PRODUCT MANUAL

CarboPac PA20

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PRODUCT MANUAL

for the

CARBOPAC PA20 GUARD COLUMN
(3 x 30 mm, P/N 060144)

CARBOPAC PA20 ANALYTICAL COLUMN
(3 x 150 mm, P/N 060142)

CARBOPAC PA20 FAST SIALIC ANALYTICAL COLUMN
(3 x 30 mm, P/N 076381)

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TABLE OF CONTENTS

SECTION 1 – INTRODUCTION 5

1.1 CarboPac PA20 5

1.2 CarboPac PA20 Anion Exchange Columns 5

1.3 CarboPac PA20 Guard Column 5

1.4 CarboPac PA20 Fast Sialic Analytical Column 5

1.5 Borate Trap 6

1.6 AminoTrap (3-mm) 7

SECTION 2 – SYSTEM REQUIREMENTS 8

2.1 BioLC Carbohydrate System (Without Columns) 8

2.2 System Requirements 8

2.3 Disposable Gold Working Electrodes 9

2.4 Electrochemical Gold Cells 10

2.5 System Start-up 10
2.5.1 AS50 Thermal Compartment Modification for 2-mm Operation 10
2.5.2 System Rinse 11

SECTION 3 – OPERATIONS 12

3.1 CarboPac PA20 Column Operational Parameters 12
3.1.1 The Most Important Rules 12
3.1.2 Initial Check List 12

3.2 Purity Requirements for Chemicals 13
3.2.1 Deionized Water 13
3.2.2 Sodium Hydroxide 13
3.2.3 Sodium Acetate 13

3.3 Preparation of Eluents and Standards 13
3.3.1 Eluent E1: Deionized Water 13
3.3.2 Eluent E2: Sodium Hydroxide 13
3.3.3 Eluent E3: Sodium Hydroxide/Sodium Acetate 14

3.4 Carbohydrate Mix of Six Monosaccharides Standard 15

3.5 Sample Preparation 15

3.6 Introduction to the Detection Methods 15

3.7 Column Start-Up Requirements 16
3.7.1 Verification of System Cleanliness 17
3.7.2 Installation of the CarboPac PA20 Column 17

SECTION 4 – APPLICATIONS 18

4.1 Runtime Optimization 18

4.2 Common Monosaccharides from Mammalian Glycoproteins 19

4.3 Sialic Acids 20

4.4 Rapid Sialic Acid Analysis 21

4.5 Carbohydrates in Foods 22

4.6 Carbohydrates in Bio- and Pharmaceutical Products and Processes 25

SECTION 5 – TROUBLESHOOTING GUIDE 27

5.1 High Background 27

5.2 Decreased Detection Sensitivity 27

5.3 Column Problems 28
5.3.1 Column Set Causing High Background 28
5.3.2 Excessive Gradient Rise 28
5.3.3 Peak Efficiency and Resolution are Decreasing 28

5.4 System Problems 29
5.4.1 High Detection Background Caused by the System 29
5.4.2 No Peaks, Poor Peak Area Reproducibility or Too Small Peak Areas 29
5.4.3 Large Baseline Dip in the Chromatogram 29
5.4.4 Incorrect or Variable Retention Times 29
5.4.5 Unidentified Peaks Appear Alongside the Expected Analyte Peaks 30

5.5 Sodium Hydroxide Cleanup 30

5.6 Nitric Acid Cleanup 30

5.7 Reconditioning of Gold Electrodes 32
5.7.1 Mechanical Polishing 32
5.7.2 Sanding of Receded Gold Working Electrodes 32
5.7.3 Chemical Reconditioning of Gold Working Electrodes 32

5.8 Failed Reference Electrode 33

APPENDIX A – QUALITY ASSURANCE REPORT 34

APPENDIX B – GOOD PRACTICES FOR SUCCESSFUL HPAE-PAD 35

APPENDIX C – PAD POSITIVE COMPOUNDS VERSUS HYDROXIDE CONCENTRATIONS 37

APPENDIX D – INSTALLATION OF A REFERENCE ELECTRODE 38

APPENDIX E – INSTALLATION OF DISPOSABLE ELECTRODES 41

APPENDIX F – WAVEFORMS FOR CARBOHYDRATE ANALYSIS 42

APPENDIX G – HPAE-PAD CARBOHYDRATE RESOURCES 43

APPENDIX H – COMPARISON OF CARBOPAC COLUMNS 44

SECTION 1 - INTRODUCTION

1.1 CarboPac PA20

The CarboPac PA20 column has been developed to give fast, efficient separations of glycoprotein monosaccharides with good spacing of monosaccharides. These columns are recommended for monosaccharide composition analysis. When combined with the AminoTrap column they are the best choice for glycoprotein monosaccharide composition analysis because the problematic amino acids are moved out of the analysis, thereby eliminating working electrode poisoning. The CarboPac PA20 may also work well in combination with the AminoTrap and EG40 eluent generator for analysis of "wood" sugars.

The CarboPac PA20 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:	6.0 µm
Pore Size:	microporous (<10 Å)
Cross-linking:	55%
Ion exchange capacity:	65 µeq per column

Latex Characteristics:

Functional Group:	difunctional quaternary ammonium ion
Latex Diameter:	130 nm
Latex Cross-linking:	5.2%

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 megohm-cm), sodium hydroxide, sodium acetate

1.2 CarboPac PA20 Anion Exchange Columns

Part Number	Product Description
060142	CarboPac PA20 Analytical Column, 3-mm
060144	CarboPac PA20 Guard Column, 3-mm
076381	CarboPac PA20 Fast Sialic Analytical Column, 3-mm
060146	AminoTrap, 3-mm
047078	BorateTrap, 4-mm

1.3 CarboPac PA20 Guard Column

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.

1.4 CarboPac PA20 Fast Sialic Analytical Column

The CarboPac PA20 Fast Sialic Analytical column has been tested specifically for sialic acid applications. A guard column or AminoTrap column is not necessary for these applications.

1.5 Borate Trap

Borate can affect peak symmetry for some monosaccharides, even when present in the low part-per-billion concentration range. Borate is one of the first ions to break through a water deionization system. Its presence in the water that is used to make up eluents for carbohydrate analysis can cause a significant loss of peak efficiency, especially for mannose and reduced monosaccharides. The BorateTrap (P/N 047078) is used immediately before the injection valve and serves to remove borate from the eluent just before chromatography.

Column:	CarboPac™ PA10
Eluent:	18 mM NaOH, 10 ng/mL borate
Flow Rate:	1.5 mL/min
Detector:	Pulsed amperometry, gold electrode

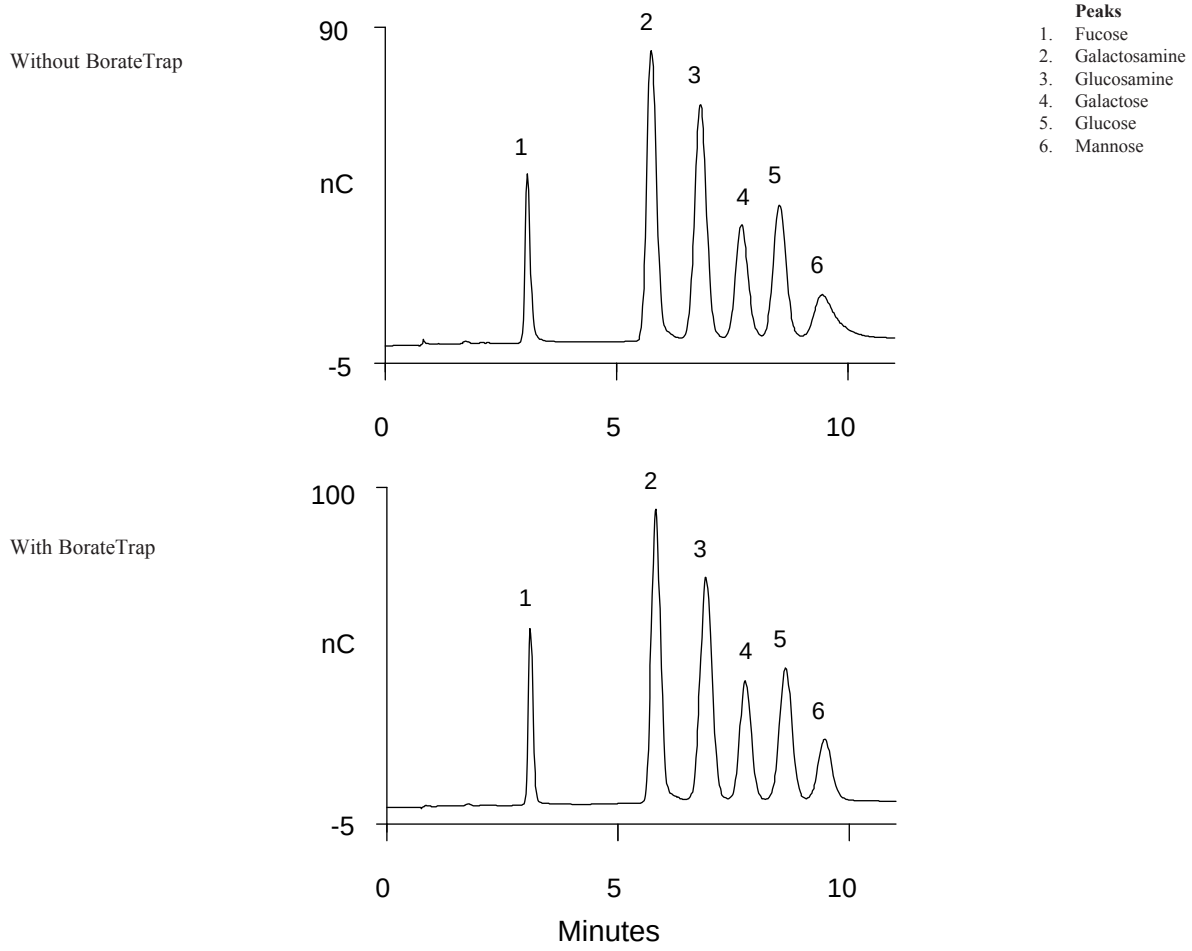


Figure 1
Effect of Borate and the BorateTrap™ on Monosaccharide Peak Symmetry

1.6 AminoTrap (3-mm)

If the samples are glycoprotein hydrolysates that have a high ratio of amino acids to carbohydrate, the AminoTrap (P/N 060146) column is the guard column of choice and replaces the standard guard column. The AminoTrap column will remove problematic amino acids from the carbohydrate elution window, reduce the working electrode poisoning, give cleaner chromatography of monosaccharides, and greatly reduce the need for correction factors.

Monosaccharide detection can be compromised by fouling of the working electrode from amino acids. This is especially apparent with amine-containing glycoconjugates with low levels of glycosylation. Lysine, which is eluted before galactosamine when the AminoTrap is not employed, tails on the gold electrode. The slow release of lysine's oxidation products inhibits detector response for later eluting monosaccharides. The AminoTrap resolves the quantitation problem by retaining lysine until after the monosaccharides have been eluted.

Sample Volume:	20 µL
Analytical Column:	CarboPac PA20 Analytical Column
Eluent:	12 mM NaOH
Eluent Flow Rate:	0.5 mL/min
Detector:	Pulsed electrochemical detection, Au electrode
Waveform:	Quadruple potential
Storage Solution:	18 mM NaOH

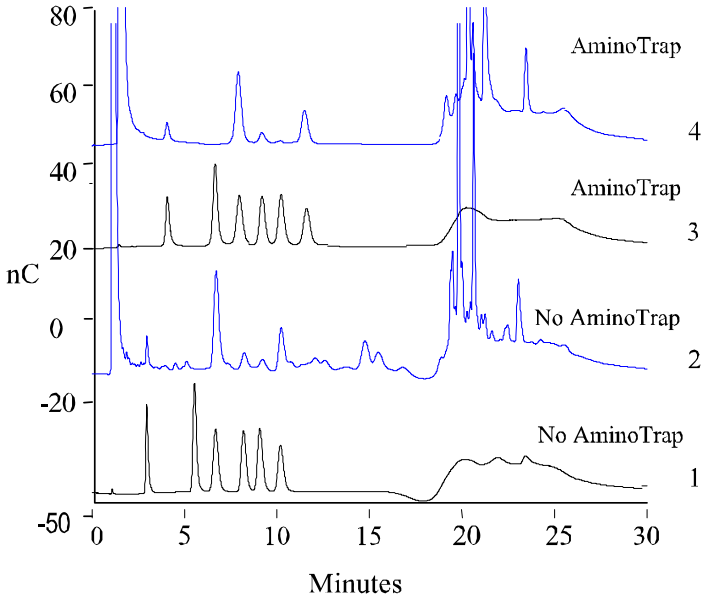


Figure 2
Profiling Mab Hydrolysate on CarboPac PA20 Column With and Without an AminoTrap

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, "DIONEX Worldwide Offices."

SECTION 2 – SYSTEM 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. On-line 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)

2.1 BioLC Carbohydrate System (Without Columns)

System Components 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 Stainless steel injection needle.
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

2.2 System Requirements

The carbohydrate separations with the CarboPac PA20 columns are optimized for use with DIONEX 2-mm systems, whether they are the 2-mm DIONEX DX-500, DX-600 or BioLC. They key issue is that 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.

In addition to the modules listed, the EG40 eluent generator (P/N 056971) can be included for CarboPac PA20 applications to deliver carbonate-free, high-purity hydroxide eluent on-line. This requires the EG40 vacuum degas conversion kit (P/N 055431) to ensure the degas unit in the pump sufficiently removes H_2 gas formed electrolytically by the EG40.

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

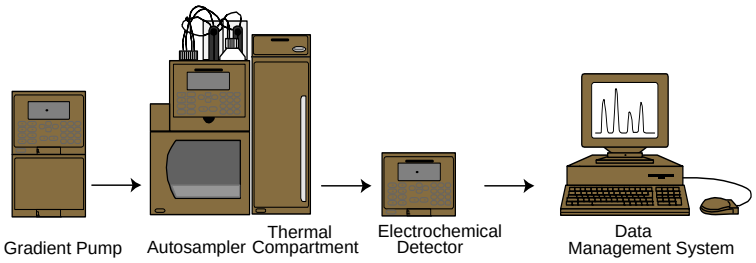


Figure 3
Carbohydrate System Configuration

2.3 Disposable Gold Working Electrodes

Carbohydrates separated by high pH anion exchange chromatography are detected by pulsed electrochemical detection and the signal is reported in coulombs (C). Electrochemical 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 DC amperometry. Pulsed amperometry and integrated amperometry employ a repeating sequence of potentials. Pulsed electrochemical detection at a gold working electrode is a reproducible and sensitive method for the detection of all carbohydrates of molecular weight up to ten-thousand.

Although carbohydrates can be oxidized at a gold working electrode, some 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 unnecessary. 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.

WARNING
Dionex Technical Note 21
You MUST USE the quadruple waveform (Waveform A) 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.

2.4 Electrochemical Gold Cells

Part Number	Product Description
060386	ED50 Electrochemical Cell with 6 disposable gold electrodes, for AS50
060385	ED50 Electrochemical Cell with 6 disposable gold electrodes, for LC25
060387	ED50 Electrochemical Cell with 6 disposable gold electrodes, for LC10/20/30
055290	Conventional ED50 Electrochemical Cell, Au, for AS50 autosampler
052556	Conventional ED50 Electrochemical Cell, Au, for LC25 chromatography oven
044108	Conventional ED50 Electrochemical Cell, Au, for LC10/20/30 chromatography ovens
060139	Disposable gold electrodes, 6 electrodes (6-pack)
060216	Disposable gold electrodes, 24 electrodes (4 bundled 6-packs)
044112	Conventional Gold Working Electrode for ED50 electrochemical detector
044198	pH-Ag/AgCl reference electrode

2.5 System Start-up

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 Thermal Compartment (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" ID PEEK tubing. The use of larger tubing will decrease peak resolution. Verify that the modules are communicating.

2.5.1 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 (black) 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 in order for the carbohydrate resolution to be optimal.

In order 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:

1. Remove the 3 Allen screws that secure the L-box to the Thermal Compartment, using a #3 metric Allen wrench.
2. 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.
3. Remove the black tubing and attach 10-12 inches of red tubing (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.
4. The groove channels have an outer diameter (OD) of 0.61". PEEK tubing has an OD of 0.62". This is so that the PEEK tubing makes good contact with the metal body of the L-box in order to maximize thermal accuracy. This means that the PEEK tubing must be fitted carefully into the groove channel so that it is seated completely. In order to complete

this, you may need to use a flat-bladed screwdriver or similar tool to push the tubing down into the channel, being careful not to bend or otherwise distress the tubing any more than in necessary.

5. When completed, you should not see any tubing sticking up above or out of the groove. Remount the L-box making sure the ends of the PEEK tubing are correctly directed through their inlets and outlets, and not crimped by the L-box when tightening. Replace the cover, making sure that the cover is tightly secured, with no obvious gaps, again to ensure temperature stability.

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

2.5.2 System Rinse

1. RINSE a new system with 2M NaOH prior to use
   DO NOT install the CarboPac PA20 column before confirming that the background < 30nC.

2. Prepare a solution of 2M 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.

3. 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.

4. Close the solvent draw-off valve and leave the pump proportioning 25/25/25/25 for 15 minutes.

5. Make sure that all surface come into contact with the sodium hydroxide; rotate the injector valve.

6. Repeat the process with 18 megohm-cm water.

7. Proceed with Section 3.

SECTION 3 - OPERATIONS

3.1 CarboPac PA20 Column and CarboPac PA20 Fast Sialic Column Operational Parameters

pH range:	pH = 0 - 14
Temperature limit:	60°C
Pressure limit:	3,500 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

3.1.1 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 ≤10 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 3,500 psi.

3.1.2 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 (for example, a 0-200 psi gauge on the low pressure side) and the appropriate size adaptors plus tubing.
- ☐ Mix of 6 carbohydrate standard
- ☐ 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

3.2 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.

3.2.1 Deionized Water

The deionized water used to prepare eluents should be Type I reagent grade water with a specific resistance of 18 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.

3.2.2 Sodium Hydroxide

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

3.2.3 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.

3.3 Preparation of Eluents and Standards

NOTE
Always sanitize the entire analyzer with 2M NaOH prior to initial start-up (see Section 2.5.2) 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.

3.3.1 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. On-line degassing is supported through the use of the GP40, GP50 and GS50 gradient pumping systems and the IS20 and IS25 isocratic pumping systems.

3.3.2 Eluent E2: Sodium 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 1M NaOH, add 80 g (52.3 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 and immediately blanket the eluent under 34-55 KPa (5-8 psi) of helium or nitrogen. 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 PA20 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)
0.1M	8.0	5.2
0.2M	16.0	10.5
0.3M	24.0	15.7
0.4M	32.0	20.9
0.5M	40.0	26.1
0.6M	48.0	31.4
0.7M	56.0	36.6
0.8M	64.0	41.8
0.9M	72.0	47.1
1.0M	80.0	52.3

3.3.3 Eluent E3: Sodium Hydroxide/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 0.1M sodium hydroxide/ 0.5 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 41.0 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

3.4 Carbohydrate Mix of Six Monosaccharides Standard

The Dionex MonoStandard, Mix of Six, P/N 043162 contains 100 nmol each of L-Fucose, D-Galactosamine, D-Glucosamine, D-Galactose, D-Glucose and D-Mannose. Dilute the standard by adding 1.0 mL DI water to the vial containing the monosaccharide standard. The concentration of each of the monosaccharides will then be 100 µM. Take a 100 µL aliquot of the diluted standard and add 900 µL to make a 10 µM solution. Inject 10 µL volumes to compare with the column test chromatogram, when installing a new column or troubleshooting a separation.

3.5 Sample Preparation

The CarboPac columns are strong anion exchangers. Thus, the sample matrix precautions applicable to ion exchange chromatography apply to these columns. High salt concentrations in the samples should be avoided where 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.). 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.

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 website at www.dionex.com.

3.6 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 5 – Troubleshooting Guide" of this manual for an overview of reconditioning techniques for conventional 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.'

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 approximately 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 3M KCl solution (224 g KCl/L) at all times.

3.7 Column Start-Up Requirements

Before installing the column, prepare a new set of eluents as described in section 3.3.1, 3.3.2 and 3.3.3. and fill the eluent bottles. Set the eluent composition to 100% for each eluent line and draw out at least 40mL of eluent from each eluent line. Perform the following background check.

3.7.1. Verification of System Cleanliness

This section is performed using the conditions of the test chromatogram (see Appendix A). Make sure that

1. the cell is not yet on
2. the pump is pumping 10mM NaOH, eg 95% A (DI water) and 5% B (200mM NaOH) at 0.5 mL/min
3. a length of yellow tubing is installed between the injector and detector cell to generate ~1000psi backpressure.
4. 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 3.6) and begin monitoring the background signal from the control panel for at least 30 minutes. Confirm that the baseline is <30nC. If the background >30 nC or the pH is out of range, see the "Troubleshooting" section at the end of this manual.

3.7.2 Installation of the CarboPac PA20 Column

Install the CarboPac PA20 column set only after the initial system test (3.7.1) determines a background level within the specified range. A premature installation on a contaminated system will cause delays during the column equilibration. The following procedure should be followed after any long term column storage (>1 week).

Remove the yellow tubing and install the CarboPac PA20, but do not connect it to the cell; instead send the effluent from the column straight to waste. The CarboPac PA20 column is shipped in 10 mM NaOH. To prepare the column for optimum performance, the CarboPac PA20 must be washed for 2 hours at 0.5 mL/min using 200 mM NaOH + 1M sodium acetate. If optimum performance is not required, 250mM NaOH for 2 hours should suffice. If using an eluent generator, rinse for 2h using 100mM KOH with the eluent line from the column directed back to the CR-ATC *Regen In* port.

Following the wash, equilibrate the column at 10 mM NaOH for 15 – 25 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. For eluent generator use, reconnect the eluent line from the cell back to the CR-ATC *Regen In* port. When using 10 mM NaOH, column washing with 200 mM NaOH for at least 10 minutes between analyses is recommended to remove carbonate. Analyses using higher NaOH concentrations may not require column washing. The use of an eluent generator can reduce column washing to 3 min with 100 mM KOH, with a periodic extended column wash for 2 hours as needed (e.g., every 24 h).

SECTION 4 – APPLICATIONS

The CarboPac columns have been designed for isocratic or gradient separation utilizing sodium hydroxide eluents up to a concentration of 1M. 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 sodium hydroxide concentration increases. If a stronger eluent is needed to elute the analytes, a sodium acetate gradient can be used.

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

4.1 Runtime Optimization

The CarboPac PA20 has been designed to give good resolution between the components in the Monostandard Mix of Six monosaccharides under a variety of sodium hydroxide conditions. These conditions can be optimized depending upon the goal of the separation. The higher the sodium hydroxide concentration, the faster the peaks will be eluted but the less the resolution between the peaks. This phenomenon is illustrated in the chromatograms below. It is important to remember that despite the fact that the peaks are separated isocratically, at low hydroxide concentration it is still necessary to regenerate the column in order to remove carbonate buildup which will shorten the peak retention times over time.

Column:	CarboPac PA20 Analytical Column
Gradient:	20 mM NaOH isocratic with a step to 200 mM NaOH at 10 min to regenerate the column.
Flow Rate:	0.5 mL/min
Temperature:	Ambient
Detection:	Pulsed electrochemical detection, Au electrode
Waveform:	Quadruple potential

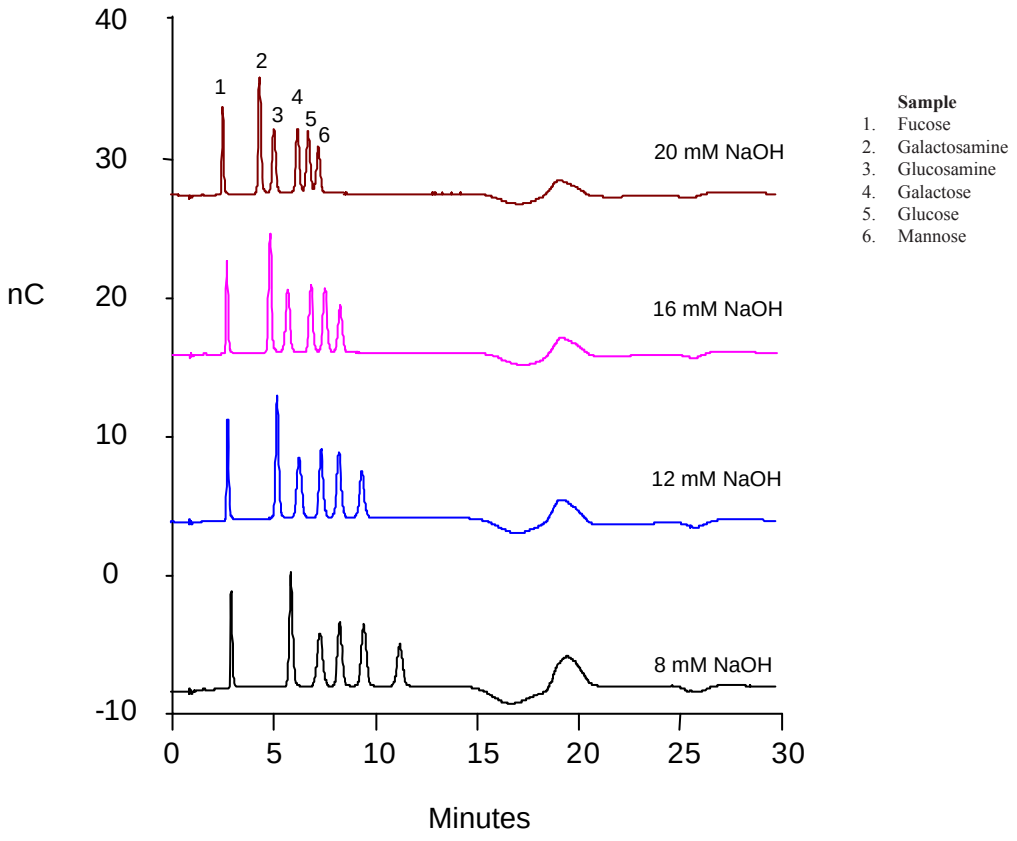


Figure 4
Runtime Optimization

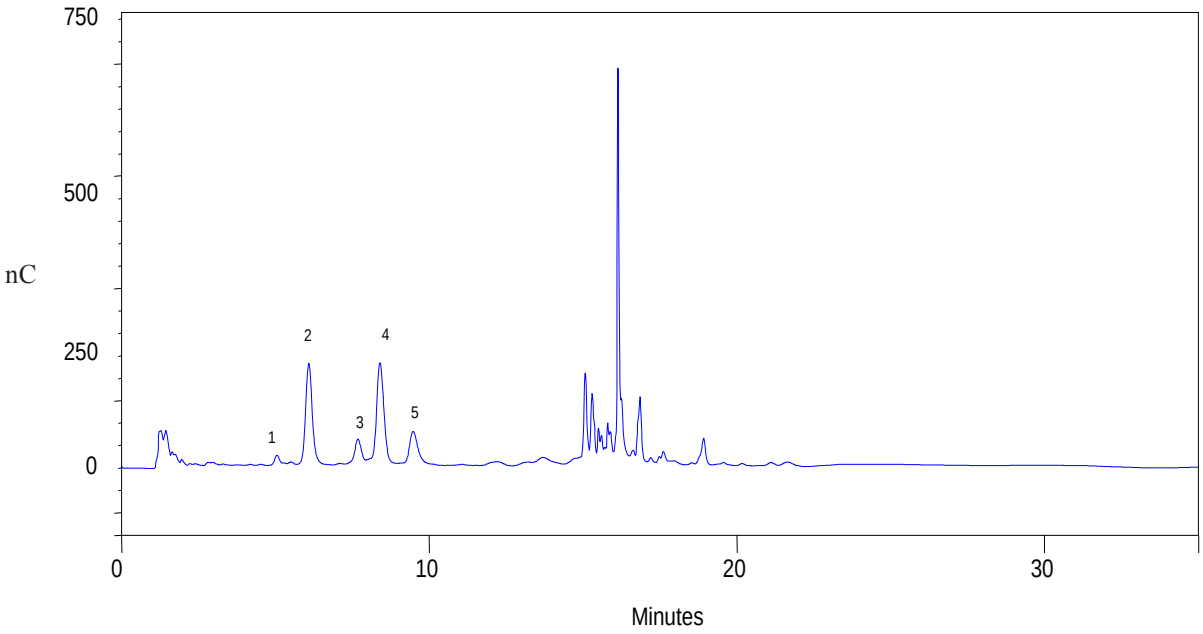
4.2 Common Monosaccharides from Mammalian Glycoproteins

Many mammalian proteins have carbohydrates attached to them. In many cases, the presence of the carbohydrate controls the biological activity of the protein or the rate at which it is cleared from the system. For example, certain glycosylated forms of tissue plasminogen activator (tPA) have more enzymatic activity than others. Erythropoeitin shows complex effects if the protein is deglycosylated or the glycosylation is altered. Failure of secretion from the body, decreased stability and decreased biological activity occurs if multiple glycosylation sites are eliminated. Desialylation and/or less branched oligosaccharides give increased activity in vitro, but decreased activity in vivo. Thus protein glycosylation is important to many scientists, including those making recombinant protein for therapeutic use.

The following conditions should be used for guidance. These conditions may be modified, as necessary, to suit your particular application needs.

Column:	CarboPac PA20 and Guard
Gradient:	12 mM NaOH, 200 mM NaOH regeneration for 6 min
Flow Rate:	0.5 mL/min
Detection:	Pulsed electrochemical detection, Au electrode
Waveform:	Quadruple potential
Sample:	30 µl reconstituted hydrolyzed serum

Peaks
1. Galactosamine
2. Glucosamine
3. Galactose
4. Glucose
5. Mannose



Sample Prep: 280 µL of 50% rabbit serum was hydrolyzed in 4M TFA for 4 h at 100°C. The sample was then speed vacuumed overnight to dryness and reconstituted in 560 µL of water and then filtered through a 0.45 µm nylon filter.

Figure 5
Analysis of Monosaccharides from Hydrolyzed Rabbit Serum

4.3 Sialic Acids

Sialic acids are a family of N- and O-substituted neuraminic acids that play an important role in physiology. They occupy terminal positions on many glycoproteins and serve as "markers" for protein removal from blood circulation. The amino group of neuraminic acid is linked to either an N-acetyl or N-glycolyl group which yields N-acetylneuraminic acid (Neu5Ac or NANA) or N-glycolylneuraminic acid (Neu5Gc or NGNA), respectively. Sialic acids are charged at neutral pH; at high pH many of the hydroxyl modifications are hydrolyzed and therefore cannot be analyzed using standard HPAE-PAD techniques. These substituted sialic acids can be separated at neutral pH and detected using PAD with the addition of post-column base. See Technical Note 41, "Analysis of Sialic Acids Using High-Performance Anion-Exchange Chromatography," for details on eluent preparation, protein hydrolysis and digestion conditions and the use of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN) as an internal standard for optimal performance.

The following conditions should be used as guidance. These may be modified, as necessary, to suit your particular application needs.

Column:	CarboPac PA20 and Guard
Gradient:	20 - 200 mM NaOAc in 100 mM NaOH over 10 min
Flow Rate:	0.5 mL/min
Detection:	Pulsed electrochemical detection, Au electrode
Waveform:	Quadruple potential
Sample:	NANA, NGNA

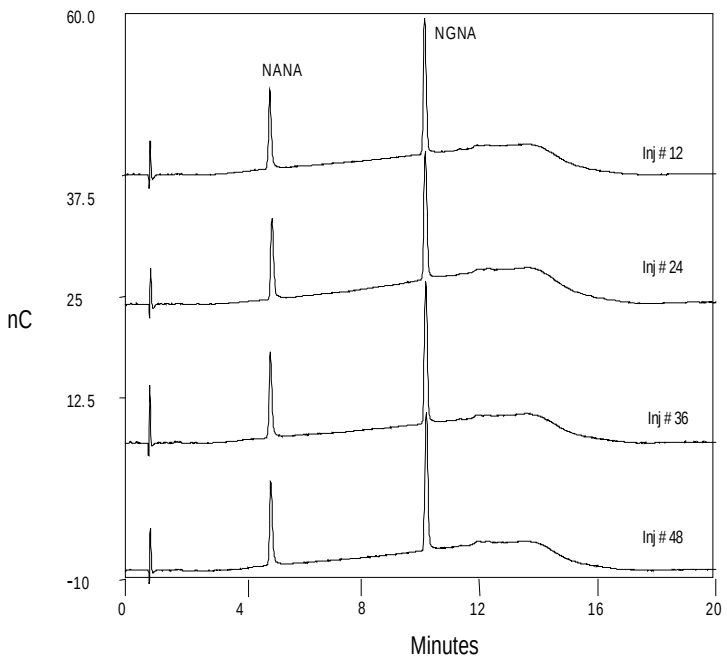


Figure 6
Sialic Acid Analysis using the CarboPac PA20

4.4 Rapid Sialic Acid Analysis

For rapid sialic acid determination in glycoprotein hydrolyzates, the CarboPac PA20 Fast Sialic column is recommended. This column requires no guard column. The conditions below were developed for acetic acid protein hydrolyzates and are presented for general guidance. They may be modified to meet the needs of other sialic acid determination applications.

Column:	CarboPac PA20 PA20 Fast Sialic Analysis Column
Gradient:	70-300 mM NaOAc in 100 mM NaOH over 2.5 min, 300 mM NaOAC in 100 mM NaOH for 0.4 min. 300-70 mM NaOAc in 100 mM NaOH over 0.1 min. 1.5 min of equilibration at 70 mM NaOAc in 100 mM NaOH.
Flow Rate:	0.5 mL/min
Injection Volume	4.5 µL, full loop
Detection:	Pulsed Amperometric Detection, disposable Au on PTFE electrode
Waveform:	Quadruple potential (Waveform A, Dionex Technical Note 21)
Sample:	A) calf fetuin, 31 ng of hydrolyzed protein on column B) sheep 1 -acid glycoprotein, 9.8 ng of hydrolyzed protein on column

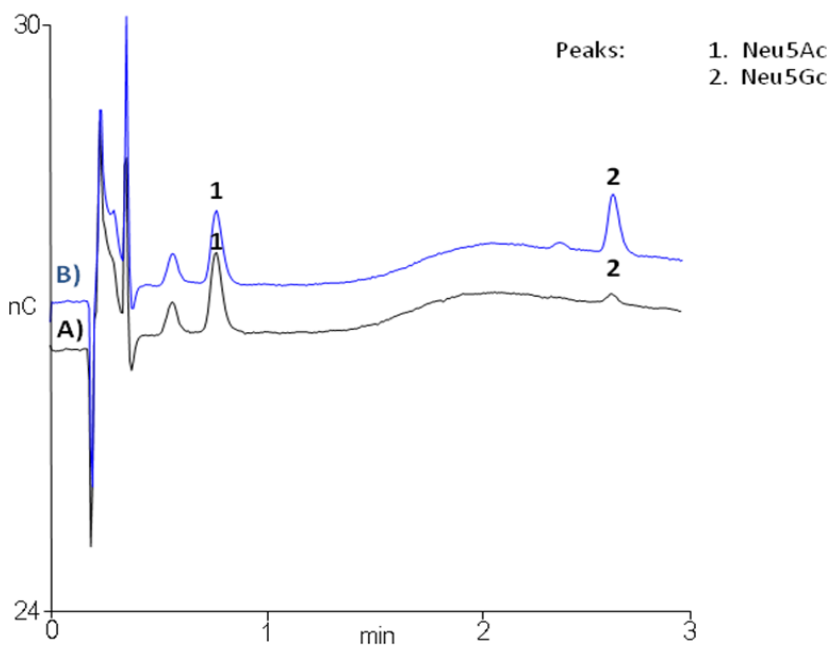


Figure 7
Sialic Acid Analysis using the CarboPac PA20 Fast Sialic Column

4.5 Carbohydrates in Foods

Isocratic sodium or potassium hydroxide eluents with concentrations on the range of 18-100 mM are typically used for the separation of monosaccharides, although for monosaccharides that are important in dietary fiber analysis, the eluent may be weaker. For all of these isocratic runs, it is important to regenerate the column using a step gradient to about 300 mM at the end of the run, to remove more strongly retained sample matrix components and ensure stable run times.

Occasionally, it may be necessary to use an extremely weak eluent or even water. Under these conditions, post column addition of 300 mM hydroxide is necessary for detection, although if the sensitivity is adequate, the added complexity of post-column hydroxide addition may not be worth the effort. For example, coffee sugars can be separated and detected with 2 mM NaOH with sufficient sensitivity to obviate the need for post-column base addition.

The following conditions should be used as guidance. These may be modified, as necessary, to suit your particular application needs.

Column:	CarboPac PA20
Eluent:	2 mM NaOH, isocratic
Flow Rate:	0.5 mL/min
Detection:	Pulsed electrochemical detection, Au electrode
Waveform:	Quadruple potential

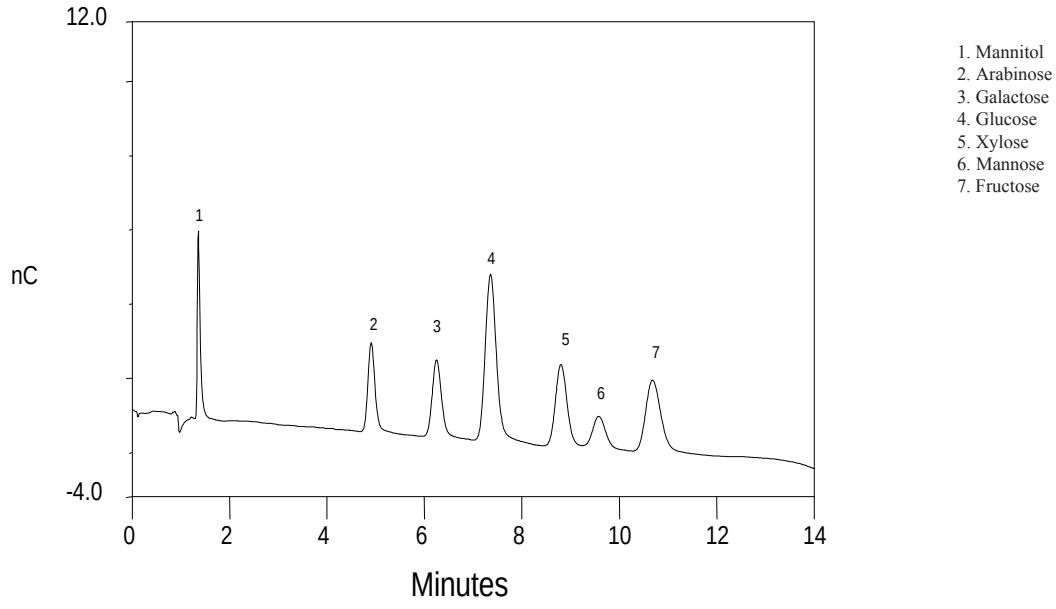


Figure 8
Separation of Coffee Sugars Using the CarboPac PA20

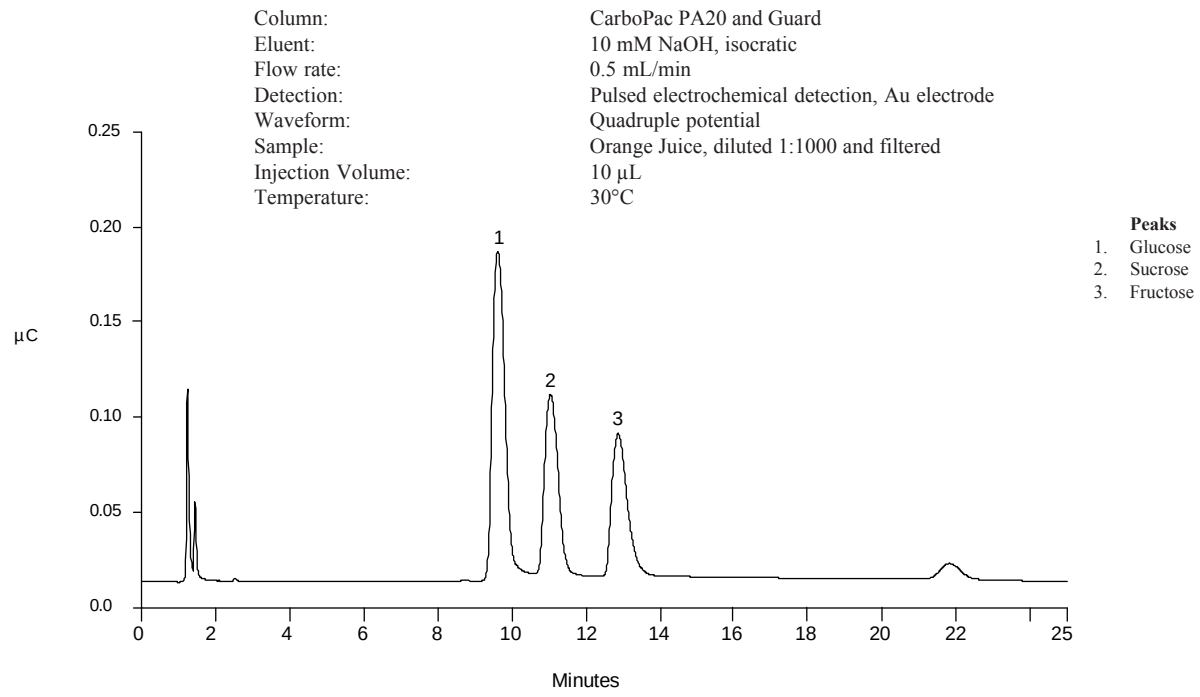


Figure 9
Analysis of Orange Juice using the CarboPac PA20

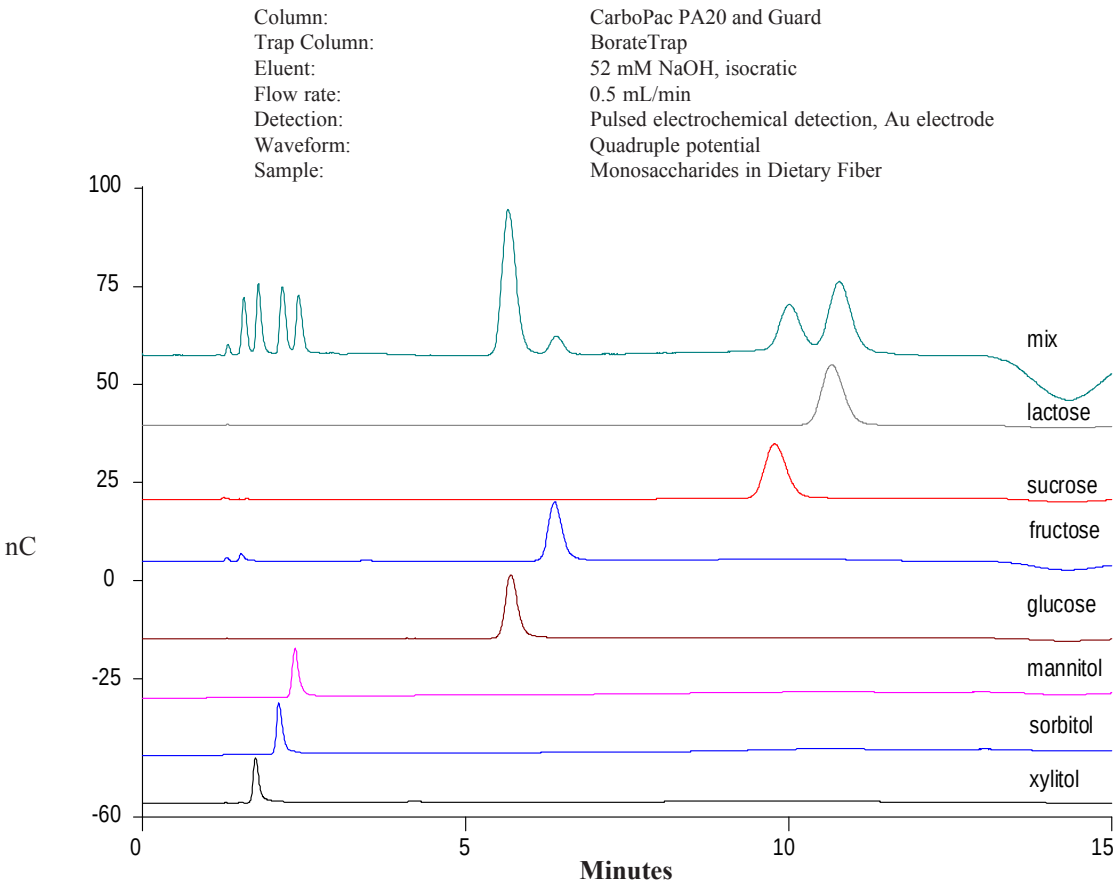


Figure 10
Separation of Monosaccharides Important in Dietary Fiber Analysis

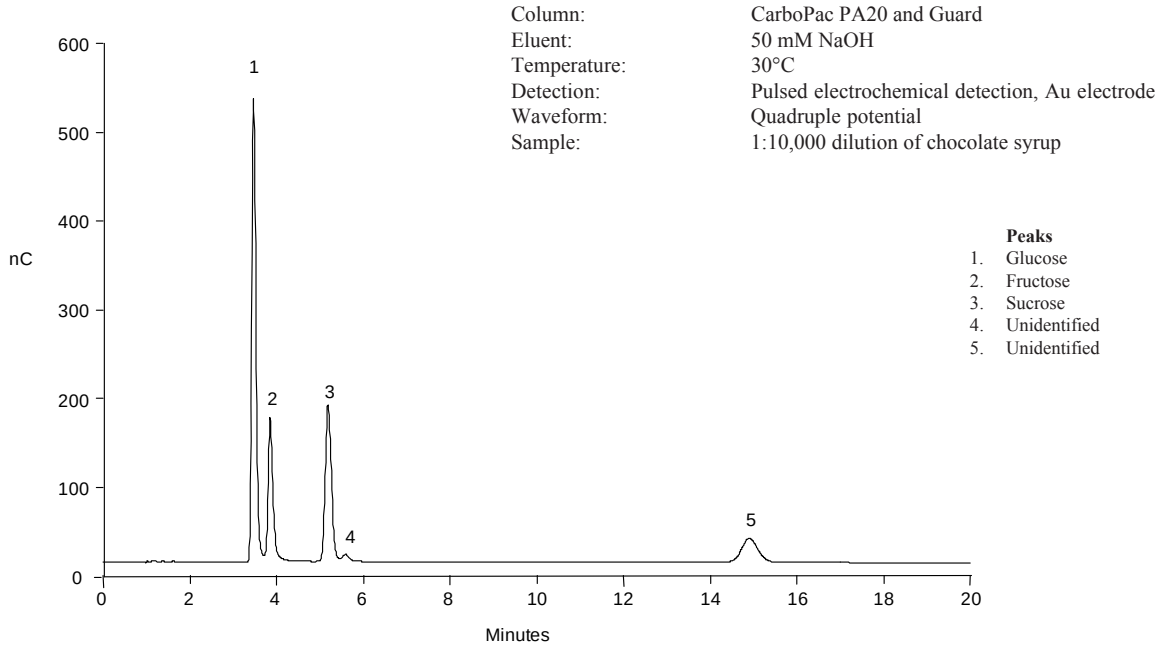


Figure 11
Analysis of Chocolate Syrup using the CarboPac PA20

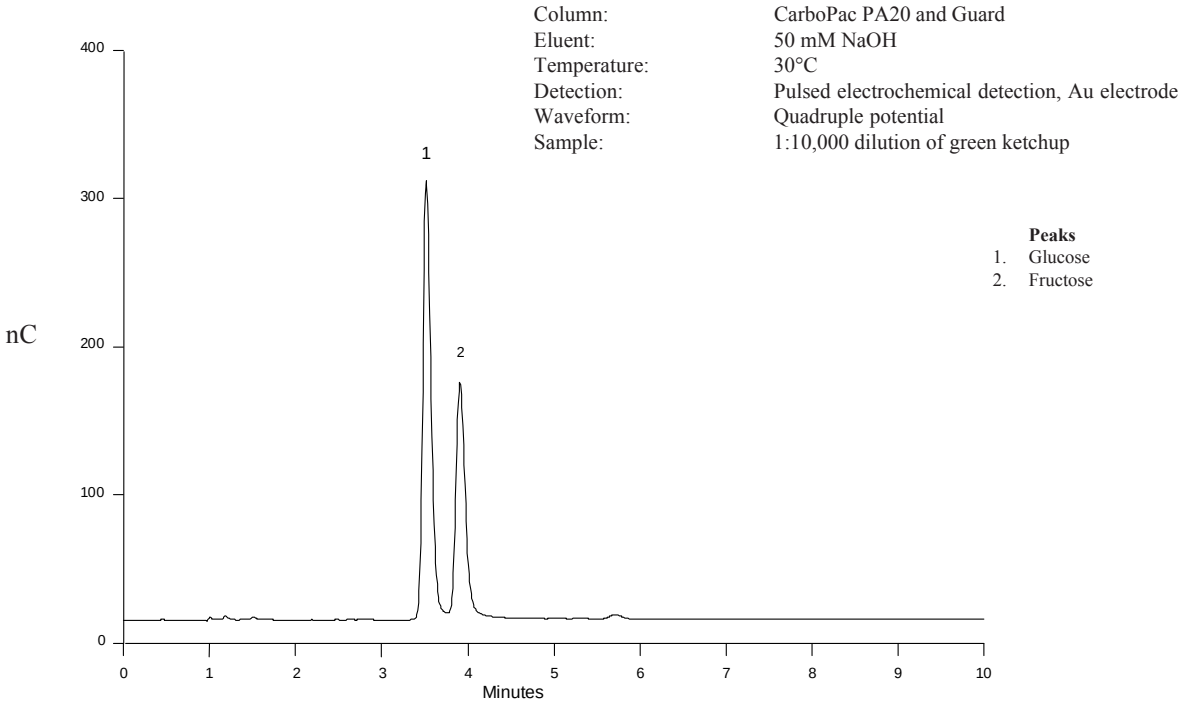


Figure 12
Analysis of Green Ketchup using the CarboPac PA20

4.6 Carbohydrates in Bio- and Pharmaceutical Products and Processes

The United States Food and Drug Administration (FDA) and the regulatory agencies in other countries require that pharmaceutical products be tested for composition to verify their identity, strength, quality and purity. Recently, attention has been given to inactive ingredients as well as active ingredients. Many of the ingredients are non-chromophoric and cannot be visualized by absorbance detection. However, carbohydrates, glycols, sugar alcohols and sulfur-containing compounds can be oxidized and therefore detected by amperometric detection.

Another area of great interest to the pharmaceutical chemist is the optimization of fermentation broths. If the correct nutrients and amino acids for a given culture are determined, and monitored, so that the correct concentrations are always maintained, then the culture has the best chance of producing the optimum yield. The CarboPac PA20 can be used on-line to monitor fermentation broths and cell cultures. Alternatively, if there is more interest in monitoring the amino acid content, then the AminoPac PA10 (P/N 055406) should be considered for direct detection, on-line.

The following conditions should be used as guidance. These may be modified, as necessary, to suit your particular application needs.

Column:	CarboPac PA20 and Guard
Eluent:	10 mM NaOH followed by 200 mM NaOH for 10 min to regenerate the column.
Temperature:	30°C
Detection:	Pulsed electrochemical detection, Au electrode
Waveform:	Quadruple potential
Sample:	1:1,000 dilution of YPD broth supernatant

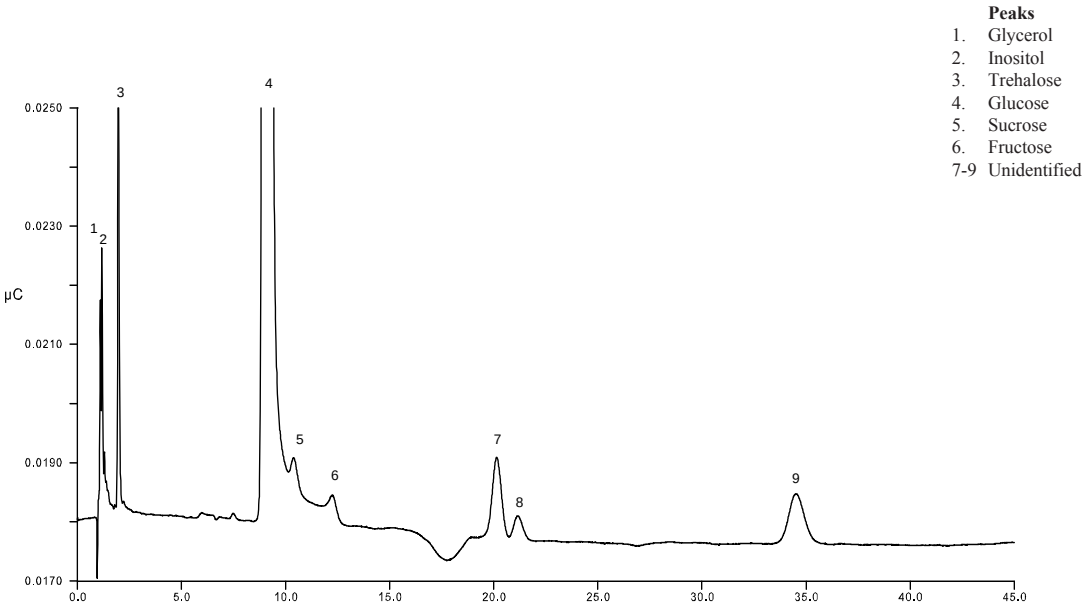


Figure 13
Analysis of Yeast Extract-Peptone-Dextrose (YPD) Broth Supernatant

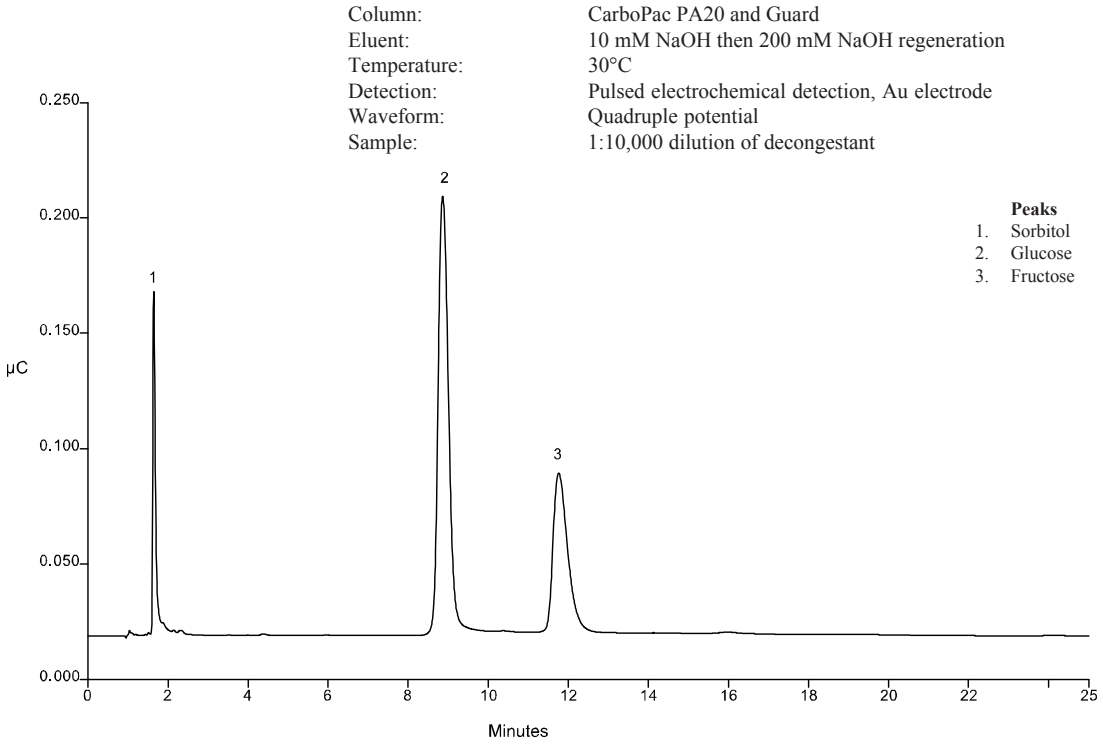


Figure 14
Analysis of a Pediatric Oral Decongestant using the CarboPac PA20

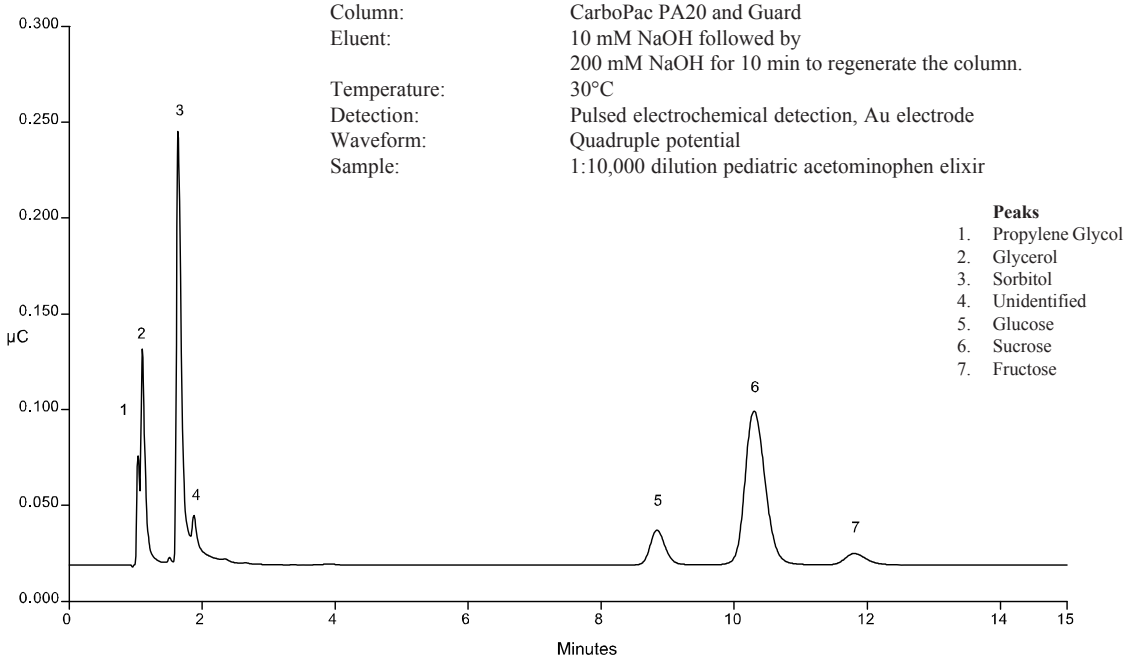


Figure 15
Analysis of a Pediatric Acetominophen Elixir

SECTION 5 - TROUBLESHOOTING GUIDE

Experimental protocol problems such as sample contamination, imprecision during sample transfer, problems during peptide or protein hydrolysis, may be related to specific experimental protocols.

Make sure to follow the rules from Section 3.1.1, "The Most Important Rules," and to recheck all of the items from Section 3.1.2, "Initial Check List."

5.1 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:

1. **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 2, "Carbohydrate Quadruple Waveform, " in Section 3.6. If the pH reading at 95/5 (%E1/%E2, i.e. 10 mM NaOH) is above 13.2 replace the reference electrode.

2. **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.

3. **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 5.3.

4. **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.

5. **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 5.5.

5.2 Decreased Detection Sensitivity

Always confirm the loss of response by performing at least one injection of the 10 µL Mix of Six monosaccharide standard mix as described in Appendix A, "Quality Assurance Report." This is to make sure that a decreased level of response is not being caused by system problems discussed in Section 5.4.2.

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 5.6, "Nitric Acid Cleanup")
The exceptions to this rule are described below.

Exceptions:

Check the pH reading in the Detail Screen of the ED50. If the value is out of range of >13.2, install a new reference electrode and then install a new gold working electrode (P/N 44112) or (P/N 060139 for 6 disposable Au electrodes). The system cleanup is not necessary. The decrease in sensitivity was caused by a gold-oxide-buildup on the electrode surface. This was because the reference potential was too high. The non-disposable gold working electrode can be reconditioned by the repair polishing described in Section 5.7.1, "Mechanical Polishing."

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

5.3 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 5.1 item 3, "Column Contamination."

5.3.1 Column Set Causing High Background

If the column has been determined to be the cause of the high background, as described above, 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.

5.3.2 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.

1. Make sure the gradient rise is not caused by the system and/or detector cell (see Section 5.4.1, "High Detection Background Caused by the System").

2. Increase column temperature to 40°C and wash the guard and column with 10 mM NaOH, 950 mM sodium acetate (5% E2, 95% E3) for at least four hours (and preferably overnight). Run a blank gradient at 30°C and if necessary repeat the 5% E2, 95% E3 wash at 40°C.

5.3.3 Peak Efficiency and Resolution are Decreasing

Always have a spare guard available.

Peak deformations may sometimes be caused by sample matrix.

1. 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 quite common to replace the Guard column several times during the lifetime of the analytical column.

2. Verify that only the 0.005" I.D. (Red) tubing is installed for all connections between injector and detector.

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

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

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

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

7. 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.

5.4 System Problems

5.4.1 High Detection Background Caused by the System

1. Verify the problem is neither the detector (see Section 5.1 item 1, “Incorrect detection parameter” and item 2, “Compromised working electrode surface”) nor the column (see Section 5.1 item 3, “Column Contamination”) related.

2. With injector, column and detector cell installed (cell voltage off) carry out the 2M NaOH wash as described in Section 5.5, “Sodium Hydroxide Cleanup.”

3. Prepare new eluents.

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

5.4.2 No Peaks, Poor Peak Area Reproducibility or Too Small Peak Areas

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

2. Check injector needle-height setting.

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

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

5.4.3 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.

5.4.4 Incorrect or Variable Retention Times

1. Check your eluent preparation procedure for possible errors.

2. Prime the pump if necessary.

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

4. 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.

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

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

7. 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.

5.4.5 Unidentified Peaks Appear Alongside the 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:

Disconnect the column and detector cell from the autosampler.

Set the pump to 100% deionized water.

Place the following solutions in the autosampler and inject in sequence. Use 25 mL fill loop injections:

1. 1 M NaOH
2. Deionized water
3. IPA
4. Deionized water
5. 1 M HCl
6. Deionized water

5.5 Sodium Hydroxide Cleanup

The sodium hydroxide (2 M) rinse used to decrease column or system-related elevated background is essentially identical to the rinse performed during an installation of a new system, Section 2.5.2, "System Rinse." 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 5.6, "Nitric Acid Cleanup." 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 5.7.1, "Mechanical Polishing."

5.6 Nitric Acid Cleanup

CAUTION

The o-ring on the priming knob must be white before proceeding with Nitric Acid cleanup. Upgrade to white o-ring if older black version is installed.

Cleaning procedure for Severely Contaminated Carbohydrate Systems:

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

2. 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.

3. Remove the CarboPac PA20 set from the system, disconnect and plug the detector cell to prevent drying out of the reference electrode chamber.

4. Replace the CarboPac PA20 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

5. 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.

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

7. 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.

8. During the rinse of step 7, 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).

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

10. 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.(It may take more than 10 hours to rinse the acid out completely. It may be convenitent to rinse overnight.)

11. The rinsing is completed when the pH at the waste outlet is approximately the same as that of the water in the reservoir containers.

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

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

13. 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 2, Section 3.6, “Carbohydrate Quadruple Waveform.” Wait until the background drops below 30nC.
14. Stop the eluent flow, turn the cell voltage off. Remove the yellow tubing and replace it with a NEW CarboPac PA20 column set (guard and analytical column).

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

16. 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. Also check the detection response by injecting the mix of six monosaccharide standard as described in Appendix A, “Quality Assurance Report.”

5.7 Reconditioning of Gold Electrodes

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

5.7.1 Mechanical Polishing

1. 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.

2. Apply several mL of water to a fresh polishing pad (P/N 36121) and 'polish' for one minute. This step removes the coarse polishing powder particles imbedded in the gold material.

3. 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.

4. 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.

5. 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.

5.7.2 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.

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

2. The only reason to sand off an electrode is to make the gold electrode flush with the KEL-F surface.

3. Use a fresh 600-grit sand paper. Make sure that the KEL-F 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.
4. Sand for less than 1 minute (continuous sanding only to bring the KEL-F 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.

5.7.3 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.

SAFETY
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 approximately 10 mL of chromic acid from the glass vessel into a 20 mL glass scintillation vial, then screw the cap on. After that, 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.

5.8 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.

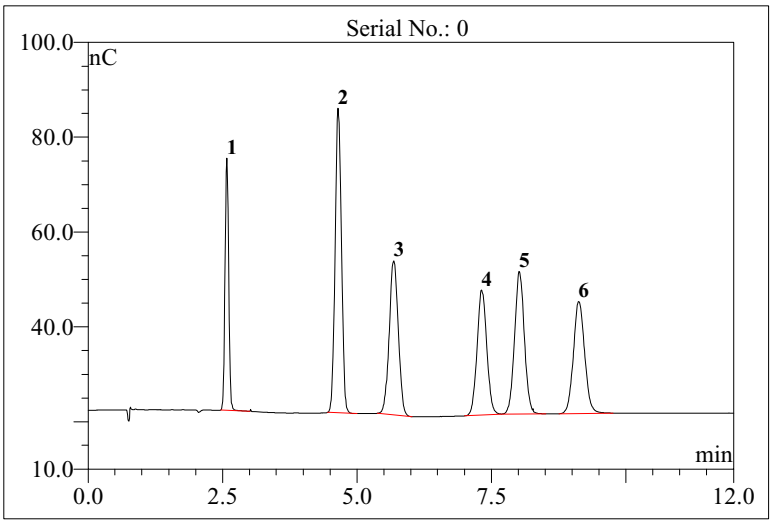
NOTE
Immediately remove the “known good” electrode and store it properly. This referencing procedure will work as long as you do not leave your “known good” electrode inside the ED50 cell for more than a few hours at a time and store it properly (immersed in 3 M KCl) in the intervening periods of time.

APPENDIX A - QUALITY ASSURANCE REPORT

Date: **20-Sep-02 14:28**
Serial No. : **00000**
Lot No. : **000-00-000**

CarboPac™ PA20
Analytical (3 X 150 mm)
Product No. 060142

Flow Rate: 0.50 mL/min
Detection: ED50
Temperature: 30°C
Injection Volume: 10 µL
Diluted Standard: 10 nmol/mL in DI Water
Each analyte is 0.1 nmoles per injection.
Recommended Trap: BorateTrap
Storage Solution: 10 mM NaOH



Eluent Composition
%A: 200 mM NaOH
%B: 10 mM NaOH

Eluent Profile

Time	%A	%B	Comment
-25.00	100	0	Regeneration
-15.05	100	0	
-15.00	0	100	Equilibration
0.00	0	100	Inject
12.00	0	100	End

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	L-Fucose	2.58	1.10	12.20	6862
2	D-Galactosamine	4.64	1.10	4.00	7439
3	D-Glucosamine	5.68	1.00	5.14	5492
4	D-Galactose	7.32	1.07	2.12	7853
5	D-Glucose	8.02	1.04	3.11	9300
6	D-Mannose	9.13	1.07	n.a.	9153

QA Results:

Analyte	Parameter	Specification	Results
D-Galactosamine	Efficiency	>=5400	PASS
D-Galactosamine	Asymmetry	0.95-1.38	PASS
D-Mannose	Retention Time	8.10-9.90	PASS
	Pressure	<=2860	2178

Production Reference:

Datasource: Product_CGrams Sample No.: 1 6.40+SP1, Build 711
Sequence: CP_PA20_QAR_Sample_for_Manual Chromeleon® Dionex Corp. 1996-2002

APPENDIX B – GOOD PRACTICES FOR SUCCESSFUL HPAE-PAD

GOOD PRACTICES

1. Always use a guard column with the CarboPac PA20 Analytical column.

2. Keep tubing lengths to a minimum and change tubing from the injector to the column, between columns, and to the detector at least once a year.

3. Change the sample loop at least once a year. Remember to calibrate the loop for proper comparison to the last loop.

4. Change the autosampler needle every 6 months to a year.

5. Regularly test the autosampler to ensure that it is functioning properly (see TN40, "Glycoprotein Monosaccharide Analysis Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)," for an example)

6. Develop all new methods with waveform A, the quadruple waveform (see TN21, "Optimal Settings for Pulsed Electrochemical Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector.").

7. Establish a system suitability standard, such as the mix of 6 monosaccharides used by Dionex for the test chromatogram.

8. Change the reference electrode every 3 months and be sure to calibrate it.

9. Disconnect the column from the cell when cleaning the column

10. Control the column and cell temperature at 30°C.

11. Use the eluent degas feature of the pump (30 seconds every 10 minutes). Without the degas, the background will eventually go negative.

12. Calibrate the pump flow rate every three months and repair (pump seals, check valves) as necessary.

13. For best reproducibility, use full loop injections.

SYSTEM PARAMETERS TO MONITOR

When your system has been installed and optimized, note the values of the following parameters. These values can be checked periodically to ensure that the system is still functioning optimally.

1. System backpressure

2. System background under your running conditions. This value is typically 20 –35 nC using the quadruple waveform, ranging from about 20 nC for only NaOH to about 35 on the addition of NaOAc.

3. Baseline noise for 1 minute intervals, should range from 30–100 pC peak-to-peak.

4. The pH reading of the reference electrode should be between 12 and 13. If there is no reading, the reference electrode has failed.

5. Mannose asymmetry should be less than 1.2, typically. If it is greater than 1.2, then the probable source of the problem is the water source. This can be confirmed by installing a BorateTrap to see if the asymmetry improves.

SHUTTING DOWN AN HPAE-PAD SYSTEM

1. For short-term (a few days) turn off the cell, stop the flow or flow a strong eluent (100 mM NaOH or stronger) at a low flow rate.

2. For long-term, fill the column with strong eluent, remove from the system and plug the ends.

3. Remove the reference electrode and put it in its container with a saturated KCl solution (see the detector manual for more information).

4. Pump water through the system (each channel used). This can be done at 5 mL/min when no column is in-line. Flush the autosampler.

RESTARTING AN HPAE-PAD SYSTEM

1. Pump water at 5.0 mL/min (no column in-line) through each eluent channel, or withdraw at least 20 mL from each line using a syringe.

2. Replace the water with the appropriate eluent and pump the eluent through each line at 5 mL/min to replace the water in the lines, or withdraw at least 20 mL from each line using a syringe.

3. Install the column, wash with strong eluent at 0.5 mL/min for 30 minutes

4. Reinstall the reference electrode.

5. Flush the autosampler, make sure the flush line is free from air bubbles.

6. Run the system with strong eluent and evaluate the background.

APPENDIX C – PAD POSITIVE COMPOUNDS VERSUS HYDROXIDE CONCENTRATIONS ON THE CARBOPAC PA20 ANALYTICAL COLUMN

Retention Times (min)

Analyte	10 mM	50 mM	100 mM	250 mM
Glycerol	1.14	1.15	1.17	1.15
Erythritol	1.24	1.24	1.25	1.22
Arabitol	1.51	1.48	1.46	1.38
Methanol	1.59	1.58	1.61	1.61
Galactitol	1.69	1.65	1.61	1.48
Ribitol	1.71	1.67	1.61	1.48
Sorbitol	1.74	1.67	1.62	1.47
Trehalose	2.23	2.00	1.87	1.62
Fucose	3.23	2.29	1.90	1.46
Maltitol	4.82	3.86	3.17	2.25
2-Deoxy-D-Glucose	5.31	3.43	2.59	1.80
Galactosamine	5.84	3.19	2.33	1.62
Rhamnose	6.03	3.00	2.19	1.57
Arabinose	7.00	3.86	2.73	1.83
Glucosamine	7.16	3.49	2.46	1.67
Galactose	8.95	4.83	3.34	2.03
Glucose	9.93	4.88	3.30	2.05
Mannose	11.40	4.86	3.18	1.97
Sucrose	11.45	7.91	5.59	3.25
Xylose	11.67	5.26	3.45	2.08
Fructose	13.29	5.78	3.64	2.20
Ribose	15.37	6.36	4.01	2.33
Oxygen Dip	17.90	18.18	N/A	18.10
Raffinose	22.04	13.84	9.06	4.45
Alpha-Lactose	22.50	9.42	5.55	2.78
Cellobiose	43.65	15.45	8.53	3.74
Melibiose		6.83	4.30	2.32
Turanose		14.77	7.95	3.57
Gentiobiose		14.79	8.56	3.69
Palatinose		15.65	8.66	3.85
Maltose	> 60	27.54	13.86	5.13
Maltotriose	> 60	> 60	50.89	12.93
Maltotetraose	> 60	> 60	> 60	34.65
Maltopentaose	> 60	> 60	> 60	> 60

APPENDIX D – INSTALLATION OF A REFERENCE ELECTRODE

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:

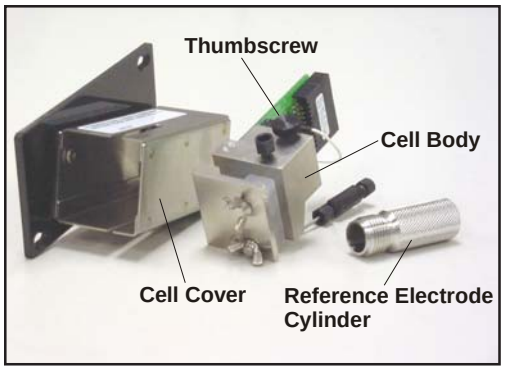
- A CHEMRAZ® O-ring (P/N 048410) (included in a bag labeled CHEMRAZ)
- An O-ring retainer (P/N 057192) (shipped in the reference electrode cavity)

NOTE

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

To complete this installation procedure, you will need:

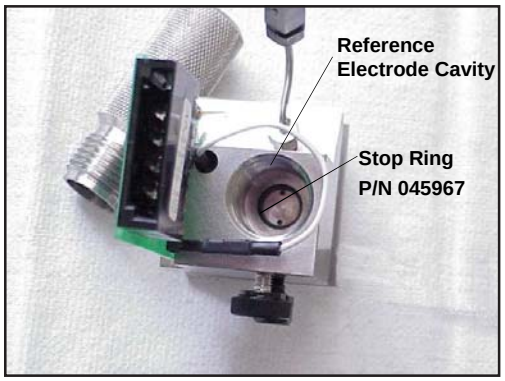
- A pair of tweezers
- Deionized water



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.

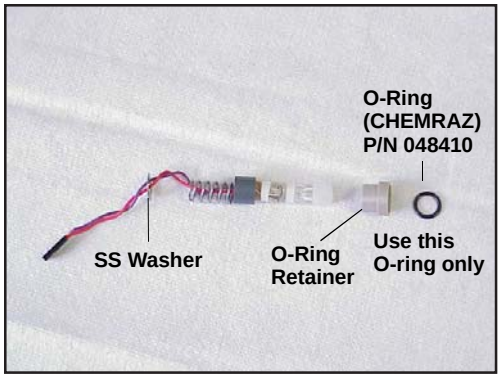


Step 2

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.

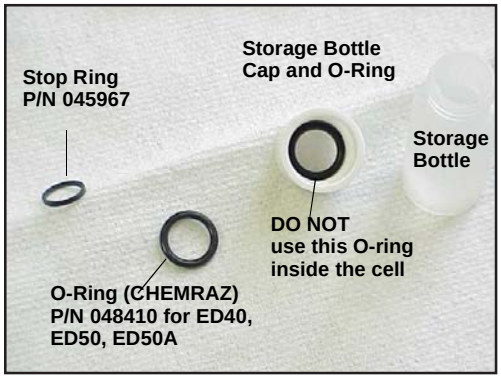
Step 3

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.



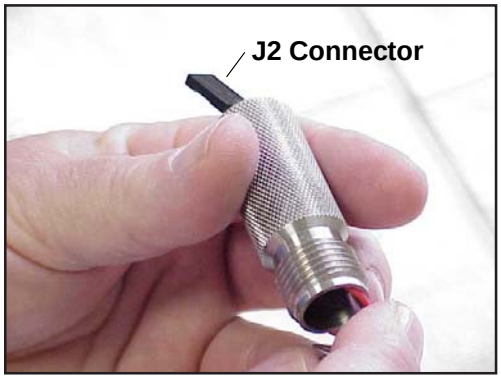
Step 4

Verify that you have all of the following parts:



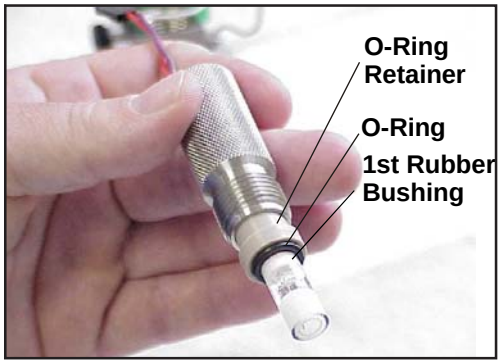
NOTE:

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

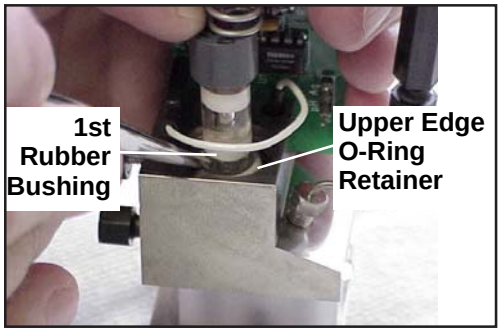
Slide the PEEK O-ring retainer and CHEMRAZ O- ring above the 1st rubber bushing.

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.

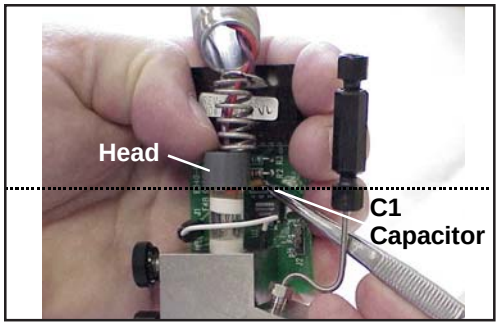
Step 7

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.



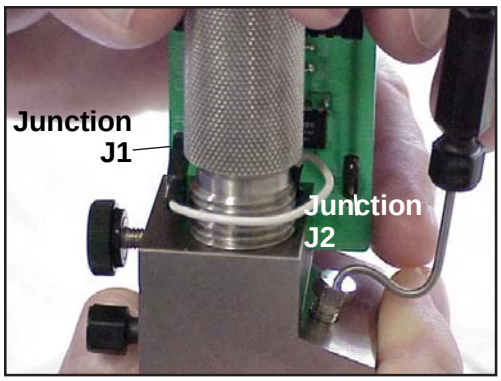
Step 8

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

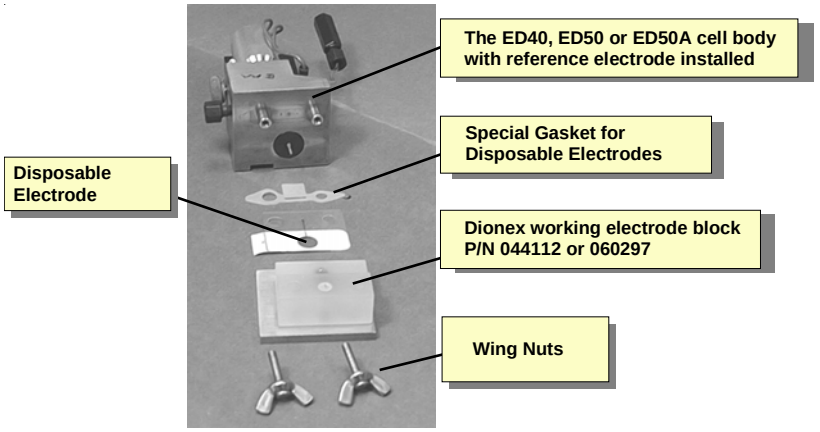
Step 12

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.

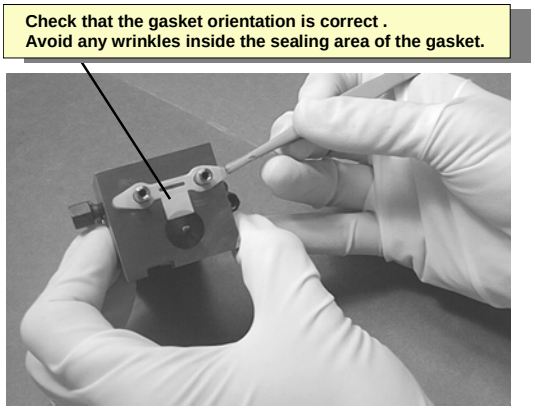
APPENDIX E - INSTALLATION OF DISPOSABLE ELECTRODES

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.

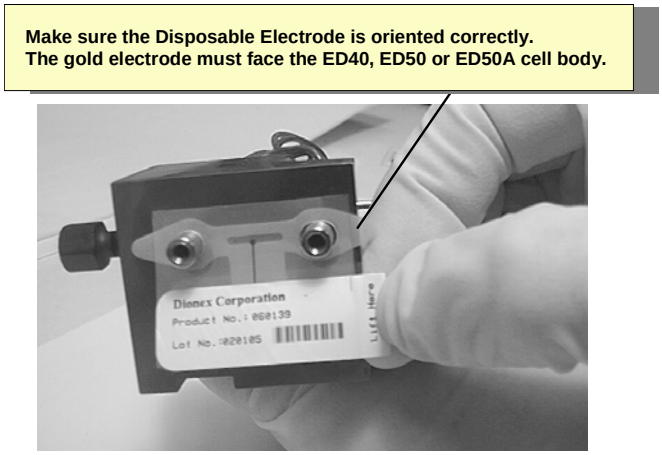
CAUTION
Read all instructions before installing.



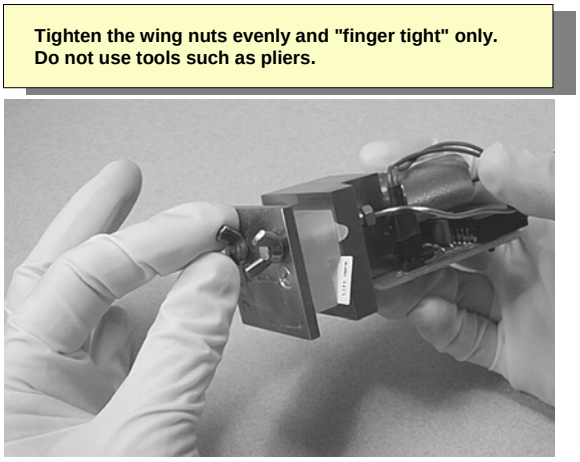
STEP 1
Check availability of all parts.



STEP 2
Install special gasket.



STEP 3
Install disposable electrode



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

APPENDIX F – 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, "Optimal Settings for Pulsed Electrochemical Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector"). 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 PA20, this dip occurs after the sugars have been eluted, and is noticeable at low detection levels. 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.

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	

APPENDIX G - HPAE-PAD CARBOHYDRATE RESOURCES

None of these resources specifically use the CarboPac PA20; however, the following Technical Notes, Application Notes and articles can be used to determine starting conditions for separations on the CarboPac PA20. The CarboPac PA20 is designed to have shorter run times and higher efficiencies.

Basic HPAE-PAD Resources

1. Dionex Technical Note 20, "Analysis of Carbohydrates by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)"

2. Dionex Technical Note 21, "Optimal Settings for Pulsed Electrochemical Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector"

3. Rocklin R.D., et al. (1998), *Anal. Chem.*, **70**, 1498-1505 (Quadruple waveform)

HPAE-PAD Monosaccharide Resources

1. Dionex Technical Notes 30, 40, 53

2. Dionex Application Notes 117, 122

3. Application Update 125

4. Weitzhandler, M., et al. (1996) *Anal. Biochem.,* **241**, 128-134 (AminoTrap Paper)

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APPENDIX H - COMPARISON OF SELECTED CARBOPAC COLUMNS

Table H1
Comparison of the CarboPac PA1, PA10 and PA20 Columns

Characteristic	CarboPac PA1	CarboPac PA10	CarboPac PA20
Recommended Applications	Monosaccharide compositional analysis, linear homopolymer separations, saccharide purification	Monosaccharide compositional analysis, linear homopolymer separations	Monosaccharide compositional analysis, linear homopolymer separations
Resin Composition	Pellicular. 10 µm diameter polystyrene/ divinyl benzene substrate agglomerated with 580 nm MicroBead quaternary ammonium functionalized latex	Pellicular. 10 µm diameter polystyrene /divinyl benzene substrate agglomerated with 460 nm MicroBead difunctional quaternary ammonium ion	Pellicullar. 6 µm diameter ethylvinyl benzene/divinyl benzene substrate agglomerated with 130 nm MicroBead quaternary ammonium functionalized latex
Substrate X-linking	2%	55%	55%
Latex X-linking	5% cross-linked	5% cross-linked	6% cross-linked
AE Capacity (4 x 250)	100 µeq	100 µeq	
Flow rate / min	1 mL/min (4-mm)	1 mL/min (4-mm)	0.5 mL/min (3-mm)
pH Compatibility	pH 0-14	pH 0-14	pH 0-14
Solvent Compatibility	0 ≤ 2%	0-90%	0-100%
Max. Backpressure	4000 psi (28 MPa)	3500 psi (25 MPa)	3500 psi (25 MPa)