarboPac PA100
Separation of Neutral N-linked Oligosaccharides (complex neutral structures)

8.5.2 High Mannose Structures

Columns:	CarboPac PA200 (3 x 250 mm) or CarboPac PA100 (4 x 250 mm)
Gradient:	0–200 mM NaOAc in 100 mM NaOH over 110 minutes
Flow Rate:	
	PA200: 0.5 mL/min
	PA100: 1mL/min
Detection:	Pulsed amperometry, QP waveform, gold electrode

Samples:

1. mann 3 standard
2. mann 5 standard
3. mann 6 standard
4. mann 7 standard
5. mann 8 standard

(All standards are from Dextra Labs)

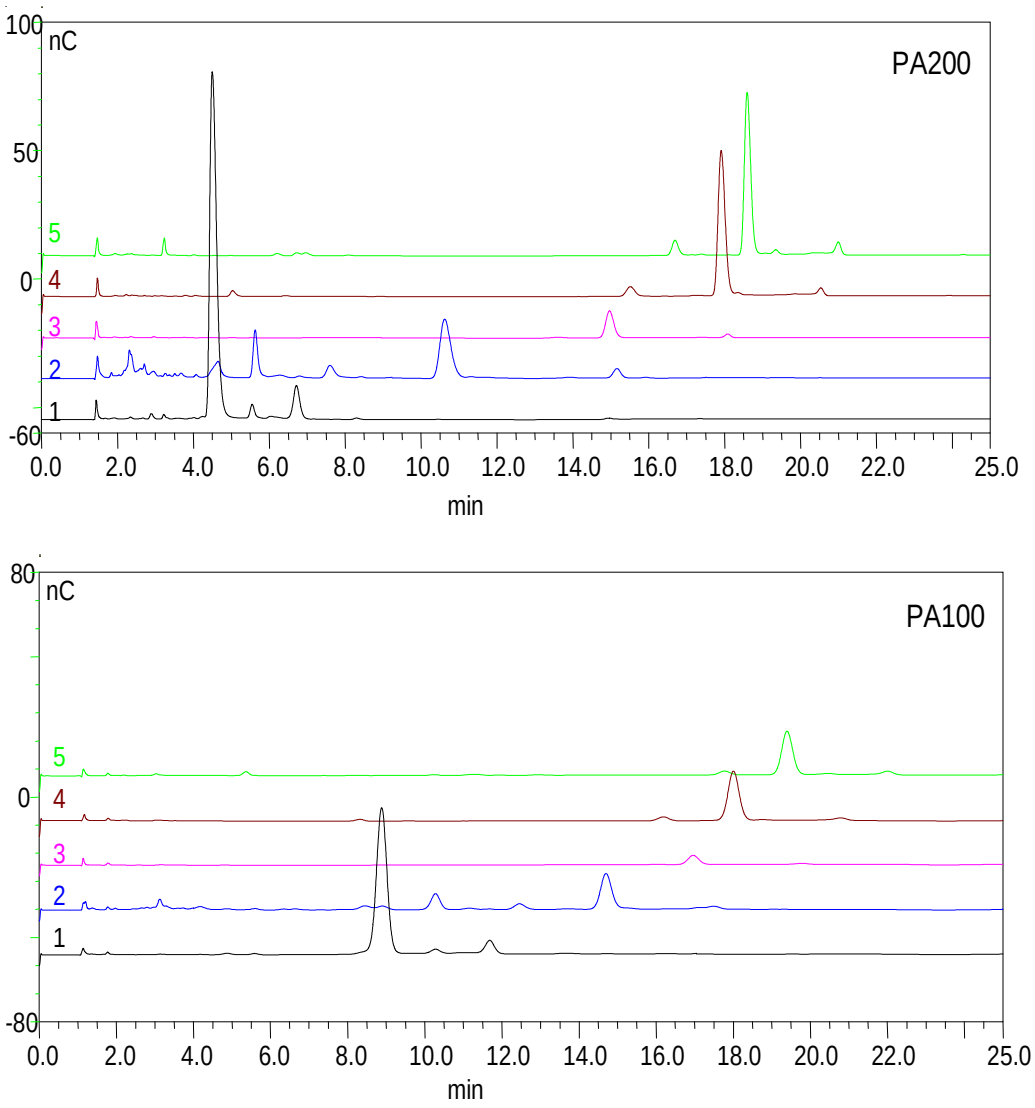


Figure 10
CarboPac PA200 vs. CarboPac PA100
Separation of Neutral N-linked Oligosaccharides (high mannose structures

8.6 HPAE-PAD Oligosaccharide Resources

This list is not intended to be comprehensive. In addition, none of these resources specifically uses the CarboPac PA200, which is a newly introduced column. However, these resources can be used to determine starting conditions for separations on the CarboPac PA200, with higher efficiencies and lower sodium acetate requirements.

8.6.1 Basic HPAE-PAD Resources - Dionex Applications

TN20	Analysis of Carbohydrates by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)
TN21	Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector
TN30	Monosaccharide and Oligosaccharide Analysis of Glycoproteins Electrotransferred onto Polyvinylidene Fluoride (PVDF) Membranes
TN36	Analysis of Exoglycosidase Digestions of N-Linked Oligosaccharides Using HPAE-PAD
TN42	Glycoprotein Oligosaccharide Analysis Using High-Performance Anion-Exchange Chromatography
AN46	Ion Chromatography: A Versatile Technique for the Analysis of Beer
AN67	Polysaccharide Analysis: Maltodextrins, Dextrans, Inulin and Other Oligosaccharides
AN82	Analysis of Fruit Juice Adulterated with Medium Invert Sugar from Beets
AN105	Glycosylation Analysis of Human Serum Transferrin Glycoforms Using Pellicular Anion-Exchange Chromatography

8.6.2 HPAE-PAD Oligosaccharide Resources

1. Thayer, J., et al. (1998) *Anal. Biochem.*, 256, 207-216 (Online desalting Paper).
2. Field, M., et al (1996) *Anal. Biochem.*, 239 92-98 (HPAE-PAD and MS to monitor oligosaccharide degradation).
3. Rohrer, J., (1995), *Glycobiology.*, 5, 359-360, (Empirical relationship between structure and detection).
4. Rohrer, J. S. et al., (1995) *Glycobiology* 5, 391-395 (Sepn of Partially Desialylated Branched Oligosaccharide Isomers).
5. Cooper, G. A. et.al. (1995) *Anal. Biochem.* 226, 182-184 (Separation of Neutral Asparagine-Linked Oligosaccharides).
6. Weitzhandler, M., et al. (1994) *J. Pharm. Sci.*, 83(12), 1670-1675 (Analysis of Carbohydrates on IgG Preparations).
7. Hardy, M. R. et al. (1988) *Proc. Natl. Acad. Sci.* (USA), 85, 3289-3293 (Separation of Positional Isomers of Oligosaccharides and Glycopeptides).
8. Durgnat, J-M, Martinez, C. Determination of fructooligosaccharides in raw materials and finished products by HPAE-PAD. *Semin Food Anal* (1997), 2, 85–97.
9. Timmermans, J.W., van Leeuwen, M.M., Tournois, H., deWit ,D. and Vliegenthart J., Quantitative analysis of the molecular weight distribution of inulin by means of anion exchange HPLC with pulsed amperometric detection. *J. Carbohydr. Chem.*, (1994), 13(6), 881-888.

SECTION 9 – TROUBLESHOOTING GUIDE

Remember that some of the problems may be related to parts of your experimental protocol (sample contamination, imprecision during sample transfer, problems during peptide or protein hydrolysis, etc.). Make sure to follow the rules from Section 4.1 and to re-check all of the items from Section 4.2. The following text should help you to locate and eliminate problems traceable to the carbohydrate hardware and chemistries. It also provides a selection of cleanup and reconditioning procedures that have been found effective by many users.

9.1 High Background

While it may be possible to obtain reasonable performance even with elevated levels of detection background according to some requirements, high background frequently brings about an increased size of gradient artifacts and can be accompanied by a presence of ghost peaks. Detection sensitivity may also change suddenly when the detection background is too high.

A background >30 nC with 10 mM sodium hydroxide at 0.5 mL/min and 30 °C using the quadruple waveform shown in Table 1 indicates one of the following possibilities:

A. Incorrect detection parameters.
Verify that "Ag" is specified in detector screen 2. Check all values of waveform in detector screen 4 against those in Table 1. If the pH reading at 95/5 (%E1/%E2, i.e. 10 mM NaOH) is above 13.2 replace the reference electrode.

B. Compromised working electrode surface.
Briefly install a new working electrode and check the background as above, If the reading remains > 30 nC, remove the new electrode within 30 minutes and continue testing for column or system contamination. Otherwise continue with your work with the new electrode installed.

C. Column contamination.
Remove the column set from the system first and replace it with a length of yellow PEEK tubing, generating a pressure drop between 1000 and 2000 psi. If the background reading improves after the column is removed from the system, go to section 9.3.

D. Water contamination.
Prepare eluents using a fresh (previously unopened) bottle of Burdick and Jackson HPLC Grade water. If the background is reduced, investigate the source of contamination in the original source of water.

E. System contamination.
If the background remains high even with fresh water and without the column, carry out the 2 M sodium hydroxide rinse described in section 9.5.

9.2 Decreased Detection Sensitivity

Always confirm the loss of response by performing at least one injection of the system suitability standard mix as described in Section 7.4.2. This is to make sure that a decreased level of response is not being caused by system problems discussed in Section 9.4.2.

Any decrease in detection sensitivity means that the working electrode surface has been affected. The operator should install a new working electrode. Spare gold working electrodes should always be available in order to avoid unnecessary delays.

IMPORTANT
Never install a new electrode without an aggressive system cleanup (section 9.6).
The exceptions to this rule are described below.

Exception:

Check the pH reading in the Detail Screen of the ED50. If the value is out of range or >13.2, install a new reference electrode and then install a new gold working electrode (P/N 55832). The system cleanup is not necessary. The decrease in sensitivity was caused

by a gold-oxide-buildup on the electrode surface because the reference potential was too high. The non-disposable gold working electrode can be reconditioned by the repair polishing described in section 9.7.1.

After installing a new working electrode (with or without the complete system cleanup), confirm the normal detection sensitivity. Carry out the monosaccharide injection test, section 6.4.2. Should the response be too low (peak height < 6 nC for 10 µL injections), immediately remove the new working electrode from the system.

9.3 Column Problems

The guard column protects the main column not only from contamination but also from excessive pressure fluctuations caused by the instrument or by operator errors. Have the guard column installed at all times, disconnect it only during some of the testing described in this section, or when priming the pump to prevent accidental over pressure.

The column set is causing the high background if the background reading decreases after the column is replaced by a section of PEEK tubing, as described in section 9.1 c.

9.3.1 Column Set Causing High Background

Disconnect the cell from the system, remove the yellow tubing and reinstall the column set. Increase the column thermostat temperature to 40 °C. Run 2 M sodium hydroxide through the column (at 0.5 mL/min) for one hour. Reset the temperature to 30 °C, pump 10 mM sodium hydroxide through the column, connect the cell and apply the quadruple waveform. If the background remains high, remove the cell from the system again and rinse the column with 10 mM NaOH, 950 mM sodium acetate (5% E2, 95% E3) for at least four hours, and preferably overnight.

9.3.2 Excessive Gradient Rise

The magnitude of the gradient rise can be minimized by running high eluent strengths during the times when the system is not in use for sample or standard analysis. This will keep the column conditioned, free from carbonate buildup, and ready for analysis.

A. Make sure the gradient rise is not caused by the system and/or detector cell (see Section 9.4.1).

B. Increase column temperature to 40 °C and wash the guard and column with 100 mM NaOH, 950 mM sodium acetate for at least four hours (and preferably overnight). Run a blank gradient at 30 °C and if necessary repeat the 100 mM NaOH, 950 mM sodium acetate wash at 40 °C.

9.3.3 Peak Efficiency and Resolution are Decreasing

Always have a spare guard available.

Peak deformations may sometimes be caused by sample matrix.

A. Run a standard separation with the Guard column removed from the system. Install a new Guard column should the separation improve with the old Guard removed. It is common to replace the Guard column several times during the lifetime of the analytical column.

B. Verify that only the 0.005" i.d. (Red) tubing is installed for all connections between injector and detector.

C.

NOTE

If you are using an AS50 thermal compartment, be sure that it has been modified for 2-mm operation. See Section 6.1.1.

D. Verify that the shortest possible length of 0.010" i.d. tubing (black) is installed between the pump and injector.

E. Check for proper installation of ferrules on all PEEK tubing starting with the injector outlet and all other connectors to the ED50 cell inlet.

F. Check temperature settings in your method and/or actual temperature in your column oven.

G. The column may be overloaded. Try to inject a smaller amount of your sample or dilute the sample more.

H. If all of the above does not lead to an improved separation, the resin bed of the main column has been damaged and the main column must be replaced.

9.4 System Problems

9.4.1 High Detection Background Caused by the System

A. Verify the problem is neither the detector (see Section 9.1 a, b) nor column (see Section 9.1 c) related.

B. With injector, column and detector cell installed (cell voltage off) carry out the 2 M NaOH wash as described in Section 9.5.

C. Prepare new eluents.

D. Rinse all three eluent lines with the new eluents (at least 40 mL by priming syringe)

9.4.2 No Peaks, Poor Peak Area Reproducibility or too Small Peak Areas

A. Check the position and filling levels of sample vials in the autosampler.

B. Check injector needle-height setting.

C. Check each line of the schedule for proper injector parameters. Revert to full loop and 10 ?L sample loop size if using other injection modes (push or pull).

D. Service the injection valve (check for leaks, Tefzel fragments, or sediments inside the valve).

9.4.3 Large Baseline Dip in the Chromatogram

A large baseline dip appearing between 17 and 19 minutes when the guard column is installed is usually caused by oxygen in the sample injected. The ‘oxygen dip’ is normal and can be reduced in magnitude with higher NaOH concentration in the eluent.

9.4.4 Incorrect or Variable Retention Times

A. Check your eluent preparation procedure for possible errors.

B. Prime the pump if necessary.

C. Measure the flow rate by weighing out the eluent collected during exactly five minutes of flow. Recalibrate the pump if necessary.

D. Your sodium hydroxide eluent bottle contains too much carbonate and/or the re-equilibration period at the end of the gradient method is too short.

E. Set the eluent composition for 100% for each eluent and draw out at least 40 mL of eluent from each of the lines.

F. Samples containing high salt content (> 50 mM) will decrease the retention times.

G. Check and/or service the pump's proportioning valve. With the pumping turned off, the flow through the pump outlet tubing (disconnected from the injector) should be zero in all eluent positions. Check this separately for each eluent line at 100% setting.

9.4.5 Unidentified Peaks Appear with Expected Analyte Peaks

During the acetate or hydroxide gradient, a number of small peaks may appear. These peaks are usually due to trace contaminants in the water supply. The contaminants accumulate on the column during the isocratic section of the chromatogram and are released, frequently as irregular baseline deformations or sharp spikes, with the increasing eluent strength.

Some trace contaminants can co-elute with monosaccharides, compromising accuracy of quantitation at lower concentrations. If extraneous peaks are observed even after the water supply is excluded as a possible cause, clean the autosampler lines and sample loop. The autosampler should be cleaned using the following protocol:

A. Disconnect the column and detector cell from the autosampler.

B. Set the pump to 100% deionized water.

C. Place the following solutions in the autosampler and inject in sequence. Use 25 µL fill loop injections:
   1. 1 M NaOH
   2. Deionized water
   3. IPA
   4. Deionized water
   5. 1 M HCl
   6. Deionized water

9.5 Sodium Hydroxide Cleanup

The sodium hydroxide (2 M) rinse used to decrease column or system-related elevated background is essentially identical with the rinse performed during an installation of a new system, Section 6.1.3. Following the rinse, check the background again while pumping the 10 mM sodium hydroxide and repeat the rinse at least once if necessary. Leave the old gold working electrode in place during the first and second checking of the detection background. Use a new or reconditioned electrode only if the background remains high even after the second rinse. Should the new electrode also produce a reading of > 30 nC, remove it from the system within 30 minutes, rinse it with water and reinstall the old electrode. In case the repeated rinse does not lower the background, perform the nitric acid cleanup described in section 9.6. Then try the background with the old electrode first and if necessary only briefly with the new electrode again. In case the new electrode delivers < 30 nC, leave it in the system, and if non-disposable electrodes are used, recondition the old electrode using the chemical cleanup described in Section 9.7.3.

9.6 Nitric Acid Cleanup

Cleaning procedure for Severely Contaminated Carbohydrate Systems:

A. Stop the run if the system is running, turn off the detector cell voltage and stop the system pump.

B. Take 500 mL each of concentrated nitric acid (65–70%) and filtered, deionized water. Mix, gently in a very clean bottle (preferably eluent reservoir A of the carbohydrate system) to give 1 L or 1:1 diluted nitric acid solution.

CAUTION
Avoid Skin Contact with Nitric Acid

NOTE
Never filter nitric acid solution as it will dissolve the filter membrane.

C. Remove the CarboPac PA200 set from the system, disconnect and plug the detector cell to prevent drying out of the reference electrode chamber.

D. Replace the CarboPac PA200 column with yellow tubing to give a backpressure of 1000–2000 psi at 1.0 mL/min, then extend the yellow tubing by a length of green or black tubing to reach a waste container. During the nitric acid cleaning, the electrochemical cell is bypassed.

CAUTION
Make sure the nitric acid waste is handled properly

E. Throw out the water, sodium hydroxide and sodium acetate in reservoirs A, B and C. Rinse each bottle with deionized water at least three times. Rinse lines A, B and C with water thoroughly.

F. Equally distribute the dilute nitric acid solution into the pre-rinsed eluent reservoirs in lines A, B and C.

G. Pump 34%A/ 33%B/ 33%C at a flow rate of 1.0 mL/min for 10–14 hours (overnight) to clean the carbohydrate system.

H. During the rinse of step G, move the injection valve between load and inject at least three times. AS50:DETAIL STATUS menu: INJECT VLV, Select I, enter: Select L, enter (x3).

I. Stop the pump and remove the nitric acid from reservoirs A, B and C into a waste container.

J. Rinse each bottle including all of the surface of PTFE tubing inside the reservoirs with deionized water at least three times, by pumping 34% A/ 33% B / 33% C at a flow rate of 1.0 mL/min to remove the acid residue from the system.

K. Consider the rinsing as completer only if the pH at the waste outlet is about 5 i.e. approximately the same as that of the water in the reservoir containers. It may take more than 10 hours to rinse the acid out completely. If necessary, rinse overnight.

NOTE
Make sure that the pH of water in the eluent reservoirs A, B and C is >5 to avoid wasting time.

L. Replace the water in the eluent reservoirs with the carbohydrate system eluents (A; water, B: 200 mM sodium hydroxide, C: 1 M sodium acetate)

M. Connect the system pump, injection valve, yellow tubing and ED50 cell. Draw at least 40 mL from each of the eluent lines before starting the pump. Start the system pump and turn on the cell using the Ag-referenced waveform of Table 1. Wait until the background drops below 30 nC.

N. Stop the eluent flow, turn the cell voltage off. Remove the yellow tubing and replace it with a NEW CarboPac PA200 column set (guard and analytical column).

O. Start the system pump (initial conditions), turn on the cell voltage and wait for the background to drop under 30 nC again.

P. Run a series of blank runs, injecting 10 µL of clean water. The success of the nitric acid rinse is indicated by achieving a background < 30 nC in steps 13-15 and by the blank gradient rise not exceeding 10 nC between the initial level and the level of the cleanup step. Check also the detection response by injecting the mix of six monosaccharide standard as described in section 6.4.2

9.7 Reconditioning of Gold Electrodes

IMPORTANT

The following procedures apply only to non-disposable gold working electrodes. Do not recondition disposable electrodes.

9.7.1 Mechanical Polishing

A. Polish with coarse polishing compound (P/N 36319) as described in the Section 5.5.2 of the ED50 manual. Polish for several minutes with as much strength as you can sustain.

B. Apply several mL of water to a fresh polishing pad and 'polish' for one minute. This step removes the coarse polishing powder particles imbedded in the gold material.

C. Polish with fine polishing compound (P/N 36318) as described in Section 5.5.2 of the ED50 manual. Polish for at least 5 minutes with as much strength as you can sustain during the entire 1 minute.

D. Apply several mL of water to a fresh polishing pad and 'polish' for I minute. This step removes the fine polishing powder particles imbedded in the gold material.

E. Reassemble the ED50 cell and apply the Table 1 waveform under initial conditions. If necessary, wait for at least 24 hours for the response to stabilize. In many cases, it is useful to wait overnight.

Repeat the entire polishing procedure until the background drops below 30 nC, or glucose response increases above 6 nC.

9.7.2 Sanding of Receded Gold Working Electrodes

IMPORTANT

This entire procedure should be used only for seriously damaged or receded non-disposable gold working electrodes. Do not sand disposable gold electrodes.

A. Sanding off of the gold electrodes is always done with a subsequent coarse and fine polishing as described above.

B. The only reason to sand off an electrode id to make the gold electrode flush with the KELF surface.

C. Use a fresh 600-grit sand paper. Make sure that the KELF surface remains planar. If the surface is not planar, the ED50 cell will leak. The cell gasket will not have the required uniform seal around the entire flow path inside the assembled cell.

D. Sand for less than 1 minute (continuous sanding only to bring the KELF to the same level as gold), rinse off the powder residue with deionized water. Polish the rinsed electrode on a clean polishing pad (P/N 36121) with deionized water to remove last traces of the powder residue. Rinse the water again.

9.7.3 Chemical Reconditioning of Gold Working Electrodes

The chemical method of reconditioning removes chemical contamination from the non-disposable working electrode surface and restores the electrode performance. Disposable electrodes should simply be replaced. If the electrode has been passivated by excessive gold oxide formation (see, for example, section 9.1, too high reference potential), the chemical cleaning will not restore the electrode performance.

NOTE

Wear gloves and safety glasses whenever handling chromic acid solutions.

A. Preparation of Chromic Acid

Dissolve/suspend 1 gram of sodium chromate in 1 mL water in a 100 mL glass beaker, slowly add 10 mL of concentrated sulfuric acid with constant stirring. Store the solution in a suitable glass vessel. When used for the first time, transfer about 10 mL of chromic acid from the glass vessel into a 20 mL glass scintillation vial, then screw the cap on. The chromic acid solution can be returned to the close glass vessel and stored for future use.

WARNING
Chromic acid is corrosive and carcinogenic.
Follow all usual precautions and proper disposal procedures.

B. Reconditioning of Electrodes

NOTE
Before, during and after the reconditioning, avoid any skin contact with the gold electrodes.

Put the working electrode on a clean filter resting on a horizontal surface. Using a fresh glass transfer pipet, apply one or two droplets of chromic acid to the electrode surface. The chromic acid should form a hemisphere (approximately 2–3 mm in diameter) covering the entire gold surface and surrounding polymeric material. Leave the reagent in place for 10 minutes. Rinse the chromic acid off with DI water, then rinse the entire electrode with water again and dry it with a clean airflow.

9.8 Failed Reference Electrode

The first indication that a reference electrode has failed is a pH readout outside of the expected range of pH 12–13, or the absence of any readout on the ED50 display. A reference electrode can be irreversibly damaged by drying out. This happens most frequently by leaving the reference electrode inside a disconnected ED50 cell. Always remove the reference cell from the ED50 cell, when the system is not in proper use (i.e. cell inlet and out let are not plugged or connected to a flowing eluent). After removal from the ED50 cell, keep the reference electrode immersed in 3 M KCl solution (224 g KCl/L) at all times.

With a “known good” reference electrode it is possible to carry out one of the following checks of the reference electrode being used in the ED50 cell.

A. Immerse the “known good” reference electrode and the tested electrode into the same 0.1 M KCl solution. Using a voltmeter, measure the potential between the two electrodes. Discard and replace any tested electrode that differs by more than 30 mV from the “known good” Ag/AgCl reference.

B. Use the procedure in the ED50 manual to measure the potential difference between two reference electrodes immersed in the same 0.1 M KCl solution.

C. Simply replace the electrode you wish to check with a “known good” reference electrode inside the ED50 cell. Apply the voltage to the cell. Discontinue using the checked electrode if insertion of the “known good” electrode decreased the background from > 20 nC to < 20 nC.

NOTE
Immediately remove the “know good” electrode and store it properly. This referencing procedure will work as long as you do not leave your “known good” electrode inside the ED50 cell for more than a few hours at a time and store it properly (immersed in 3 M KCL) in the intervening periods of time.

SECTION 10 – INSTALLING A REFERENCE ELECTRODE

10.1 What Has Changed

The amperometry cell for the Dionex ED40, ED50 and ED50A detectors has been redesigned to facilitate installation of the reference electrode. Two new parts are included with the cell:

- A CHEMRAZ® O-ring (P/N 048410) (included in a bag labeled CHEMRAZ)
- An O-ring retainer (P/N 057192) (shipped in the reference electrode cavity)

NOTE

These instruction replace the reference electrode installation instructions in your detector operator's manual.

10.2 What Is Needed

To complete this installation procedure, you will need:

- A pair of tweezers
- Deionized water

10.3 Installation Procedure

Step 1

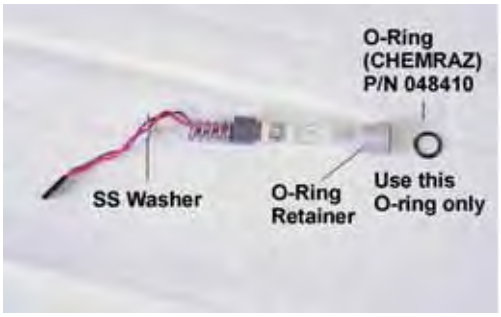
Remove the cell from the box. Loosen the cell cover thumbscrew and remove the cover. Unscrew the reference electrode cylinder and remove it from the cell body. NOTE: The photo shows the mounting plate for an AS50 autosampler compartment. Mounting hardware varies, depending on where the cell is installed.

Step 2

Remove the O-ring retainer from the reference electrode cavity. Verify that the stop ring is at the bottom of the reference electrode cavity. If you used the cell previously, rinse and dry the cavity to remove any particulate matter such as salt crystals, etc. Make sure that the inlet and outlet are open to avoid any hydraulic pressure buildup when inserting the reference electrode.

Step 3

Remove the reference electrode from its box. Remove the electrode from the storage bottle by partially unscrewing the bottle cap and pulling the electrode out of the opening in the cap. Rinse the electrode thoroughly in deionized water to remove any precipitated salt.



Step 4
Verify that you have all of the following parts:

NOTE:
The O-ring inside the storage bottle cap and the CHEMRAZ O-ring are made from different materials. To prevent leaks, use only the CHEMRAZ O-ring.

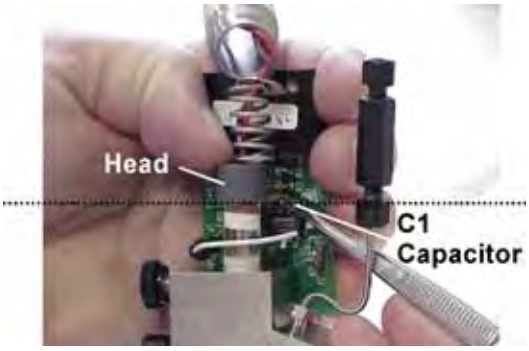
Step 5
Pull the J2 connector through the opening in the electrode cylinder.

Step 6
Slide the PEEK O-ring retainer and CHEMRAZ O- ring above the 1st rubber bushing.

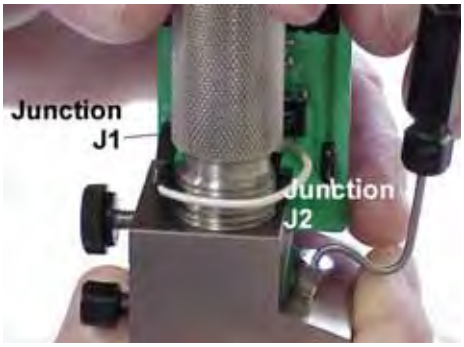
IMPORTANT:
While installing the electrode, maintain all parts in a vertical orientation, with the bottom of the electrode pointing down. This avoids bubble formation and helps ensure correct installation of the electrode.

Step 7
Carefully insert the reference electrode into the reference electrode cavity until it touches the stop ring. The 1st rubber bushing will be visible above the cell body.

Step 8
Using tweezers, slide the O-ring retainer and O- ring all the way down until there is about 2 to 3 mm between the upper edge of the O-ring retainer and the bushing.



Step 9
When the reference electrode is in the correct position, the lower edge of the head is roughly at the same height as the C1 capacitor on the electronics card.



Step 10
While still keeping all parts in a vertical orientation, screw the electrode cylinder into the cell body and finger tighten.

Step 11
Connect the J2 connector to junction J2 on the electronics card and verify that the white working

Step 12
Slide the cell cover back over the cell body, making sure that the cable connector on the end of the electronics card lines up with the opening in the cell cover. Tighten the thumbscrew.

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SECTION 11 – WAVEFORMS FOR CARBOHYDRATE ANALYSIS

Two waveforms have been developed for the ED40 for carbohydrate analysis. These are the triple potential waveform and the quadrupole potential waveform. The triple potential waveform uses a positive potential to clean the gold working electrode, while the quadrupole potential waveform uses a negative potential. Please refer to the ED50 manual for instructions on how to access these waveforms.

The triple potential waveform has been widely used since its introduction with the ED40 electrochemical detector (waveform B in Dionex Technical Note 21). However, because this waveform uses oxidative electrode cleaning (positive cleaning potential), the working electrode becomes eroded over time. This is accompanied by a gradual decrease in carbohydrate peak response. Quantitative analysis is still possible by using internal standards and regularly spaced injections of external standards.

NOTE

Never use the triple waveform with the disposable electrodes. The triple waveform with dramatically reduce the lifetime of the disposable electrodes.

A better alternative is to use the quadruple potential waveform (waveform A in Dionex Technical Note 21), which was developed to minimize electrode wear and to optimize long term reproducibility. Minor disadvantages of using this waveform include a slightly noisier signal and an increased sensitivity to dissolved oxygen. Oxygen causes a dip in the baseline as it passes through the detector. With the CarboPac PA1 this occurs between galactose and mannose. This effect is noticeable at low detection levels.

Dionex recommends using the quadrupole waveform in order to take advantage of its long-term detection stability. Both waveforms are pre-programmed into the ED50 detector. A more extensive discussion of waveforms for carbohydrate detection can be found in Technical Note 21, “Optimal Settings for Pulsed Electrochemical Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector.”

Triple Waveform			Quadruple Waveform		
Time (sec)	**Potential (V) vs. Ag/AgCl**	**Integration**	**Time (sec)**	**Potential (V) vs. Ag/AgCl**	**Integration**
0.00	+0.05		0.00	+0.1	
0.20	+0.05	Begin	0.20	+0.1	Begin
0.40	+0.05	End	0.40	+0.1	End
0.41	+0.75		0.41	–2.0	
0.60	+0.75		0.42	–2.0	
0.61	-0.15		0.43	+0.6	
1.00	-0.15		0.44	–0.1	
			0.50	–0.1	

Table 3
Comparison of the CarboPac PA100 and PA200 Columns

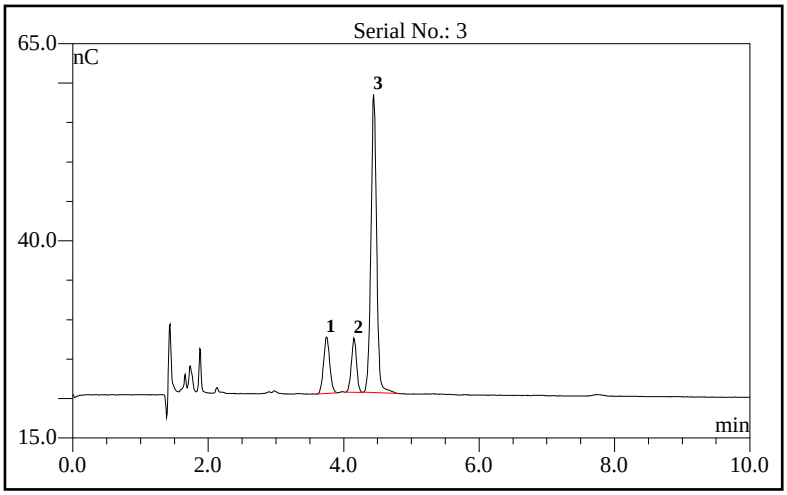
Characteristic	CarboPac PA100	CarboPac PA200
Recommended Applications	Oligosaccharide mapping and analysis	Higher resolution oligosaccharide mapping and analysis
Resin Composition	Pellicular. 8.5 µm diameter ethylvinylbenzene/divinyl benzene substrate agglomerated with 275 nm MicroBead quaternary ammonium functionalized latex	Pellicular. 5.5 µm diameter ethylvinylbenzene/divinyl benzene substrate agglomerated with 43 nm MicroBead quaternary ammonium functionalized latex
Substrate X-linking	55%	55%
Latex X-linking	6% cross-linked	6% cross-linked
AE Capacity (4 x 250)	90 µeq	35 µeq
Flow rate/min	1 mL (4-mm)	0.5 mL (3-mm)
pH Compatibility	pH 0–14	pH 0–14
Solvent Compatibility	0 < 90%	0–100%
Max. Backpressure	4000 psi (28 MPa)	3500 psi (22 MPa)

CarboPac™ PA200
Analytical (3 X 250 mm)
Product No. 062896

Date: 10-Mar-04 17:00
Serial No. : 000003
Lot No. : 014-01-086E

Flow Rate: 0.50 mL/min
Detection: ED50
Temperature: 30°C
Injection Volume: 10 µL
Diluted Standard: 12 nmol/mL in DI Water
Each analyte is 0.12 nmoles per injection.
Storage Solution: Eluent

Eluent Composition
%A: 100 mM NaOH + 50 mM Sodium acetate



Quadruple Waveform

Time	Potential	Integration
0.00	0.10	
0.20	0.10	Begin
0.40	0.10	End
0.41	-2.00	
0.42	-2.00	
0.43	0.60	
0.44	-0.10	
0.50	-0.10	

Reference Electrode: Ag/AgCl
Electrode Mode: Ag

No.	Peak Name	Ret.Time (min)	Asymmetry (EP @ 10%)	Resolution (EP)	Efficiency (EP)
1	Unknown	3.74	1.11	2.68	8076
2	alpha-(2,6)-NAN-Lac	4.15	1.01	2.05	14365
3	alpha-(2,3)-NAN-Lac	4.44	1.02	n.a.	14721

QA Results:

Analyte	Parameter	Specification	Results
alpha-(2,3)-NAN-Lac	Efficiency	>=10800	PASS
alpha-(2,3)-NAN-Lac	Asymmetry	1.00-1.54	PASS
alpha-(2,3)-NAN-Lac	Retention Time	4.17-4.83	PASS
	Pressure	<=2640	1740

Production Reference:

Datasource: CON_SQL_local
Sequence: CP_PA200
Sample No.: 1
6.50 SP3 Build 980