DIONEX

Technical Note 20

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Analysis of Carbohydrates by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)

INTRODUCTION

Methods for the liquid chromatographic analysis of carbohydrates have often employed silica-based amino-bonded or polymer-based, metal-loaded, cation-exchange columns, with refractive index (RI) or low-wavelength ultraviolet (UV) detection. These analytical methods require attention to sample solubility, sample concentration and, in the case of the metal-loaded cation-exchange columns, also require column heating. In addition, RI and low-wavelength UV detection methods are sensitive to eluent and sample matrix components. This usually precludes the use of gradients and often requires stringent sample cleanup prior to injection.

As a result, an improved chromatographic technique known as *high-performance anion exchange* (HPAE) was developed to separate carbohydrates. Coupled with pulsed amperometric detection (PAD), it permits direct quantification of nonderivatized carbohydrates at low-picomole levels with minimal sample preparation and cleanup. HPAE chromatography takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH using a strong anion-exchange stationary phase. This technical note is intended as an introduction to HPAE-PAD carbohydrate analysis. The technique has been reviewed extensively,[1–4] and those articles should be consulted for more specific details.

HPAE-PAD is extremely selective and specific for carbohydrates because:

1. Pulsed amperometry detects only those compounds that contain functional groups that are oxidizable at the detection voltage employed (in this case, sensitivity for carbohydrates is orders of magnitude greater than for other classes of analytes).
2. Neutral or cationic sample components in the matrix elute in, or close to, the void volume of the column. Therefore, even if such species are oxidizable, they do not usually interfere with analysis of the carbohydrate components of interest.

ANION-EXCHANGE CHROMATOGRAPHY

I. Mechanism of Separation

Although anion-exchange chromatography has been used extensively to analyze acidic carbohydrates and glycopeptides, it has not been commonly used for analysis of neutral sugars. However, examination of the pK_a values of the neutral monosaccharides listed in Table 1 shows that carbohydrates are in fact weak acids. At high pH, they are at least partially ionized, and thus can be separated by anion-exchange mechanisms. This approach cannot be used with classical silica-based columns because these matrices dissolve at high pH. Anion exchange at high pH is, however, ideally suited to base-stable polymer anion-exchange columns.

Table 1. Dissociation Constants of Some Common Carbohydrates[5] (in water at 25 °C)

Sugar	pK_a
Fructose	12.03
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Dulcitol	13.43
Sorbitol	13.60
α-Methyl glucoside	13.71

II. CarboPac™ Columns

A. CarboPac PA1 and PA-100 Columns

Dionex designed the CarboPac series of columns specifically for carbohydrate anion-exchange chromatography. These columns permit the separation and analysis of mono-, oligo-, and polysaccharides. The CarboPac PA1 and CarboPac PA100 are packed with a unique polymeric, nonporous, MicroBead™ pellicular resin. MicroBead resins exhibit rapid mass transfer, high pH stability (pH 0–14), and excellent mechanical stability that permits back pressures of more than 4000 psi (28 MPa). Column reequilibration after gradient analysis is fast, generally taking 10 min or less. A diagram of a typical pellicular anion-exchange resin bead is shown in Figure 1.

Both the CarboPac PA1 and the CarboPac PA100 are designed for the rapid analysis of mono- and oligosaccharides. The CarboPac PA1 is particularly well-suited to the analysis of monosaccharides and the separation of linear homopolymers, while the CarboPac PA100 is optimized for oligosaccharide resolution and separation. Several examples of separations obtained using these columns are shown in the "Applications" section of this technical note.

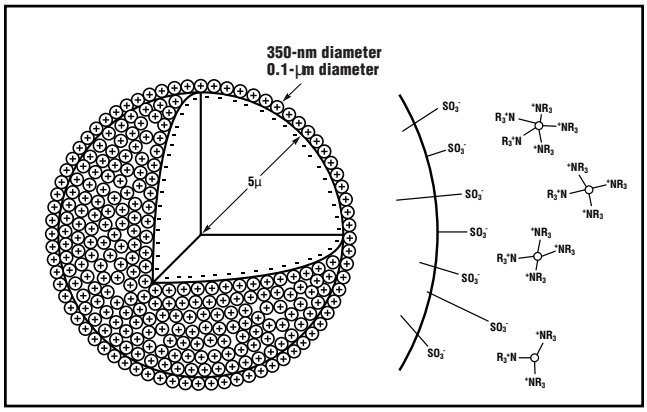

Figure 1. *Pellicular anion-exchange resin bead.*

B. CarboPac MA1 Column

Reduced carbohydrates (also called sugar alcohols) have traditionally been a difficult class of carbohydrates to separate by liquid chromatography. They are weaker acids than their nonreduced counterparts (compare the pK_as of glucose and sorbitol or galactose and dulcitol in Table 1), and are therefore poorly retained on the CarboPac PA1 and PA100 columns. The CarboPac MA1 was developed to address the challenge of retaining and separating extremely weak acids. This column is packed with a macroporous polymeric resin which has an ion-exchange capacity 45 times that of the CarboPac PA1. As a result, weak anions bind more strongly to the column, requiring higher sodium hydroxide concentrations for elution. The increase in hydroxide ion concentration leads to greater ionization of the sugar alcohols, with greatly improved retention and resolution on the column.

Nonreduced neutral oligosaccharides can also be analyzed on the CarboPac MA1 column, although their analysis times are longer than on the CarboPac PA1 and PA100 columns. Retention of carbohydrates on the CarboPac MA1 can be manipulated by altering the sodium hydroxide concentration of the eluent (see Table 2). Note that the elution order of several of the compounds changes with the sodium hydroxide concentration. This can be used to design separation strategies for specific sets of analytes. Examples of separations obtained with the CarboPac MA1 column are shown in the "Applications" section of this technical note.

Table 2. k´ Values of Selected Analytes on the CarboPac MA1 Column[a]

Analyte	Eluent Concentration (M NaOH)				
	0.05	0.14	0.25	0.38	0.50
Glycerol	1.13	0.99	0.89	0.80	0.72
m-Inositol	1.32	1.08	0.86	0.69	0.56
s-Inositol	1.63	1.30	1.02	0.81	0.64
GlcNol	1.81	1.40	1.09	0.89	0.75
Fucitol	1.94	1.63	1.40	1.18	1.05
Erythritol	2.02	1.71	1.44	1.25	1.13
GalNol	2.29	1.81	1.39	1.13	0.95
GalNAcol	2.35	1.81	1.38	1.12	0.95
GlcNAcol	2.61	1.96	1.48	1.18	0.95
Xylitol	3.09	2.48	1.95	1.59	1.35
Arabitol	4.69	3.62	2.76	2.24	1.92
Sorbitol	6.43	4.72	3.33	2.55	2.06
Dulcitol	6.52	5.04	3.73	2.87	2.26
Adonitol	7.09	5.31	3.83	2.96	2.43
Mannitol	8.98	6.38	4.37	3.28	2.63
Fucose	10.34	4.72	2.52	1.69	1.25
Isomaltitol	12.22	8.15	4.89	3.30	2.43
Lactitol	14.97	9.61	5.49	3.57	2.43
Gp-Man	15.66	10.36	6.18	4.15	3.05
GalN	18.56	7.16	3.39	2.13	1.48
GlcN	20.88	7.71	3.61	2.24	1.55
Maltitol	31.21	17.25	8.80	5.44	3.67
Glucose		15.70	7.19	4.31	2.91
Mannose		13.55	6.15	3.72	2.53
Galactose		17.82	8.25	4.99	3.43

[a] The capacity factor, k´, is defined as: $k' = (V_A - V_0)/V_0$, where V_A is the retention volume of the analyte on the column and V_0 is the void volume.

Table 3. Comparison of the CarboPac MA1, PA1, and PA100			
Characteristic	**CarboPac MA1**	**CarboPac PA1**	**CarboPac PA100**
Recommended applications	Mono- and disaccharide alcohol analysis in food products, physiological fluids, tissues, and reduced glycoconjugate saccharides[b]	Monosaccharide compositional analysis, linear homopolymer separations, saccharide purification	Oligosaccharide mapping and analysis
Resin composition	8.5-µm-diameter vinylbenzyl-chloride/divinylbenzene macroporous substrate fully functionalized with an alkyl quaternary ammonium group	10-µm-diameter polystyrene/ divinylbenzene substrate agglomerated with 350-nm MicroBead quaternary amine functionalized latex	10-µm-diameter ethylvinylben-zene/divinylbenzene substrate agglomerated with 350-nm MicroBead quaternary amine functionalized latex
MicroBead latex cross-linking	N/A, no latex	5% cross-linked	6% cross-linked
Anion-exchange capacity	4500 µeq per 4 × 250-mm column	100 µeq per 4 × 250-mm column	90 µeq per 4 × 250-mm column
Recommended flow rate	0.4 mL/min (4 × 250-mm column)	1 mL/min (4 × 250-mm column)	1 mL/min (4 × 250-mm column)
pH compatibility	pH 0–14	pH 0–14	pH 0–14
Organic solvent compatibility	0%	0–2%	0–100%
Maximum back pressure	2000 psi (14 MPa)	4000 psi (28 MPa)	4000 psi (28 MPa)

[b] Note that sialylated and other acidic mono- and oligosaccharides may not be recovered from the CarboPac MA1 column. It is not recommended that this column be used with these analytes.

III. Guidelines for CarboPac Column Selection

Table 3 provides a comparison of the three CarboPac columns. The following guidelines are useful in selecting the right CarboPac column for a particular application.

A. Monosaccharides

For reducing monosaccharides, the recommended column is the CarboPac PA1, while the MA1 is recommended for sugar alcohols. The CarboPac MA1 column also generates excellent neutral monosaccharide separations, although retention times are longer than on the PA1. Amino-sugars are better resolved on the CarboPac PA1 than on the MA1, but the reverse is true for N-acetamido sugars.

B. Neutral Oligosaccharides

The CarboPac PA100 is the most appropriate column for the oligosaccharide mixtures characteristic of glycoprotein-derived oligosaccharides, although these compounds are only slightly less well-resolved on the CarboPac PA1 column than on the PA100. Neutral oligosaccharides up to nine monosaccharide units in size are separable on the CarboPac MA1. However, the CarboPac MA1 will usually have longer retention times than the PA100, and selectivities of the two columns are almost identical.

Oligosaccharides cleaved by reductive β-elimination from glycoproteins contain a reduced terminal and generally elute earlier than the same oligosaccharide with a reducing terminal. Reduced di- and trisaccharides will elute significantly earlier than their nonreduced counterparts, and may be poorly resolved on the CarboPac PA1 and PA100. These compounds are readily separated on the CarboPac MA1 column.

C. Charged Oligosaccharides

Charged oligosaccharides (for example, those that are sialylated, phosphorylated, sulfated, or contain carboxyl groups) are separated based on their composition, linkage, and the level of formal negative charge. They can be separated at both high (13) and low (4.6) pH. At low pH, the separations are largely dependent on the charge-to-mass ratio of the oligosaccharide but may also be influenced by linkage. Selectivity for sialylated oligosaccharides will change with pH as a result of oxyanion formation. The CarboPac PA100 is recommended for sialylated oligosaccharides, although in many cases the PA1 performs adequate separations.

D. Glycosaminoglycans

Oligosaccharides derived from glycosaminoglycans, such as nonsulfated chondroitin disaccharides, are separable on the CarboPac PA1.[6]

E. Linear Polysaccharides

Linear polysaccharides can be separated on the basis of length almost equally well on the CarboPac PA1 and PA100. The CarboPac PA1 has a slightly higher capacity than the PA100 and is the better column to use for linear homopolymers. The CarboPac PA100 was designed for nonlinear and heterogeneous polysaccharides. N, N – 1 resolution of linear polysaccharides has been demonstrated on the CarboPac PA1 and PA100 columns with inulin polymers to over 60 monosaccharide units. The CarboPac PA1 requires a higher sodium acetate concentration than the PA100 to elute species of the same length.

Table 4 summarizes the applications for which the three CarboPac columns are the most appropriate.

The CarboPac PA1 and PA100 are available in guard (4 × 50 mm), analytical (4 × 250 mm), semi-preparative (9 × 250 mm) and preparative sizes (22 × 250 mm). A guard column should be used in front of an analytical column to prolong the analytical column life. The CarboPac MA1 column is available in analytical and guard sizes. A partial list of column part numbers follows. Please contact your local Dionex office to order any column not listed below.

Part No.	Description
35391	CarboPac PA1 Analytical (4 × 250 mm)
43096	CarboPac PA1 Guard (4 × 50 mm)
39686	CarboPac PA1 Semipreparative (9 × 250 mm)
43055	CarboPac PA100 Analytical (4 × 250 mm)
43054	CarboPac PA100 Guard (4 × 50 mm)
44066	CarboPac MA1 Analytical (4 × 250 mm)
44067	CarboPac MA1 Guard (4 × 50 mm)

IV. Sample Stability at High pH

Carbohydrates undergo a number of well documented reactions at high pH that can potentially interfere with chromatography. However, in most cases these reactions are slow at room temperature and do not appear to occur to any noticeable extent over the time course of the chromatography. Some of these reactions are discussed below:

A. The Lowbry de Bruyn, van Ekenstein Transformations[7] (epimerization and keto-enol tautomerization)

D-fructose elutes as a single sharp peak with no evidence of formation of D-glucose or D-mannose via the Lowbry de Bruyn, van Ekenstein transformation. In addition, when glucose is left in 150 mM sodium hydroxide for four days at room temperature, there is no evidence for the presence of any mannose or fructose.

Table 4. CarboPac Columns Recommended by Application

	CarboPac PA1	CarboPac PA100	CarboPac MA1
Monosaccharides	+++	+/–	++
Sialylated branched oligosaccharides	++	+++	–
Neutral branched oligosaccharides	++	+++	+
Linear oligo- and polysaccharides	+++	+++	–
Reduced mono- and disaccharides	+	–	+++

+++ indicates most suitable
— indicates that the column is not recommended for this application.

Epimerization of *N*-acetyl glucosamine (GlcNAc) to *N*-acetyl mannosamine (ManNAc) has been demonstrated for solutions of GlcNAc in 100 mM sodium hydroxide. The equilibrium ratio of GlcNAc: ManNAc was 80:20 after 2–3 hours of exposure. This epimerization is not observed in separations using the CarboPac PA1 column, presumably because the sodium hydroxide concentration is 16 mM and the chromatography is sufficiently rapid (16 min) that exposure to alkali is minimized. Oligosaccharides are separated in 100 mM sodium hydroxide and are also retained longer on the column, particularly when sialylated. Under these conditions, oligosaccharides may exhibit 0 to 15% epimerization. As alditols do not epimerize in alkali, oligosaccharide epimerization can be eliminated if the oligosaccharide is reduced to the alditol prior to chromatography. For the same reason, monosaccharide alcohols are not epimerized in the high concentrations of alkali needed to elute them from the CarboPac MA1 column.

B. De-acetylation of N-acetylated Sugars

The hydrolysis of acylated sugars at high pH is another potential problem. Approximately 20% of a sample of *N*-acetylglucosamine is hydrolyzed to free glucosamine by exposure to 150 mM sodium hydroxide overnight at room temperature. However, chromatography of *N*-acetyl glucosamine at high pH generates a single sharp peak with no evidence of formation of the

(well resolved) free-base analog. Likewise, samples of *N*-acetyl neuraminic acid and *N*-glycolyl neuraminic acid are easily separated as sharp symmetrical peaks[8].

C. ß-Elimination or Peeling of 3-O-Substituents on Reducing Sugars

The ß-elimination of 3-O-substituents on reducing sugars is also a potentially serious side reaction that proceeds, in most cases, too slowly at room temperature to be a problem. The treatment of laminaribiose (glucopyranosyl ß-1-3 glucopyranose) with 150 mM sodium hydroxide for 4 h destroys more than 80% of the disaccharide, producing glucose and a second unidentified peak. However, laminaribiose generates a single peak during chromatography by HPAE with no evidence of glucose or other breakdown products.[8] Conversely, D-glucose-3-sulfate, which has a very good leaving group, decomposes rapidly during chromatography.

PULSED AMPEROMETRIC DETECTION

I. Theory of Operation

Pulsed amperometry permits detection of carbohydrates with excellent signal-to-noise ratios down to approximately 10 picomoles without requiring derivatization. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode. The products of this oxidation reaction also poison the surface of the electrode, which means that it has to be cleaned between measurements. This is accomplished by first raising the potential to a level sufficient to oxidize the gold surface. This causes desorption of the carbohydrate oxidation products. The electrode potential is then lowered to reduce the electrode surface back to gold. The sequence of potentials is illustrated in Figure 2.

Pulsed amperometric detection thus employs a repeating sequence of three potentials. Current from carbohydrate oxidation is measured at the first potential, E_1. The second, E_2, is a more positive potential that oxidizes the gold electrode and cleans it of products from the carbohydrate oxidation. The third potential, E_3, reduces the gold oxide on the electrode surface back to gold, thus permitting detection during the next cycle at E_1.

The three potentials are applied for fixed durations referred to as t_1, t_2, and t_3. The step from one potential to the next produces a charging current that is not part of the analyte oxidation current, so the analyte oxidation current is measured after a delay that allows the charging current to decay. The carbohydrate oxidation current is measured by integrating the cell current after the delay. Current integrated over time is charge, so the detector response is measured in coulombs. Alternatively, the average current during the integration period can be reported. In this case, the units used are amperes.

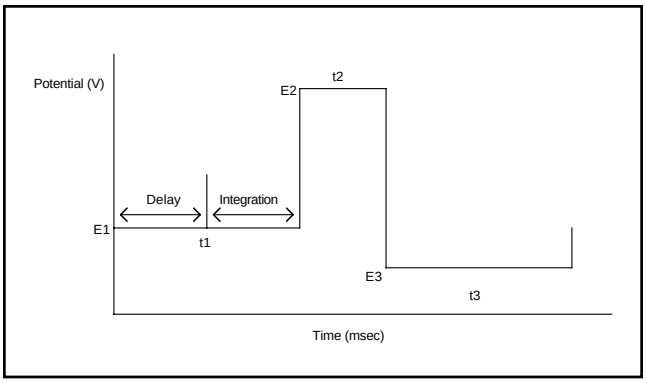

Figure 2. *Diagram of the pulse sequence for carbohydrate detection.*

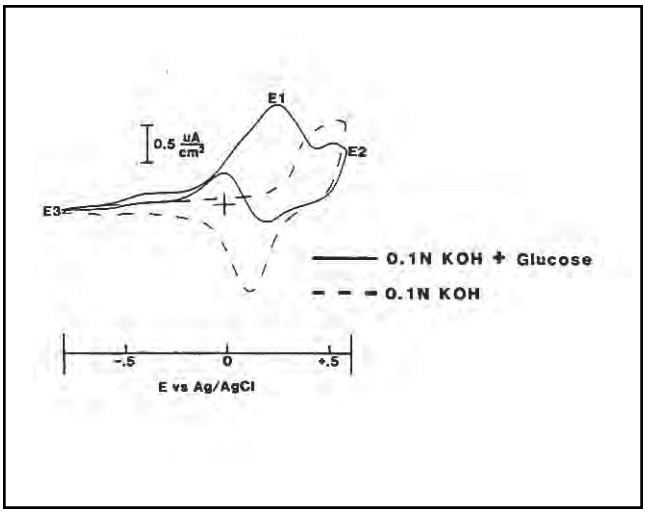

Figure 3. *Cyclic voltammetry of glucose on a gold electrode.*

Optimal potentials can be determined by electrochemical experiments such as cyclic voltammetry, in which the applied potentials are slowly scanned back and forth between positive and negative potential limits. The resulting current is plotted on the Y-axis with oxidation (anodic) currents up and reduction (cathodic) currents down. Figure 3 shows the cyclic voltammogram of glucose in a 100 mM potassium hydroxide solution on a gold electrode. The dashed line is a background scan of a solution of 100 mM potassium hydroxide. As the potential is raised, the current starts to rise at about 0.2 V (see Figure 3, upper dashed

line). This is caused by oxidation of the gold surface. Reduction of the surface gold oxide back to gold occurs on the reverse scan (lower dashed line) with a cathodic (negative) current peak at about 0.1 V.

When glucose is present (solid line), its oxidation peaks at about 0.25 V (upper solid trace), which is also the potential at which formation of gold oxide begins. The glucose oxidation current drops as gold oxidation continues to increase, demonstrating that the formation of gold oxide inhibits oxidation of glucose. On the reverse scan, the current actually reverses from negative to positive at the onset of gold oxide reduction, further evidence of the inhibiting effect of gold oxide on the oxidation of glucose. It is thus important to use a measuring potential (E_1) below that required for gold oxidation.

All three potentials are important. However, the most important is E_1 — the potential at which the carbohydrate oxidation current is measured. A plot of detector response as a function of E_1 is shown in Figure 4. The background current is also shown. The maximum response is shown to occur at about 0.2 V for the three sugars tested, although the best signal-to-noise ratio actually occurs at a slightly lower potential. Figure 4 shows that the voltage at which the maximum response occurs is the same for three very different sugars: xylitol, a nonreducing sugar alcohol; glucose, a reducing monosaccharide; and sucrose, a nonreducing disaccharide. This is because the oxidation of the sugars

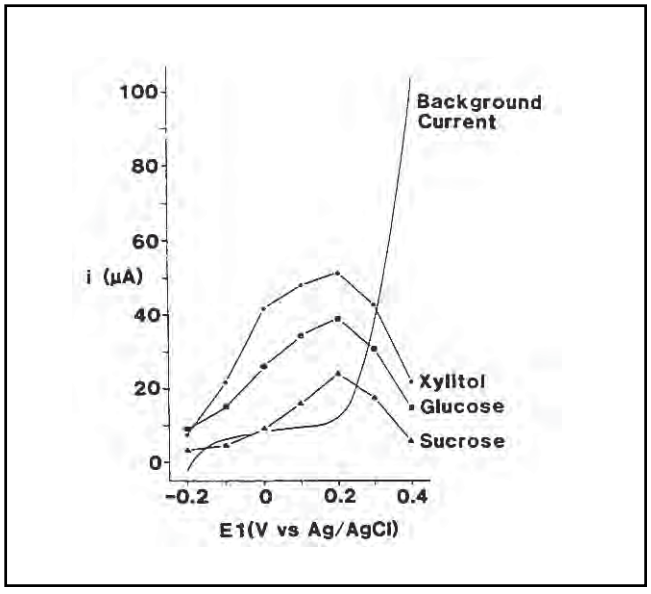

Figure 4. *The oxidation current generated at different values of E_1 for three different carbohydrates.*

at the electrode is catalyzed by the electrode surface. As a result, the amperometric response of a class of compounds is controlled primarily by the dependence of the catalytic surface state on the electrode potential and not on the redox potentials of the compounds themselves. Pulsed amperometric detection is thus a universal detection method for all carbohydrates, although derivatization of two or more hydroxyl groups will decrease (and may even abolish) detection.

Potential E_2 must be high enough and long enough to oxidize the electrode surface fully so that the carbohydrate oxidation products are completely removed. This potential cannot be too high, however, or excessive gold oxidation will occur and the electrode will wear too rapidly. The third potential, E_3, must be low enough to reduce the oxidized surface of the gold electrode completely without being so low that chemical reductions (for example, of oxygen to hydrogen peroxide) will occur. The results of these reactions may cause baseline disturbances during subsequent measurement at E_1.

Recommended pulse sequences for the Dionex pulsed amperometric detectors are given in Technical Note 21, which is available from your local Dionex representative.

APPLICATIONS

I. Eluent Preparation for Carbohydrate Analysis

When making eluents for carbohydrate analysis, it is important to use reagents of the grade listed:

- 50% (w/w) Sodium hydroxide solution
  Fisher Cat. No. SS254-1
- Anhydrous sodium acetate
  Fluka Cat. No. 71179

- Sodium Hydroxide: It is extremely important to minimize contamination of the eluent solutions with carbonate. Carbonate, being a divalent anion at pH ≥12, binds strongly to the columns and interferes with carbohydrate binding, causing a drastic decrease in column selectivity and a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should *not* be used. A 50% w/w sodium hydroxide solution is much lower in carbonate. Any carbonate present will precipitate to the bottom of the container and can be avoided. The concentration of

the 50% sodium hydroxide solution is approximately 19.3 M, so diluting 20.8 mL of a 50% solution into 2 L of water yields a 0.2 M sodium hydroxide solution.

- Distilled Water: It is essential to use high-quality water. It is critical that there be as little dissolved carbon dioxide as possible in the water. It should also be of high resistivity (18 MΩ) and biological contamination should be absent. The use of fresh Pyrex® glass-distilled water is recommended. The still should be fed with high-resistivity (18 MΩ) water, and the use of plastic tubing should be avoided because it often supports microbial growth. Biological contamination is often the source of unexpected glucose peaks after acid hydrolysis.

A. Guidelines to Handling the 50% (w/w) Sodium Hydroxide Solution

1. To avoid mixing of the sodium carbonate precipitate into the solution, store the 50% sodium hydroxide close to where the eluent will be prepared. *Do not shake or stir this solution.*
2. Never pour the solution from the bottle.
3. Pipet sodium hydroxide from the center of the solution, not from edges or bottom. Do not allow the pipet to stir the solution at the bottom.
4. Use only plastic pipets, as sodium hydroxide leaches borate and silicate out of glass. Borate will complex with carbohydrates and will thus alter their chromatographic behavior.
5. Never return unused liquid to the bottle.
6. Close the bottle immediately after each use and leave open for the shortest time possible to avoid carbon dioxide absorption.
7. Discard the bottle of 50% sodium hydroxide when 2 to 3 cm or less of solution remains.

B. Eluent Preparation

It is impossible to completely eliminate all carbonate from eluents. Therefore, to ensure reproducible chromatography, it is essential to use the same methods consistently in preparing the solutions. Once eluents have been prepared, they should be kept blanketed under helium (5–7 psi/34–48 kPa) at all times.

i. Distilled Water

High-quality water should be degassed by one of the following two methods:

1. Sparging with helium for 20–30 min. Degassing is complete when all of the small bubbles first formed upon degassing disappear.
2. Sonication for 30–60 s while degassing with a water vacuum aspirator, followed by a 10-min helium sparge. Degassing is again complete when all of the small bubbles first formed upon degassing disappear.

ii. Sodium Hydroxide

Degas the required volume of water, as described above. After degassing is complete, use a plastic pipet to add the appropriate amount of 50% sodium hydroxide solution to give the required concentration. Avoid bubbling air into the eluent when expelling the 50% sodium hydroxide solution from the pipet. Rinse the pipet by drawing some of the sodium hydroxide/water mixture into the pipet and expelling it back into the solution. Repeat this several times. Add a stirring bar to the mixture and stir gently without agitating the surface for about 2 min.

As an alternative, the 50% sodium hydroxide can be pipetted directly into the distilled water as it is being sparged by the Dionex Eluent Degas Module (EDM). Sparge the water for 15 min, add the sodium hydroxide, rinse the pipet, and swirl the solution in the bottle to mix. Then sparge the solution for an additional 5 min. The sparging will complete the mixing.

Both methods work well. It is most important to be consistent in the method used. Store sodium hydroxide solutions in plastic containers, as they will leach borate and silicate out of glass.

iii. Sodium Hydroxide/Sodium Acetate Solutions

Degas the required volume of water as described above, and transfer it to a graduated cylinder. Add a stir bar and start stirring, while steadily adding the anhydrous crystalline sodium acetate. After the salt dissolves, retrieve the stir bar and add the appropriate volume of 50% (w/w) sodium hydroxide to the graduated cylinder in the same manner as described previously. Bring the volume to the requisite level (e.g., 1–2 L). Vacuum filter the mixture through a 0.2-µm nylon filter. Alternatively, sparge the filtered acetate solution for 15 min, add the sodium hydroxide, swirl the solution to mix, and continue to sparge for 5 min. Once again, it is important to be consistent in the method used to make up the solution.

Sodium acetate solutions should last about one week. The most consistent chromatography has been obtained using sodium acetate purchased from Fluka.

II. Sample Preparation

It is recommended that all samples other than pure standards be passed through a 0.45-µm nylon filter prior to injection to remove particulates. Cellulose acetate and other filters should be avoided because they may leach carbohydrates. Filters of a type not previously verified as "clean" should be evaluated for contribution of "PAD-active" components before use.

Sample preparation is obviously dependent on sample matrix complexity and, as such, the recommendations that follow should be considered as guidelines only. In particular, the effect of sample pretreatment cartridges on the carbohydrate analytes themselves should be predetermined using standard solutions. It may be found that some carbohydrates have a strong affinity for particular cartridge packing materials. This is obviously of importance for quantification and in the detection of low levels of carbohydrates.

A. Samples Containing High Levels of Protein or Peptides

Physiological fluids such as plasma, urine, or other samples containing high levels of proteins should be deproteinized first. This may be achieved by standard precipitation procedures or by passing the analyte solution through a hydrophobic filter cartridge such as the Dionex OnGuard® RP Cartridge (P/N 39595).

B. Samples Containing High Levels of Humic Acids or Phenolics

To remove the phenolic fraction of humic acids, tannic acids, or lignins found in food samples (such as wine), the sample may be passed through a polyvinylpyrrolidone (PVP) filter cartridge, such as the Dionex OnGuard-P Cartridge (P/N 39597).

C. Samples Containing Halides

To remove halides, the sample may be passed through a Dionex OnGuard-Ag cartridge (P/N 39637). This cartridge selectively removes Cl^-, Br^- and I^- in preference to other anionic species. This cartridge is, however, a cation exchanger, so amino sugars will be extracted unless they are *N*-acetylated.

D. Samples Containing Sulfate and Other Anions

Sulfate may be precipitated as the barium salt by addition of barium hydroxide solution. However, it should be noted that some carbohydrates may co-precipitate with the barium sulfate in this procedure, especially carbohydrates bearing sulfate esters. The Dionex OnGuard A cartridge (P/N 42102) is designed specifically to remove anion contaminants from sample matrices. OnGuard A cartridges contain styrene-based anion-exchange resin in the bicarbonate form. They should not be used with samples that contain sialic acids, or sugars with other acid substituents.

III. Standard Chromatography Conditions for the Analysis of Carbohydrates

The conditions described in this section have been found to give reliable separations of the common classes of carbohydrates using HPAE chromatography. Samples and their matrices vary, therefore these conditions are intended to be used as guidelines only.

A. Monosaccharides—Neutral and Amino Sugars

These sugars can be successfully separated on the CarboPac PA1 column using isocratic conditions with 16 mM sodium hydroxide as the eluent. A representative chromatogram is shown in Figure 5. Because the concentration of sodium hydroxide used for the separation is only 16 mM, the column should be regenerated after each run. Otherwise, carbonate will start to contaminate the column, irrespective of the care taken to eliminate it from eluents and samples. Regenerate the column by washing it with 200 mM sodium hydroxide for 10 min at a flow rate of 1.0 mL/min. This procedure will also remove other strongly bound contaminants such as peptides and amino acids. *This step is extremely important and should not be omitted.* After washing, the column should be reequilibrated with 16 mM sodium hydroxide at a flow rate of 1.0 mL/min for 10 min. It is very important to keep the rinse and reequilibration times consistent from run to run.

B. Sugar Alcohols

Mono- and oligosaccharide sugar alcohols can be separated using the CarboPac MA1 column with sodium hydroxide eluents. Examples of isocratic separations are shown in Figures 6 and 7. Gradients can be used to improve separations (Figure 8) or to acceler-

ate the elution of late-eluting components (Figure 9). Table 2 shows that the elution order of certain carbohydrates may be altered by changing the sodium hydroxide concentration.

C. Sialic Acids, Sialylated, and Phosphorylated Oligosaccharides

The elution of acidic sugars from the CarboPac PA1 or the CarboPac PA100 columns requires stronger eluents than those used with neutral sugars. This is usually accom-plished by the addition of sodium acetate to the sodium hydroxide eluent. Sodium acetate accelerates the elution of strongly bound species without compromising selectivity and without interfering with pulsed amperometric detection. Sodium acetate/sodium hydroxide solutions can be used isocratically (Figure 10) or in gradients (Figsure 11 and 12). Sodium acetate gradients are *not recommended* for the CarboPac MA1 column because column regeneration will require several hours. The use of sodium acetate gradients is not a problem with the CarboPac PA1 and CarboPac PA100, because these columns have a lower anion-exchange capacity and thus regenerate quickly.

To maintain baseline stability, it is helpful 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 eluents as follows:

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

When devising gradients for the analysis of a carbohydrate sample of unknown composition, it is generally good practice to run a "scouting gradient". This gradient consists of a rapid linear gradient (t ≤ 15 min) from a low to a sufficiently high acetate concentration that it will elute all of the components. It is then possible to fine tune the separation, with the assurance that the gradient is sufficiently broad to include all of the sample components.

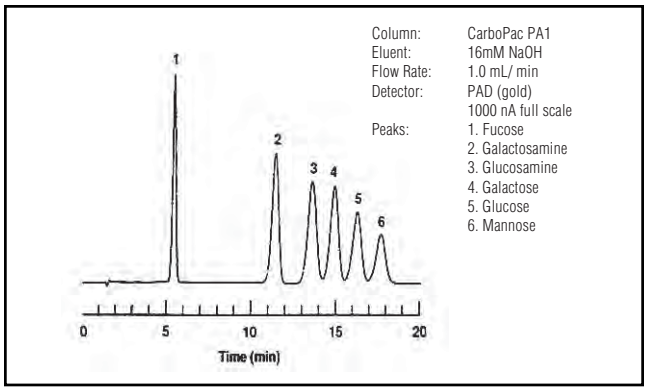

***Figure 5**. Separation of neutral and amino monosaccharides derived from glycoproteins.*

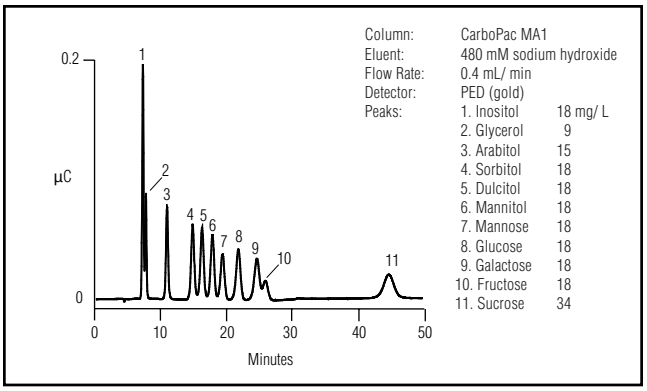

***Figure 7**. Separation of reducing and nonreducing carbohydrates. Food alditols and aldoses are separable under isocratic conditions on the CarboPac MA1.*

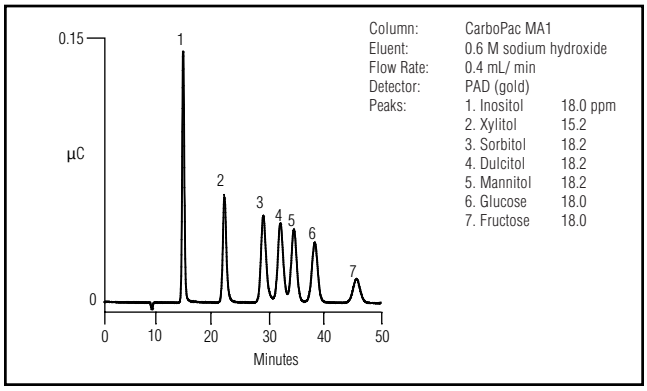

***Figure 6.** Isocratic separation of a group of alditols plus glucose and fructose on the CarboPac MA1 column.*

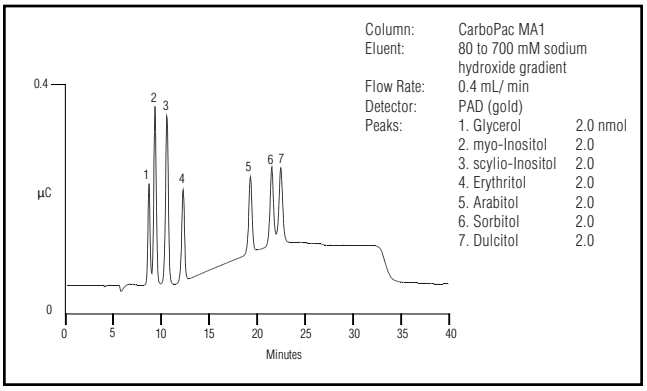

***Figure 8**. Separation of alditols found in biological fluids. The NaOH gradient improves the separation of sorbitol and dulcitol, which are poorly resolved at NaOH concentrations that permit resolution of glycerol from inositol.*

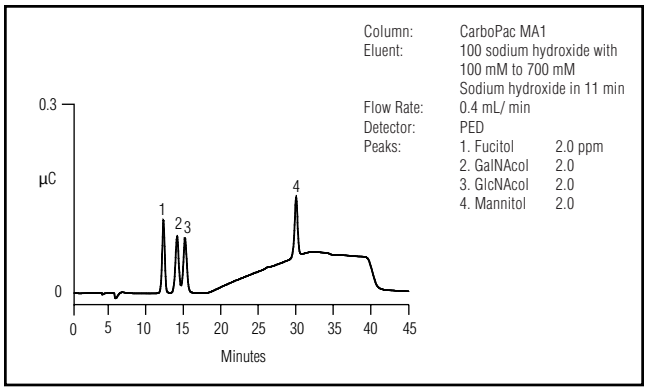

***Figure 9**. Separation of monosaccharide alditols released by direct ß-elimination from glycoproteins. The hydroxide gradient following the isocratic separation of the first three components accelerates the elution of mannitol as well as any oligosaccharide alcohols that may have been released during the ß-elimination process.*

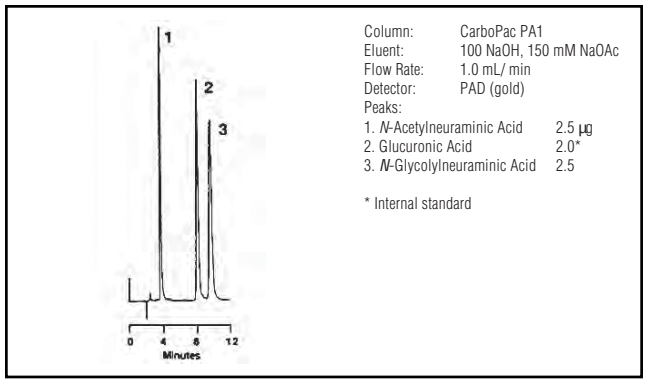

***Figure 10.** Isocratic separation of sialic acids using a sodium hydroxide/sodium acetate mixture.*

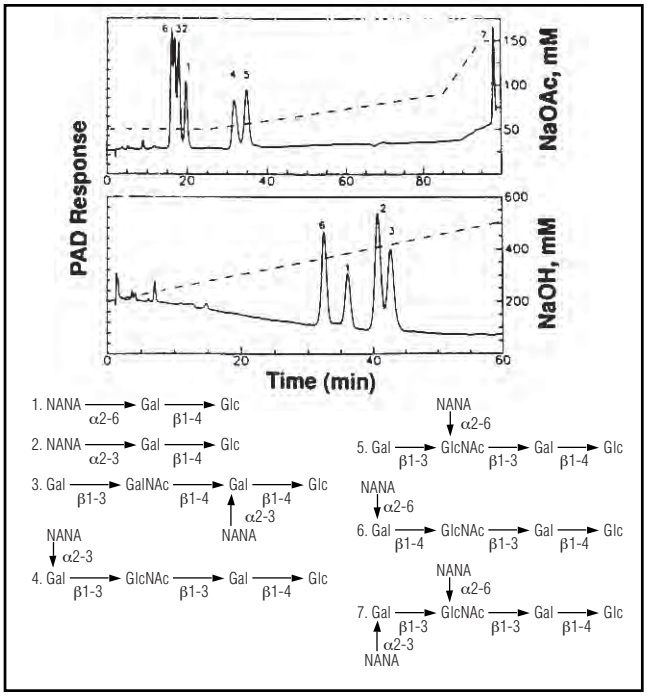

***Figure 11**. Gradient separation of sialylated oligosaccharides using the CarboPac PA1 column.*

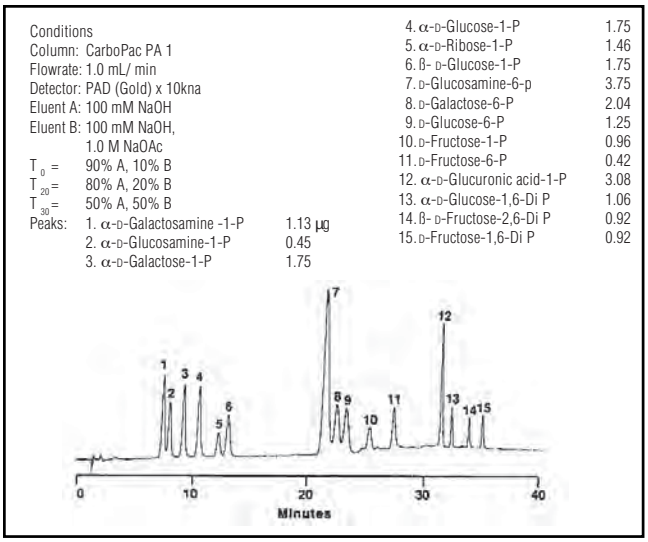

***Figure 12**. Analysis of mono- and diphosphorylated monosaccharides.*

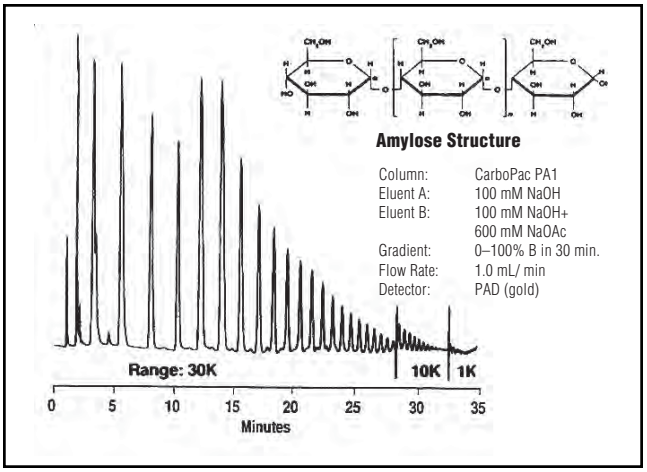

***Figure 13**. Analysis of "Dextrin 7" glucose polymer.*

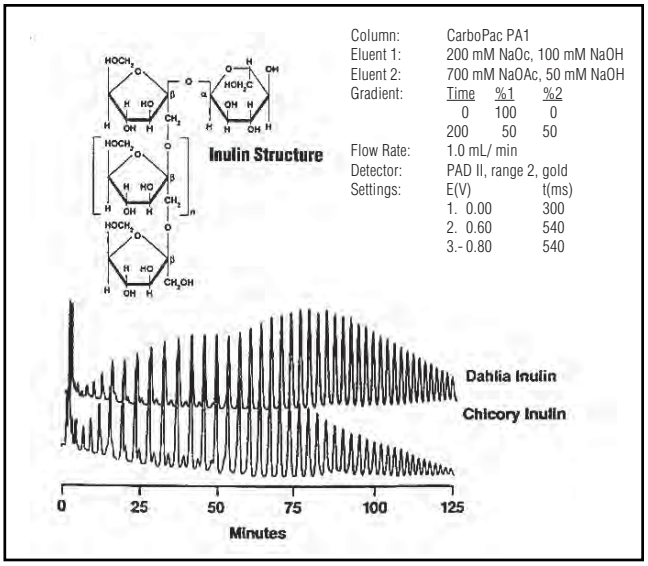

***Figure 14**. Comparison of water washed inulins (Cichorium intybus vs Dahlia sp.) using the CarboPac PA1.*

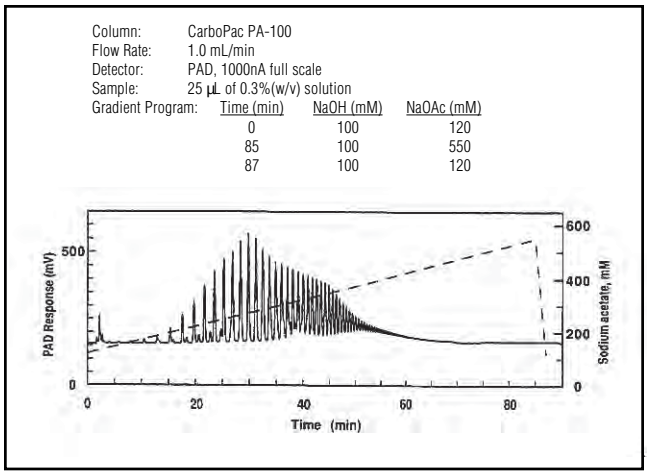

Figure 15. *Gradient separation of chicory inulin using the CarboPac PA100.*

Sialic acids can also be separated at neutral pH. This is particularly useful for O-acetylated species, which are unstable at high pH[9–11].

D. Oligo- and Polysaccharides

Separations of high mannose, hybrid, and complex oligosaccharides are best accomplished using the CarboPac PA100 column. Linear homopolymers are successfully separated on the CarboPac PA1 or PA100. In all cases, separations are accelerated and improved by using sodium acetate gradients in sodium hydroxide. Sodium hydroxide gradients are also useful. Separation of several polysaccharides using either the CarboPac PA1 or the PA100 are shown in Figures 13 through 15. Figure 14 also shows the structure of inulin.

Figure 16 shows the separation of a group of neutral glycoprotein-derived oligosaccharides on the CarboPac PA100, while Figures 17 through 20 show examples of how HPAE-PAD analysis on the CarboPac PA100 column can be used to map oligosaccharides released from glycoproteins by enzyme digestion. Note that in Figure 18 the two oligosaccharide peaks marked 1 and 2 differ only in the linkage position of the sialic acid on one branch of the triantennary structure ($\alpha 2 \rightarrow 6$ versus $\alpha 2 \rightarrow 3$).

Neutral O-linked oligosaccharides released by reductive elimination are alditols and may be best separated on the CarboPac PA1 column (≥3 carbohydrate units) or on the CarboPac MA1 column (<3 carbohydrate units).

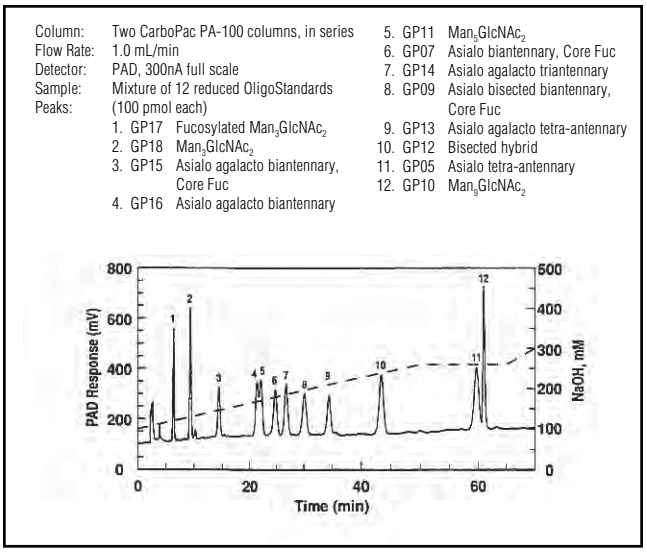

Figure 16*. Separation of Dionex OligoStandard™ glycoprotein-derived oligosaccharides.*

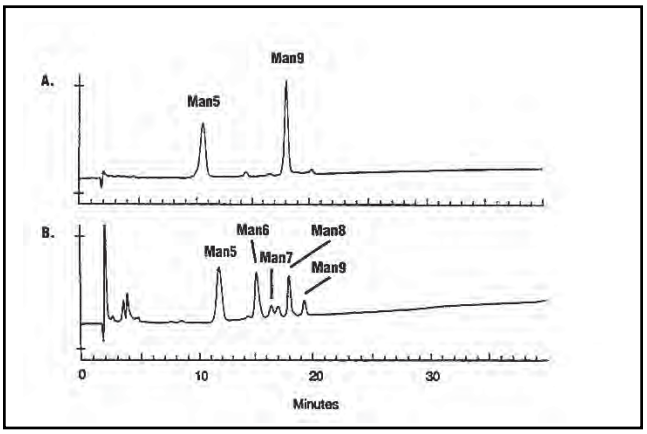

Figure 17*. Separation of glycoprotein-derived mannose oligosaccharides using the CarboPac PA100 column. Panel A: Dionex OligoStandards GP11 and GP10, $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$, respectively. Panel B: Endonuclease H digest of ribonuclease B.*

V. Postcolumn Addition of Base

To optimize baseline stability and detector sensitivity, it is sometimes necessary to add strong base to the eluent stream postcolumn, particularly when using neutral pH eluents or when the eluent contains a low concentration sodium hydroxide. Postcolumn addition of base can help with quantification, in part by maintaining a more constant hydroxide concentration at the electrode. Users have found, however, that postcolumn addition of base is often unnecessary with routine isocratic and gradient separations at sodium hydroxide concentrations ≥15 mM.

A. Separations at Low pH

Sialic acids, sialylated oligosaccharides, and other carbohydrates bearing strongly acidic substituents can be separated by anion exchange at lower pH values.[10,11] This option is particularly useful when analyzing oligosaccharides that possess O-acetylated sialyl groups, because these groups are unstable at high pH. When low-pH eluents are used, sodium hydroxide must be added to the eluent after it has left the column and before it enters the detector, because carbohydrates are best detected at gold electrodes when pH ≥12.

B. Sodium Hydroxide Gradients

Changing the sodium hydroxide concentration alters the pH of the solution, which can affect the detector electrode response. While the PED and the solvent compatible PAD are affected very little by pH changes, the standard PAD-2 cell is fairly sensitive. A gradient from 10 mM sodium hydroxide to 100 mM sodium hydroxide results in an effective change of 1 pH unit during the gradient. The effect on detector response can be minimized by the use of optimized pulse sequence settings. Optimized pulse sequence settings are discussed in detail in Technical Note 21, which can be obtained from your local Dionex representative. If necessary, however, a solution of sodium hydroxide can be added postcolumn to minimize the pH shift. For example, the addition of 300 mM sodium hydroxide to the column effluent of a gradient of 10 to 100 mM sodium hydroxide would result in a total pH change of only 0.11 units (if the flow rates of the postcolumn base and eluent were equal). A pH change of this magnitude would generate a negligible baseline shift.

Postcolumn base can be delivered through a mixing T using the Dionex Postcolumn Pneumatic Controller. To ensure run-to-run reproducibility, the Controller should be adjusted so that:

1. The flow rate is constant.
2. The mixture entering the detector has a pH ≈ 13.
3. The flow rate of the mixture of eluent plus postcolumn base stays the same from run to run.

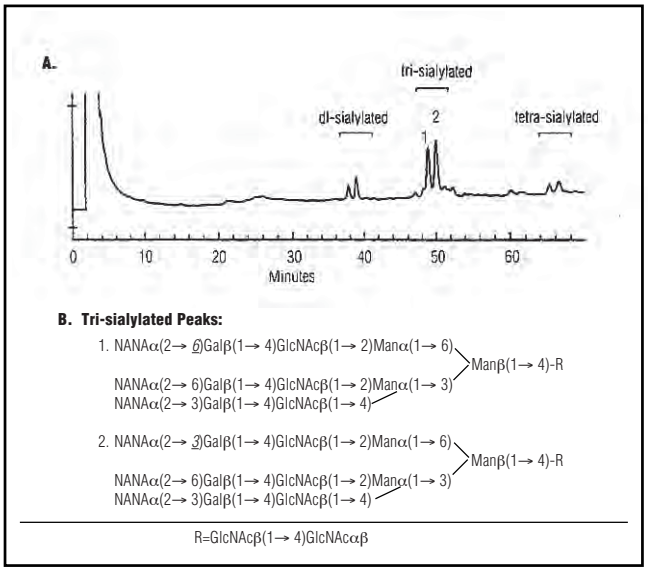

***Figure 18**. Separation of fetuin N-linked oligosaccharides. Panel A: HPAE-PAD analysis using a CarboPac PA100. Panel B: Structures of the trisialylated species, peaks 1 and 2.*

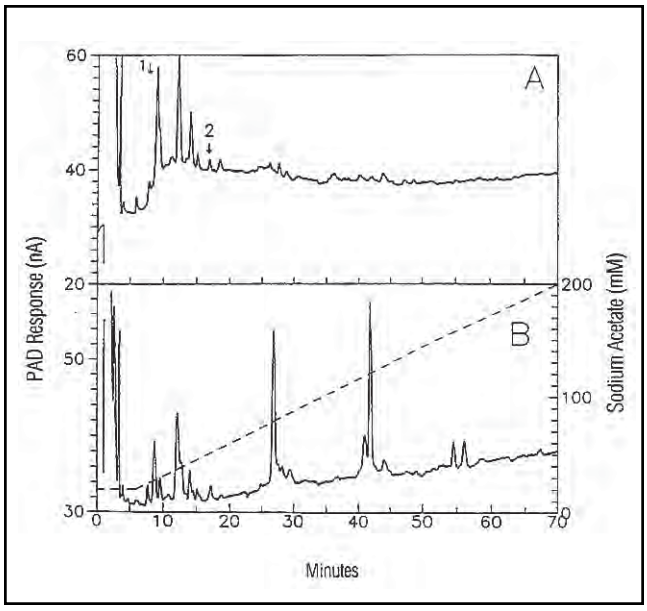

***Figure 19**. Gradient separation (using the CarboPac PA100 column) of oligosaccharides released by sequential enzyme digestion of recombinant tissue plasminogen activator (rtPA). Panel A: High-mannose oligosaccharides released from rtPA by digestion with endonuclease H. The elution positions of $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$ are indicated by the numbered arrows. Panel B: Oligosaccharides released from rtPA by endonuclease F_2 (cleaves predominantly biantennary-type chains). (From Weitzhandler et al.[12] Reproduced with permission.)*

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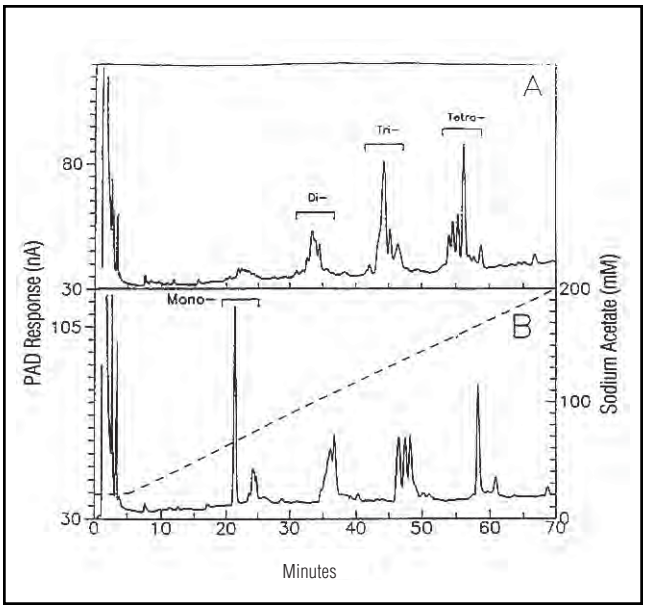

Figure 20. *Gradient separation (using the CarboPac PA100 column) of oligosaccharides in recombinant erythropoietin (rEPO). Panel A: Oligosaccharides released by PNGase F digestion.*
Panel B: Digestion of the mixture in panel A by endo-ß-galactosidase shows that three of the four major tetrasialylated species contain polylactosamine structures.
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