Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

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Goal

Update Dionex Technical Note 20 to show the current status of HPAE-PAD while still providing a description of its basic principles

Introduction

Nearly 40 years after it was first introduced, HPAE-PAD is a well-established technique for separating and quantifying a wide range of carbohydrates. HPAE-PAD has replaced or augmented many of the methods used 40 years ago. There are numerous reasons for the acceptance and popularity of HPAE-PAD for carbohydrate analysis, but undoubtedly the two main reasons are high-resolution separations combined with sensitive (low-picomole quantities) direct detection. In other words, more carbohydrates can be determined with small amounts of sample and no laborious sample derivatization. With these benefits, HPAE-PAD has been applied to a broad range of carbohydrates in a wide variety of samples. HPAE-PAD is selective and specific for carbohydrates because:

1. Pulsed amperometry detects only those compounds that contain functional groups that are oxidizable at the detection voltage applied. Even when non-carbohydrates are detected under the conditions used for carbohydrates, the sensitivity for carbohydrates is typically orders of magnitude greater than for the other oxidized compounds.
2. Neutral or cationic sample components elute in, or close to, the void volume of the column. Therefore, even if such species are oxidizable, they do not usually interfere with the determination of the carbohydrates of interest.

The success and popularity of HPAE-PAD have resulted in numerous published reviews. References one through ten are a sampling of older and newer reviews.[1-10] This Technical Note discusses the basics of the separation and detection mechanisms of HPAE-PAD, the anion-exchange columns used for HPAE-PAD, good practices for successful HPAE-PAD, limitations of the technique, eluents for HPAE-PAD, sample preparation considerations, and a brief review of some of the major applications of HPAE-PAD.

Anion-exchange chromatography

Prior to the introduction of HPAE-PAD, the only types of carbohydrates that anion-exchange chromatography was used for were acidic carbohydrates, which are negatively charged, and negatively charged glycoconjugates such as glycopeptides. High-performance anion-exchange chromatography of neutral (uncharged) carbohydrates is possible because they are weak acids, and thus at high pH they ionize and can be separated as anions. Table 1 lists dissociation constants of some common carbohydrates, revealing the need for a mobile phase pH>11 to produce oxyanions. Researchers found that for mono- through tetrasaccharides a carbohydrate's acidity directly impacted its HPAE-PAD retention.[11] The more acidic the carbohydrate, the more tightly it was bound to the HPAE column. Deuteration has a small impact on a carbohydrate's acidity, and it was shown that this impact, while small, was large enough to separate deuterated glucoses by HPAE.[12] Closer inspection also shows the importance of a carbohydrate's anomeric hydroxyl group to its acidity. Glucose has a pKa of 12.28. When its anomeric hydroxyl is reduced to form sorbitol, its pKa increases to 13.60, and when its anomeric hydroxyl is methylated to form α-methylglucoside, its pKa increases to 13.71. This means that the mobile phase pH will need to be higher for oxyanion formation for sorbitol and α-methylglucoside than for glucose. Classical silica-based HPLC columns are rapidly destroyed by high pH, so a new type of column had to be developed for HPAE-PAD. This column type is discussed in the next section.

Table 1. Dissociation constants of some common carbohydrates[13] (in water at 25 °C)

Sugar	pK_a
Frutose	12.03
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Dulcitol	13.43
Sorbitol	13.60
α-Methylgluoside	13.71

Columns for HPAE

To take advantage of the fact that neutral carbohydrates are anions at high pH, Thermo Fisher Scientific (then Dionex Corporation) designed polymeric anion-exchange columns that can be used with pH 0–14 mobile phases. The first of these columns was the Thermo Scientific™ Dionex™ CarboPac™ PA1 column. The resin in this column has the basic structure shown in Figure 1. For the Dionex CarboPac PA1 column, the resin is prepared from polymeric nonporous 10 µm diameter beads that are surface sulfonated and then coated (latexed) with small beads containing the anion-exchange group (a quaternary amine). The small beads (latex) are electrostatically bound to the larger beads (substrate particles). This process creates a pellicular high-performance anion-exchange resin. The small beads create short diffusion pathlengths and thus rapid mass transfer that yields sharp chromatography peaks. These sharp peaks and the resin selectivity yield high-resolution carbohydrate separations. Most of the Dionex CarboPac family of columns are created with the same synthetic strategy with variation in the substate and latex bead diameters, the crosslinking of those beads, and the quaternary amine anion-exchange groups. Three Dionex CarboPac columns have a different column architecture, the Dionex CarboPac MA1, the Dionex CarboPac SA10, and Dionex CarboPac PA300 columns.

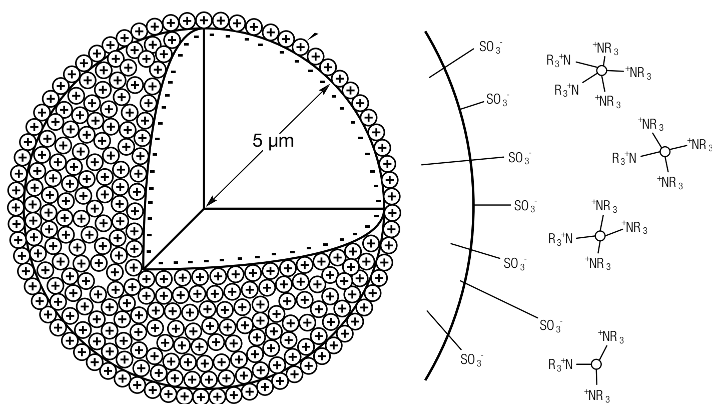

Figure 1. Pellicular anion-exchange resin bead

The Dionex CarboPac MA1 column is packed with a macroporous polymeric resin, which, as a result of surface amination, has the anion-exchange groups on the particle surface (Figure 2). Thus, this is not a latexed resin. This column has 45 times the anion-exchange capacity of the Dionex CarboPac PA1 column. Therefore, 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 sugar alcohols and other carbohydrates with pK_a values higher than typical monosaccharides (e.g., glucose), yielding improved retention and resolution of these compounds compared to the Dionex CarboPac PA1 column and similarly constructed Dionex CarboPac columns.

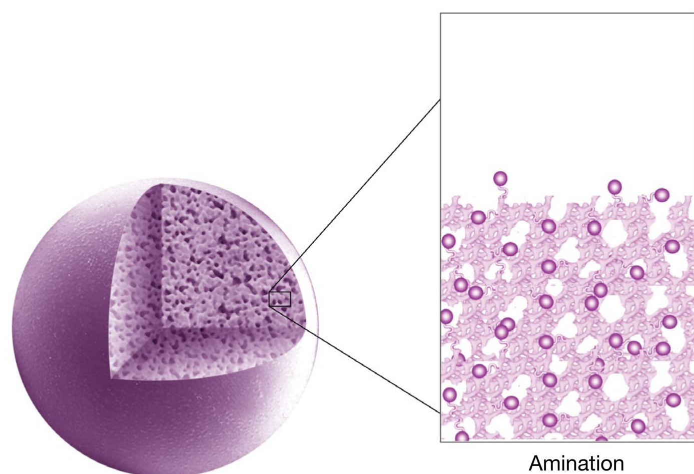

Figure 2. Dionex CarboPac MA1 column construction

The Dionex CarboPac SA10 column is packed with a supermacroporous (2,000 Å pores) polymeric resin that is latexed (Figure 3). This results in a high-capacity column for fast separations of monosaccharides derived from plants that are difficult to resolve with the other members of the Dionex CarboPac column family. The Dionex CarboPac SA10 column is packed with resin having 6 µm beads but is also available with 4 µm beads. The Dionex CarboPac PA300 column uses the same column architecture but is only available with 4 µm beads.

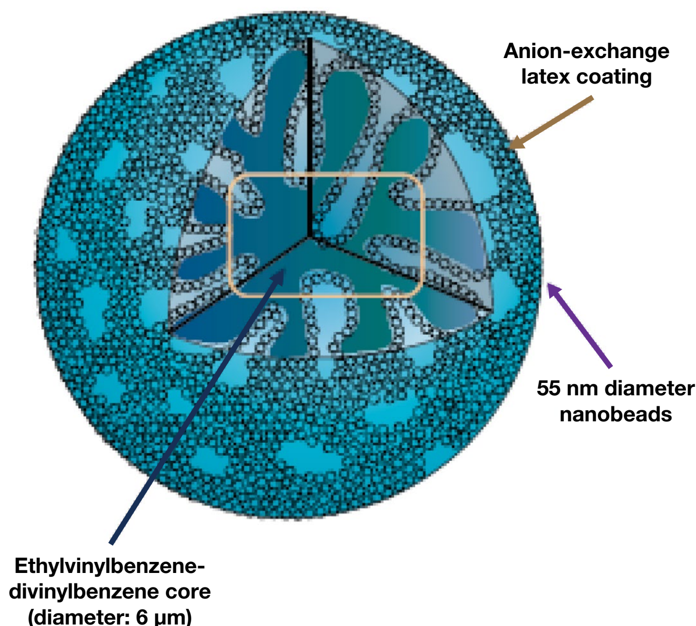

Figure 3. Dionex CarboPac SA10 column construction

Table 2 shows the complete family of Dionex CarboPac columns with their major applications and other notes. The reader should look at the column manuals for details on the column's construction, limitations, and maintenance. When designing a new carbohydrate separation, the analyst should start with the following columns for specific classes of carbohydrates. To separate monosaccharides and negatively charged monosaccharides (e.g., sialic acids and sugar phosphates), the analyst should start with the Dionex CarboPac PA20 column. Analysts seeking a fast sialic acid analysis solution should use the Dionex CarboPac PA20 Fast Sialic Acid column. For oligosaccharide separations, the Dionex CarboPac PA200 column is the first choice. The Dionex CarboPac MA1 column is best used for mono- and disaccharide alditols, especially when they need to be determined simultaneously with mono- and disaccharides. There are three newer columns for applications not well covered by these four columns.

Table 2. Columns for HPAE-PAD listed in the order of their introduction

Column*	Major application(s)	Comment(s)
Dionex CarboPac PA1	Mono- and oligosaccharides, sialic acids	The original HPAE-PAD column, a general-purpose column
Dionex CarboPac PA100	Oligosaccharides	Improvement for the Dionex CarboPac PA1 column but has been replaced by the Dionex CarboPac PA200 and PA300 columns.
Dionex CarboPac MA1	Sugar alcohols, monosaccharides, anhydrosugars	Dionex CarboPac PA300 column replaces the Dionex CarboPac MA1 column for some sugar alcohol applications.
Dionex CarboPac PA10	Monosaccharides, sialic acids	Improvement for the Dionex CarboPac PA1 column but has been replaced by the Dionex CarboPac PA20 column
Dionex CarboPac PA20	Monosaccharides, sialic acids	New monosaccharide and sialic acid applications should start with this column.
Dionex CarboPac PA200	Oligosaccharides	New oligosaccharide applications should start with this column.
Dionex CarboPac PA20 Fast Sialic Acid	Sialic acids	Sialic acid determinations in less than five minutes
Dionex CarboPac SA10	Monosaccharides from plants and in biofuel samples	This column provides the best separation of monosaccharides derived from plants.
Dionex CarboPac PA210	Mono- to tetrasaccharides in food samples	
Dionex CarboPac PA300	*O*-linked glycans, other oligosaccharides weakly retained on the Dionex CarboPac PA200 column, sugar alcohols, anhydrosugars	

* These columns are available in a variety of formats and sometimes particle sizes. For more information about these columns, including their manuals and part numbers, please see www.thermofisher.com.

For monosaccharides derived from plants, the Dionex CarboPac SA10 column is the best choice. Using only weak hydroxide eluents, it can better resolve these carbohydrates than the Dionex CarboPac PA20 column, and it has the column capacity to handle the sulfate from sulfuric acid hydrolysis of plant material used to produce monosaccharides. The Dionex CarboPac PA210 column is the best choice when a set of mono- to tetrasaccharides from foods or plant material must be determined. The newest column, the Dionex CarboPac PA300 column, is best for uncharged oligosaccharides poorly retained on the Dionex CarboPac PA200 column, including those that are linked to a serine or threonine (*O*-linked oligosaccharides (glycans)) in a glycoprotein. The Dionex CarboPac PA300 column can also separate monosaccharide alditols with lower hydroxide concentrations, those suitable for electrolytic eluent generation, though it cannot resolve as many monosaccharide alditols and monosaccharides as the Dionex CarboPac MA1 column.

The remaining three columns, the Dionex CarboPac PA1, PA10, and PA100 columns are older generation columns. These are best used for established methods, including methods published by standards setting or regulatory bodies in which the column is specified by name.

Sample stability at high pH

Carbohydrates undergo some 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.

The Lobry de Bruyn-Van Ekenstein transformation (epimerization and keto-enol tautomerization)[14]

Using HPAE-PAD methods at room temperature and hydroxide concentrations 100 mM and less, D-fructose elutes as a single sharp peak with no evidence for the formation of D-glucose or D-mannose via the Lobry de Bruyn-Van Ekenstein transformation. However, some fructose degradation is evident when separated on a Dionex CarboPac MA1 column using 480 mM and greater hydroxide. The fructose peak has a shoulder, indicating some degradation. This was observed by Anderson et al. using a gradient from 300 to 800 mM NaOH.[15] They noted that to quantify fructose accurately, it should be measured with the Dionex CarboPac PA1, PA10, or PA100 columns, which use lower hydroxide concentrations. Some carbohydrates, including common ones like glucose, will degrade if incubated at high alkaline pH, especially with increased temperature, but, as noted above, when injected in a neutral pH solution, degradation is not observed during chromatography.

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 h of exposure. This epimerization is not observed in separations using the Dionex 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. This epimerization is observed as a small peak (the ManNAc form) eluting just after the main oligosaccharide peak. 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 Dionex CarboPac MA1 column.

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*-acetylglucosamine at high pH generates a single sharp peak with no evidence of formation of the (well-resolved) free-base analog. Likewise, samples of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are easily separated as sharp symmetrical peaks.[5]

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-PAD with no evidence of glucose or other breakdown products.[16] Conversely, D-glucose-3-sulfate, which has a very good leaving group, decomposes rapidly during chromatography.

O-acetylated sialic acids and *O*-methylated galacturonic acids

For the titled compounds, these modifications are rapidly lost in basic conditions. While there are many sialic acids, they are either based on Neu5Ac or Neu5Gc. Therefore, HPAE-PAD of either of these sialic acids that have acetylated hydroxyl groups (e.g., *N*-acetyl 9-*O*-acetylneuraminic acid (Neu5,9Ac_2)) will yield a chromatogram with either Neu5Ac or Neu5Gc. Pectin is composed mainly of polygalacturonic acid that has methyl esterification. To preserve these methyl groups, the polygalacturonic acid is separated at neutral pH, and sodium hydroxide is added post column prior to the electrochemical cell.[17]

Pulsed amperometric detection

Pulsed amperometry permits detection of carbohydrates with excellent signal-to-noise ratios down to single-digit picomole levels without requiring derivatization of the carbohydrate. Therefore, it is a direct detection technique. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold working electrode. That current is integrated over a set time period to yield a charge. Because the products of this oxidation reaction foul the surface of the working electrode, it must be cleaned. This is accomplished by lowering the potential to clean the gold surface. While this cleans the working electrode surface of the carbohydrate oxidation products, it yields an inactive electrode. To reactivate the electrode, the potential is raised for a very short time, lowered again for a very short time, and returned to the detection potential. The sequence of potentials is called a waveform and the waveform typically used for HPAE-PAD of carbohydrates is shown in Figure 4. The development of this waveform and the science behind the waveform's potential choices have been published.[18,19] Before the development of the waveform in Figure 4, the common waveform for carbohydrate detection used oxidative rather than reductive cleaning. Oxidative cleaning does remove carbohydrate detection products, but it also removes some gold oxide from the surface. This leads to a gradual loss of electrochemical response. During a few days of analysis, this loss is insignificant, and thus, quality results were generated with that waveform, which is perhaps the reason some analysts still use it. A discussion of oxidative versus reductive cleaning waveforms for carbohydrate analysis can be found in Thermo Scientific Dionex Technical Note 21.[20]

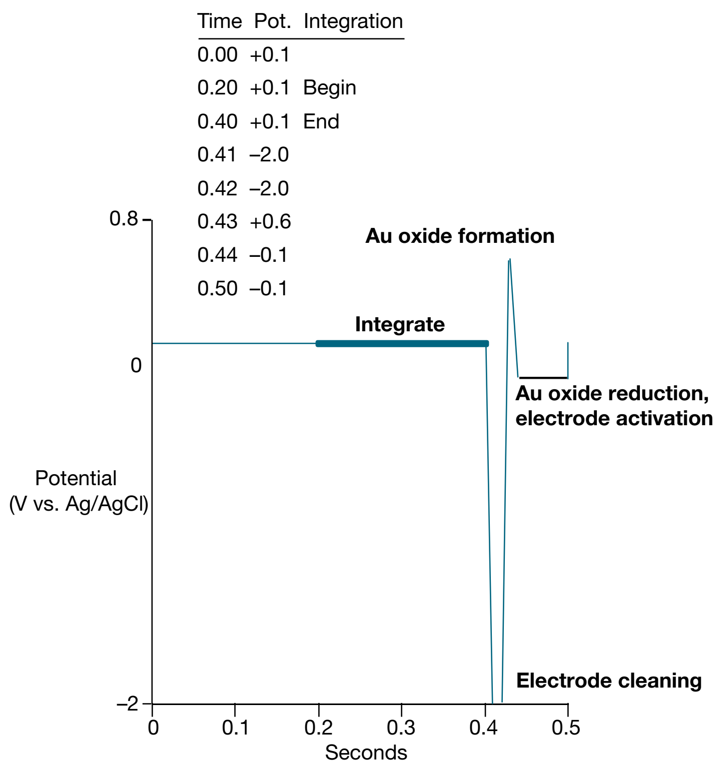

Figure 4. Automated four-potential waveform for carbohydrates

The waveform in Figure 4 starts with a potential of 0.1 V (versus a reference of Ag/AgCl – to be discussed below). This potential is maintained for 0.4 s with the current measured for the last 0.2 s. The first 0.2 s allows the charging current, which is the current generated by changing potentials and thus not analyte signal, to decay. At 0.41 s, the potential is quickly reduced to -2.0 V, where it remains for 0.01 s to clean the working electrode. At 0.43 s, the potential is raised to 0.6 V to reactivate the electrode and then lowered to -0.1 V at 0.44 s, where it remains until 0.5 s. The time at 0.6 V is too short to allow the gold oxide bond to form, and thus, no gold is lost.[21] As the waveform requires 0.5 s, data can be collected at 2 Hz. A faster version of this waveform is available in Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software that allows data to be collected at 3 Hz, which is better for fast methods and methods with early eluting analytes. The waveform with oxidative cleaning required 1 s; therefore, data must be collected at 1 Hz.

The waveform in Figure 4 can be used to detect all carbohydrates. Figure 5 shows a fundamental study from the time of PAD introduction. In a DC amperometry experiment, a sugar alcohol, monosaccharide, and disaccharide are analyzed at different oxidation potentials. At 0.2 V, each has its maximum response, providing experimental support for the use of a single waveform for all carbohydrates. The figure shows that above 0.2 V the background current increases rapidly. This is due to the formation of gold oxide. We detect at a potential less than 0.2 V because the S/N is better as the start of oxide formation contributes to noise. While the waveform in Figure 4 was optimized at 100 mM sodium hydroxide, it does not need to be adjusted for lower or higher pH eluents.

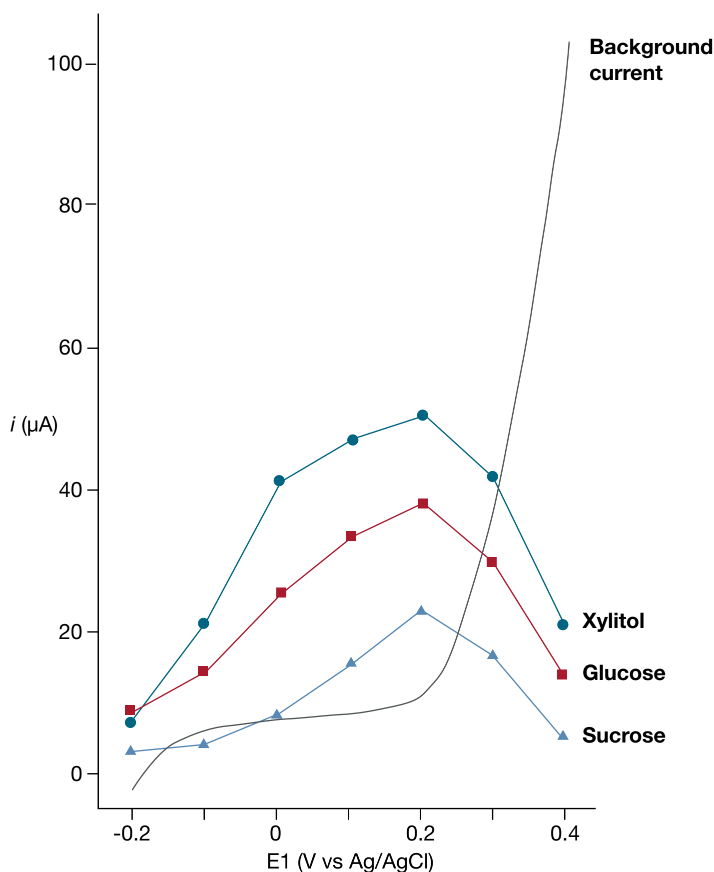

Figure 5. Peak height vs. E1 for carbohydrates

The electrochemical cell consists of three electrodes—the working electrode, reference electrode, and the counter electrode. These detect the analyte, ensure the correct potential is set at the working electrode, and prevent current generated at the working electrode from passing through the reference electrode, respectively. A gold working electrode is used for HPAE-PAD. Disposable gold working electrodes are also available. Traditionally, a silver/silver chloride (Ag/AgCl) reference electrode has been used for HPAE-PAD, but recently a palladium hydrogen (PdH) reference electrode was introduced. The cell body acts as the counter electrode. The cell also has a working electrode gasket. The gasket seals the flow path of the cell and establishes the cell volume. A thinner gasket results in a smaller cell volume and greater detection sensitivity. Thus, the analyst has some control of sensitivity with the choice of working electrode gasket thickness. The impact of gasket thickness on electrochemical response is discussed in Technical Note 186.[22]

When complex (i.e., "dirty") samples are analyzed, the working electrode can be fouled and response reduced. To restore response, the working electrode is polished. While this is effective, it can take up to a day for response to stabilize so samples can be analyzed. To eliminate this problem, disposable electrodes were introduced. The first disposable working electrodes had gold on a polyester surface. These are still available to fulfill the needs of analysts that included them in a standard operating procedure, but they have been largely replaced by gold on a PTFE surface. These newer disposable electrodes have longer lifetimes and can be used with hydroxide concentrations greater than 100 mM. The gold on PTFE disposable electrodes typically have at least a four-week lifetime, compared to a 1- or 2-week lifetime of the original disposable electrodes. Technical Note 110 provides more information on disposable gold working electrodes and how to effectively use them.[23]

A properly functioning reference electrode is vital to successful HPAE-PAD. If the reference electrode is not functioning as intended, the wrong potentials will be set at the working electrode. This leads to poor detection and even non-detection, but more critically, it can lead to cell damage. For this reason, we recommend replacing the Ag/AgCl reference electrode every six months of use and storing it properly (in saturated KCl) when not in use.

This reference electrode is a combination Ag/AgCl and pH electrode. The pH reading it provides (even when using the Ag/AgCl half), should be within a few tenths of a pH unit of the mobile phase pH. An incorrect reading indicates a bad reference electrode. The Ag/AgCl reference electrode is a liquid reference electrode that changes over time and needs to be replaced periodically. The PdH reference electrode is a solid-state reference electrode and at this writing, not long after its introduction, we believe it to have a long lifetime. Technical Note 73348 compares the performance of the two reference electrodes and discusses how to properly use the PdH reference electrode.[24] The Users' Electrochemical Guide provides additional information on running an HPAE-PAD system with electrochemical detection.[25]

Eluent preparation for HPAE-PAD

Proper preparation of eluents is the most important action an analyst can take for successful HPAE-PAD. In other words, most HPAE-PAD problems are created by improper eluent preparation. For a detailed report and instructions on this topic, please see Technical Note 71 - *Eluent preparation for high-performance anion-exchange chromatography with pulsed amperometric detection.*[26]

Briefly, nearly all HPAE-PAD eluents are composed of water, sodium hydroxide, and sodium acetate. Each must be of the appropriate quality. The water should be deionized water with 18 MΩ·cm resistivity and is free of borate and microbial contamination. Bottled HPLC water is not appropriate. All hydroxide eluents should be prepared from commercially available 50% NaOH solution. Sodium acetate eluents should be made with high quality sodium acetate, including that sold by Thermo Fisher Scientific that has been tested specifically for electrochemical applications, passed through a 0.2 µm nylon filter, and must always include hydroxide to prevent microbial contamination of the prepared eluent. Improperly prepared eluents can lead to high detection background, high noise, loss of sensitivity, loss of analyte retention, and a contaminated system that can cause long-term issues that cannot simply be fixed by properly prepared new eluents. Other eluent components have been used for HPAE-PAD carbohydrate analysis, including sodium nitrate and potassium oxalate. These have not been used enough to know the important aspects of their preparation, but we recommend choosing high purity chemicals.

Electrolytic eluent generation for HPAE-PAD

For some carbohydrate applications, eluents can be prepared electrolytically by the chromatography system. There are two approaches. One approach uses an electrolytic eluent generator to produce potassium hydroxide (chromatographically equivalent to sodium hydroxide) eluents up to 100 mM. This approach has been used for monosaccharide analysis and will be discussed more in the applications section. In the second approach, an eluent generator equipped with a KOH cartridge is connected in series with an eluent generator equipped with a methanesulfonic acid (MSA) cartridge to produce eluents containing KOH, KOH and KMSA, or MSA. The total concentration can be up to 200 mM. Therefore, this approach, named Dual Eluent Generation Cartridge (Dual EGC) Mode, can be used to produce KOH eluents up to 200 mM. Perhaps more importantly, it can be used to produce KOH/KMSA eluents that can be used to execute many applications that use NaOH/sodium acetate eluents. Methanesulfonate as an eluant is approximately three times stronger than acetate. Production of only MSA (the acidic mode) can be used for column cleaning. Reference 27 provides a detailed description of Dual EGC Mode and its application.[27] More on the application of Dual EGC Mode will be discussed in the applications section.

Sample preparation for HPAE-PAD carbohydrate analysis

Samples that not transparent (i.e., cloudy samples or those containing particulate matter) should be passed through a 0.45 µm nylon filter prior to injection. 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 releasing PAD-active components before use. Sample preparation depends on sample matrix complexity and, as such, the recommendations that follow should be considered guidelines only. In particular, the effect of sample pretreatment cartridges, or any other treatment, on the carbohydrate analytes themselves, should be predetermined using standard solutions. Some carbohydrates may have a strong affinity for some cartridge packing materials. This is important for the quantification 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 protein levels 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 Thermo Scientific™ Dionex™ OnGuard II™ RP Cartridge (2.5 cc, P/N 057084, 48 pack – 1 cc also available). Note that some procedures will increase the ionic strength of the resulting solution, possibly impacting the subsequent chromatography. See section D for anion removal.

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 and beverage samples (e.g., wine), the sample may be passed through a polyvinylpyrrolidone (PVP) filter cartridge, such as the Dionex OnGuard II P (1 cc, P/N 057087, 48 pack). These compounds can bind to the anion-exchange column and eventually consume enough column capacity to interfere with carbohydrate chromatography. Removing these compounds from the column to restore capacity may not be possible, even with the strongest column cleaning protocols.

C. Samples containing halides

To remove halides, the sample may be passed through a Dionex OnGuard II Ag cartridge (2.5 cc, P/N 057089, 48 pack – 1 cc also available). 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. Note that *N*-acetylated amino sugars will lose their acetylation during acid hydrolysis, often used to release monosaccharides from glycoconjugates. Halides will consume column capacity and limit the amount of sample that can be detected.

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 coprecipitate with the barium sulfate in this procedure, especially carbohydrates bearing sulfate esters. The Dionex OnGuard II-A cartridge (2.5 cc, P/N 057092, 48 pack – 1 cc also available) is designed specifically to remove anionic contaminants from sample matrices. These 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 acidic substituents (e.g., sugar phosphates). Removing anions from a sample will allow larger sample volumes to be injected if lower detection limits are desired.

HPAE-PAD separations and applications

After more than 40 years, there have been HPAE-PAD applications and the separations necessary for those applications developed for nearly all alkaline-stable carbohydrates. Table 2 provides general guidance for Dionex CarboPac column choice based on the type of carbohydrate. Table 3 lists all the HPAE-PAD carbohydrate applications developed at Thermo Fisher Scientific. If an application uses one of the older Dionex CarboPac columns, Table 2 suggests a new column for the application. For your application, first search to determine if the application was previously published or look for a similar application that can be adjusted for your needs. The carbohydrates that have been analyzed by HPAE-PAD include monosaccharides, disaccharides, sialic acids, oligosaccharides, small polysaccharides, sugar alcohols, sugar phosphates, sulfated sugars—including sulfated disaccharides from glycosaminoglycans, sugar acids (e.g., glucuronic acid), anhydrosugars, aminoglycosides, and synthetic and modified carbohydrates. These carbohydrates have been determined in a wide variety of samples. This section will discuss essential considerations in HPAE-PAD method development for carbohydrates and show some popular applications.

Table 3 (part 1). HPAE-PAD application documents

Application document number	Title	Column(s) used
TN30	Monosaccharide and oligosaccharide analysis of glycoproteins electrotransferred onto polyvinylidene fluoride (PVDF) membranes	Dionex CarboPac PA1, Dionex CarboPac PA100
TN36	Analysis of exoglycosidase digestions of *N*-linked oligosaccharides using HPAE-PAD	Dionex CarboPac PA1
TN40	Glycoprotein monosaccharide analysis using HPAE-PAD with eluent generation	Dionex CarboPac PA20
TN41	Analysis of sialic acids using high-performance anion-exchange chromatography	Dionex CarboPac PA10
TN42	Glycoprotein oligosaccharide analysis using high-performance anion-exchange chromatography	Dionex CarboPac PA100
TN53	Determination of glycoprotein monosaccharide composition by HPAE-PAD using on-line electrolytically generated eluents	Dionex CarboPac PA10
AN61	Determination of tobramycin and impurities using HPAE-PAD	Dionex CarboPac PA1
AN66	Determination of neomycin B and impurities using HPAE-IPAD	Dionex CarboPac PA1
AN67	Determination of plant-derived neutral oligo- and polysaccharides	Dionex CarboPac PA1, Dionex CarboPac PA100
AN82	Analysis of fruit juices adulterated with medium invert sugar from beets	Dionex CarboPac PA100
AN87	Determination of sugar alcohols in confections and fruit juices by HPAE-PAD	Dionex CarboPac MA1
AN92	The determination of sugars in molasses by high-performance anion exchange with pulsed amperometric detection	Dionex CarboPac PA1
AN105	Glycosylation analysis of human serum transferrin glycoforms using pellicular anion-exchange chromatography	Dionex CarboPac PA100
AN117	Quantification of carbohydrates and glycols in pharmaceuticals	Dionex CarboPac MA1, Dionex CarboPac PA10, Thermo Scientific™ Dionex™ IonPac™ ICE-AS1
AN122	The determination of carbohydrates, alcohols, and glycols in fermentation broths	Dionex CarboPac MA1, Dionex CarboPac PA1
AU125	Monosaccharide analysis of serum	Dionex CarboPac PA1
TN125	Guidelines for successful use of Thermo Scientific Dionex AminoTrap columns	Thermo Scientific™ Dionex™ AminoTrap™, Dionex CarboPac PA10, Dionex CarboPac PA20
TN133	HPAE-PAD peak area response of glycoprotein oligosaccharides	Not applicable
AU141	Improved long-term stability of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid peak area responses using Waveform A, a quadruple potential waveform	Dionex CarboPac PA10
TN146	Fast determinations of lactose and lactulose in milk products using HPAE-PAD	Dionex CarboPac SA10-4µm

Table 3 (part 2). HPAE-PAD application documents

Application document number	Title	Column(s) used
AN147	Determination of polydextrose in foods by AOAC Method 2000.11	Dionex CarboPac PA1
AU150	Determination of plant-derived neutral oligo- and polysaccharides	Dionex CarboPac PA200
AU151	Determination of sucralose in reduced- carbohydrate colas using high-performance anion-exchange chromatography with pulsed amperometric detection	Dionex CarboPac PA20
AN155	Determination of trans-galactooligosaccharides in foods by AOAC Method 2001.02	Dionex CarboPac PA1
AN159	Determination of sucralose using HPAE-PAD	Dionex CarboPac PA20
AU164	Determination of glucosamine in chondroitin sulfate-containing dietary supplements using HPAE-PAD	Dionex CarboPac PA20
AU176	Preparation of peptide *N*-glycosidase F digests for HPAE-PAD analysis	Dionex CarboPac PA200
AU180	Direct determination of sialic acids in glycoprotein hydrolyzates by HPAE-PAD	Dionex CarboPac PA200
AN181	Determination of streptomycin and impurities using HPAE-PAD	Dionex CarboPac PA1
AU181	Rapid screening of sialic acids in glycoproteins by HPAE-PAD	Dionex CarboPac PA20 Fast Sialic Acid
AN186	Analysis of paromomycin by HPAE-IPAD	Dionex CarboPac PA1
TN186	The effect of working electrode gasket thickness on the sensitivity and linearity of carbohydrate response by PAD	Dionex CarboPac PA20, Dionex CarboPac SA10
AU192	Carbohydrate determination of biofuel samples	Dionex CarboPac SA10
AN197	Determination of glucosamine in dietary supplements using HPAE-PAD	Dionex CarboPac PA20
AN202	High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) analysis of mannose-6-phosphate	Dionex CarboPac PA200
AU202	A fast method for sugar analysis of instant coffee samples	Dionex CarboPac SA10-4µm
AN225	Rapid method for the estimation of total free monosaccharide content of corn stover hydrolysate using HPAE-PAD	Dionex CarboPac PA1
AN233	Determination of galactosamine containing organic impurities in heparin by HPAE-PAD	Dionex CarboPac PA20
AN248	Determination of lactose in lactose-free milk products by high-performance anion-exchange chromatography with pulsed amperometric detection	Dionex CarboPac PA20
AN253	HPAE-PAD determination of infant formula sialic acid	Dionex CarboPac PA20
AN255	Determination of free and total glycerol in biodiesel samples by HPAE-PAD chromatography	Dionex CarboPac MA1
AN267	Analysis of the aminoglycoside antibiotics kanamycin and amikacin matches USP requirements	Dionex CarboPac MA1
AN270	Determination of hydroxymethylfurfural (HMF) in honey and biomass	Dionex CarboPac PA1
AN280	Carbohydrate in coffee: AOAC Method 995.13 vs. a new fast ion chromatography method	Dionex CarboPac PA1, Dionex CarboPac SA10
AN282	Rapid and sensitive determination of biofuel sugars by ion chromatography	Dionex CarboPac SA10
AN1006	Determination of carbohydrates in urine by HPAE-PAD	Dionex CarboPac MA1, Dionex CarboPac PA20
AN1013	Polysialic acid analysis: Separating polymers with high degrees of polymerization	Dionex CarboPac PA200
AN1050	Evaluating protein glycosylation in limited-quantity samples by HPAE-PAD	Dionex CarboPac PA20, Dionex CarboPac PA200
AN1083	Determination of myo-inositol (free and bound as phosphatidylinositol) in infant formula and adult nutritionals	Dionex CarboPac MA1, Dionex CarboPac PA1
AN1089	Determination of carbohydrates in acid hydrolysates of wood	Dionex CarboPac SA10-4µm

Table 3 (part 3). HPAE-PAD application documents

Application document number	Title	Column(s) used
AN1091	Determination of uronic acids and wood sugars in wood-based hydrolysates	Dionex CarboPac PA20, Dionex CarboPac PA200
AN1149	Profiling fructosyloligosaccharides (FOS)-containing samples by HPAE-PAD	Dionex CarboPac PA200
AN1152	Determination of carbohydrates in kombucha using HPAE-PAD	Dionex CarboPac PA20
AN1158	HPAE-PAD determination of carbohydrates in honey to evaluate samples for quality and adulteration	Dionex CarboPac PA210
AN1161	Improved method for determination of biofuel sugars by HPAE-PAD	Dionex CarboPac SA10-4µm
AN71410	Ion chromatography: A versatile technique for the analysis of beer	Dionex CarboPac PA1, Dionex CarboPac PA100
AN71993	Profiling galactosyloligosaccharide-containing samples by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)	Dionex CarboPac PA200
AN72210	Fast determination of biofuel sugars by HPAE-PAD	Dionex CarboPac SA10-4µm
TN72225	Glycoprotein monosaccharide analysis using HPAE-PAD with manually prepared eluent	Dionex CarboPac PA20
TN72264	HPAE-PAD *N*-linked oligosaccharide profiling of IgG	Dionex CarboPac PA200
TN72478	Instrument Configuration for native *N*-linked oligosaccharide characterization by HPAE-PAD/MS	Dionex CarboPac PA200
AN72580	An improved HPAE-PAD method for glycoprotein monosaccharide determination	Dionex CarboPac PA20-4µm
AN72610	Determination of carbohydrates in algal biofuel samples	Dionex CarboPac PA20-4µm
AN72632	Fast and sensitive determination of lactose in lactose-free products using HPAE-PAD	Dionex CarboPac PA210
AB72720	A glycopeptide standard for improving glycoprotein hydrolysis reaction yield for accurate monosaccharide content determination	Dionex CarboPac PA20-4µm
AN72779	Limit of β-cyclodextrin (Betadex) in Betadex sulfobutyl ether sodium	Dionex IonPac AS11
AN72780	Determination of lactose in lactose-free dairy products using HPAE coupled with PAD and MS dual detection	Dionex CarboPac PA210
AU72829	HPAE-PAD analysis of *N*-linked glycans: Improving glycan resolution	Dionex CarboPac PA200
AN72914	HPAE-PAD profiling of *N*-linked oligosaccharides from glycoproteins using dual eluent generation cartridges	Dionex CarboPac PA200
AN73009	An HPAE-PAD method for determination of saccharides in atmospheric aerosol samples	Dionex CarboPac MA1
AN73011	HPAE-PAD determination of cyclodextrins	Dionex CarboPac PA200
AN73132	Carbohydrate analysis of agave syrup using HPAE-PAD	Dionex CarboPac PA1
AN73341	Determination of sugars in dairy products using HPAE-PAD	Dionex CarboPac PA1
TN73348	Carbohydrate determinations by HPAE-PAD using a PdH reference electrode	Dionex CarboPac PA20, Dionex CarboPac PA210
AN73605	Determination of low- and non-caloric sweeteners in food and beverages by HPAE-PAD	Dionex CarboPac PA20
AN73896	Carbohydrate analysis of agave syrup using HPAE-PAD in Dual Eluent Generation Cartridge Mode	Dionex CarboPac PA1
AN73980	Improved carbohydrate analysis of agave syrup using HPAE-PAD in Dual Eluent Generation Cartridge Mode	Dionex CarboPac PA200
AN73986	Determination of trans-galactooligosaccharides in foods using HPAE-PAD in Dual Eluent Generation Cartridge Mode	Dionex CarboPac PA1
AN74042	Structural characterization of mucin *O*-linked glycans using a novel anion exchange column in HPAE-PAD-MS	Dionex CarboPac PA300
AN74121	Determination of glycoprotein sialic acid composition using HPAE-PAD in Dual Eluent Generation Cartridge Mode	Dionex CarboPac PA20

Small carbohydrates that are neutral (uncharged at pH 7) will only require hydroxide eluent for separation. If those carbohydrates are in samples containing strongly anionic material, column washing with a sodium acetate containing eluent may be occasionally needed to maintain column capacity. Charged carbohydrates (e.g., sialic acids) and large carbohydrates require sodium acetate containing eluents. When developing gradient separations with sodium acetate, it is best to start with some acetate in the eluent (i.e., do not start the separation with only hydroxide). This will avoid a small upset in the baseline when the stationary phase switches from the hydroxide form to the acetate form. Starting with as little as 10 mM sodium acetate prevents this and shortens column equilibration between injections. If your separation uses only hydroxide eluents with concentrations less than 100 mM, it may be possible to use an eluent generator. If your separation uses acetate-containing eluents, it may be possible to use Dual EGC Mode.

Some of the more popular HPAE-PAD carbohydrate applications are monosaccharide compositional analysis, sialic acid compositional analysis, oligosaccharide profiling from glycoproteins, oligo/polysaccharide profiling from plant materials, analysis of biofuel production material for monosaccharides, sugar alcohol analysis, and the determination of anhydrosugars in air samples. Some applications will be discussed below. The featured applications are all covered in more detail in the referenced application notes. To find an application note, search by the application note number in the Thermo Scientific AppsLab Library of Analytical Applications online resource (https://appslab.thermofisher.com/). For example, for Application Note 72580, search "AN72580". For many of these important applications, there is more than one application document. In general, this document will discuss the most recent application document for the application. The other application documents can be found either in Table 3 or by searching AppsLab.

Monosaccharide compositional analysis of glycoconjugates

This common application of HPAE-PAD typically works as follows. The sample, usually a purified glycoconjugate, is treated with a volatile acid and heated for a set amount of time. It is then cooled and the acid removed by drying in a Thermo Scientific™ SpeedVac™ system. The dried sample is suspended in DI water and injected into the HPAE-PAD system for analysis. Figure 6 shows separations of the HCl and TFA hydrolysates of human α_1-acid glycoprotein using a Dionex CarboPac PA20-4µm column in a short format that allows faster separations and thus improves productivity. Because the separation uses only low concentration hydroxide eluents, they are produced by an electrolytic eluent generator. The eluent generator produces eluent that is essentially carbonate-free, allowing a shorter column wash to be used after each injection. This reduces the injection-to-injection time compared to using manually prepared eluents. More details on this analysis can be found in Application Note 72580. Monosaccharide analysis with other Dionex CarboPac columns with either eluent generation or manually prepared eluents can be found by searching AppsLab.

Column:	Dionex CarboPac PA20-4µm (2 × 100 mm) and guard (2 × 30 mm)
Eluent source:	Dionex EGC 500 KOH
Eluent:	0–8 min, 10 mM KOH, 8–14 min, 100 mM 14–20 min 10 mM KOH
Flow rate:	0.22 mL/min
Temp.:	30 °C
Detection:	PAD (disposable Au)

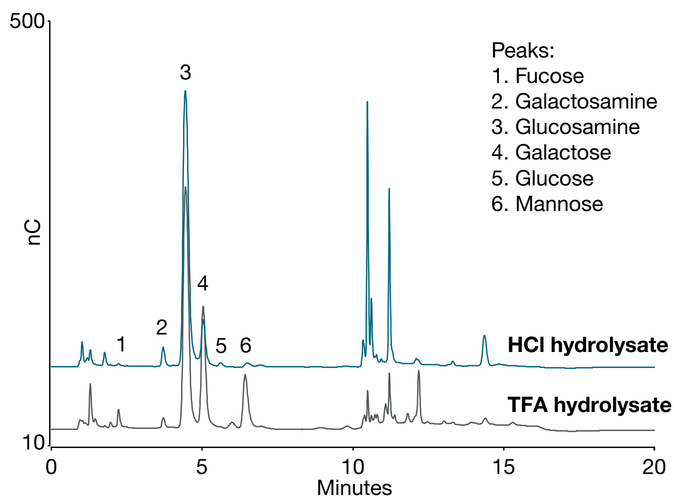

Figure 6. Analysis of human α_1 acid glycoprotein TFA and HCl hydrolysates

Sialic acid compositional analysis of glycoconjugates

This analysis also begins by treating the glycoconjugate with a volatile acid to release the sialic acids, though the conditions are much milder than those used for monosaccharide analysis. Instead of using acid for release, a neuraminidase, an exoglycosidase, can be used to

release sialic acids. Figure 7 shows the separation of Neu5Ac and Neu5Gc on a Dionex CarboPac PA20 column. While there are other sialic acids that are modified versions of Neu5Ac and Neu5Gc (e.g., acetylated at hydroxyl groups of the sialic acid), they are not stable in alkaline eluents, and thus, we only determine Neu5Ac and Neu5Gc. As sialic acids are negatively charged at neutral pH, acetate is required for their separation. A gradient separation is used to ensure an efficient peak for Neu5Gc, which is often present at low concentrations. Its peak may be too broad with an isocratic separation and thus hamper its detection. More details on this analysis can be found in Application Update 180. AN74121 shows it is possible to do this application in Dual EGC Mode. Sialic acid analysis with other Dionex CarboPac columns can be found by searching AppsLab.

For both monosaccharides and sialic acids, there are samples where these carbohydrates are already free in solution (i.e., no acid hydrolysis or enzyme treatment is necessary). In these cases, little if any sample preparation beyond sample dilution and filtration may be needed for HPAE-PAD analysis.

Column: Dionex CarboPac PA20 guard, 3 × 30 mm
Dionex CarboPac PA20, 3 × 150 mm
Eluent: 70–300 mM acetate in 100 mM NaOH from 0 to 7.5 min,
300 mM acetate in 100 mM NaOH from 7.5 to 9.0 min,
300–70 mM acetate from 9.0 to 9.5 min.
7 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature: 30 °C
Flow rate: 0.5 mL/min
Inj. volume: 10 µL
Detection: PAD, Au (Disposable)
Samples: Neu5Ac and Neu5Gc standards
Peaks: 1. Neu5Ac 75 pmol
2. Neu5Gc 5.8

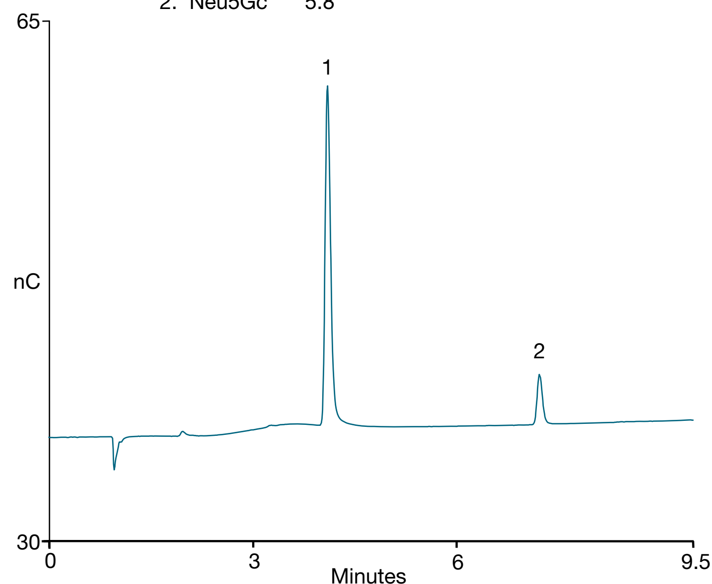

Figure 7. Separation of sialic acids

Determination of carbohydrates in instant coffee

This is an example of a determination where the carbohydrates are free in solution. This determination is the subject of an AOAC Method that was approved in 1995. Table 4 lists that method as well as other official HPAE-PAD methods for food analysis. The method for instant coffee uses a Dionex CarboPac PA1 column and a separation with DI water after first conditioning the column with hydroxide eluent. Because the elution is with water, we need to add sodium hydroxide post-column to achieve the proper pH for carbohydrate detection. For a 1 mL/min eluent flow rate, we typically add 0.3 M sodium hydroxide at a flow rate of 0.5 mL/min through a tee with the outlet going to the electrochemical cell. This is best done with a second pump (e.g., the second pump of dual pump module of the Thermo Scientific™ Dionex™ ICS-6000 system), but it can be done pneumatically with a pressurized container containing a bottle with 0.3 M sodium hydroxide. There are other methods where the analyst has included post-column addition of sodium hydroxide. When the eluent contains a low concentration of hydroxide (e.g., <5 mM), the post-column addition of hydroxide will extend the linear calibration range.

Table 4. Official food methods using HPAE-PAD

Analysis	Dionex CarboPac column	Official method
Sugars in molasses	Dionex CarboPac PA1	AOAC Method 996.04 ICUMSA Method approval 1994
Carbohydrates in soluble coffee	Dionex CarboPac PA1	AOAC Method 995.13 ISO 11292 approved
Fructans in food and food products	Dionex CarboPac PA1	AOAC Method 997.08
Polydextrose	Dionex CarboPac PA1	AOAC Method 2000.11
Transgalacto-oligosaccharides	Dionex CarboPac PA1	AOAC Method 2001.02
Low level glucose and fructose in raw and refined sugar	Dionex CarboPac PA1	AOAC Method 2000.17
Myo-inositiol in infant formula	Dionex CarboPac PA1 guard and Dionex CarboPac MA1	AOAC Method 2011.18

Figure 8 shows a separation of carbohydrates in coffee samples according to the AOAC method, though with the column temperature set at 15 °C to improve resolution of some of the carbohydrates. The AOAC method specifies post-column addition of 0.3 M sodium hydroxide at 0.6 mL/min. More details on this analysis can be found in Application Note 280, including a significantly shorter method that uses a Dionex CarboPac SA10 column.

Column: Dionex CarboPac PA1 (4 × 250 mm) and guard (4 × 50 mm)
Eluent: DI water from 0 to 50 min
300 mM NaOH from 50 to 65 min
DI water from 65 to 80 min (re-equilibration)
Flow rate: 1.0 mL/min
Temperature: 15 °C
Inj.volume: 10 µL
Detection: PAD

Peaks:
1. Mannitol
2. Fucose
3. Arabinose
4. Rhamnose
5. Galactose
6. Glucose
7. Xylose
8. Sucrose
9. Mannose
10. Fructose
11. Ribose

A. Standards
B. Free carbohydrates in green coffee
C. Free carbohydrates in instant coffee
D. Total carbohydrates in instant coffee

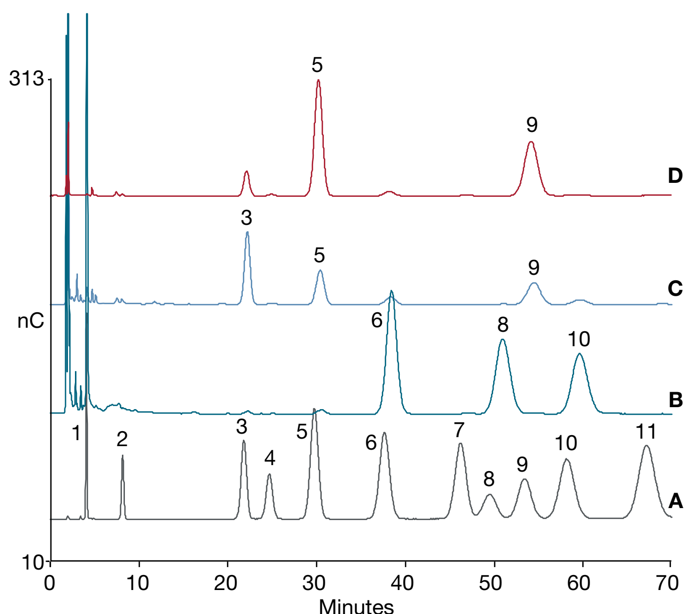

Figure 8. Determination of carbohydrates in coffee

Determination of carbohydrates in samples for biofuel production

One of the major processes for producing biofuels is to treat a carbohydrate-containing biological material to yield monosaccharides that are fermented to yield the biofuel. To optimize this process, the carbohydrate content is determined prior to fermentation. The typical monosaccharides include arabinose, rhamnose, galactose, glucose, mannose, and xylose, and they can be difficult to separate with the Dionex CarboPac columns usually used for monosaccharide analysis. The Dionex CarboPac SA10 column was developed to address this need.

Figure 9 shows a separation of an acid hydrolysis of a wood sample. More details on this analysis can be found in Application Note 1089. Other examples of determining carbohydrates in samples for biofuel production can be found by searching AppsLab.

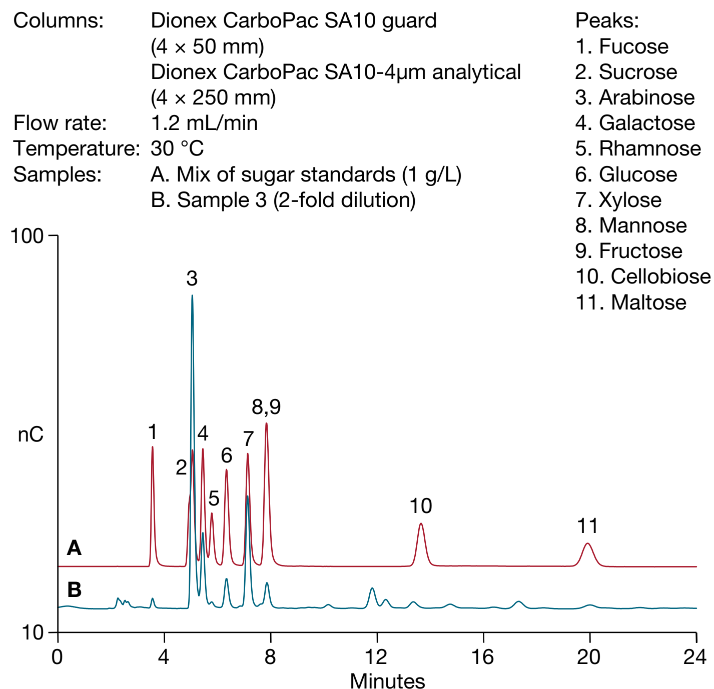

Figure 9. Separation of carbohydrates in a wood hydrolysate

Determination of carbohydrates in air samples

Air can contain carbohydrates from wood burning and those emitted by plants. Burning wood yields anhydrosugars (e.g., levoglucosan, mannosan, and galactosan). Though these compounds are less retained on Dionex CarboPac columns used for monosaccharide analysis, if these are the only analytes of interest, the columns typically used for monosaccharide analysis (e.g., Dionex CarboPac PA20 columns) are used for analysis. When determining a larger range of carbohydrates, the Dionex CarboPac MA1 column is often the choice. Figure 10 shows a separation of eleven carbohydrates found in air. More details on this analysis can be found in Application Note 73009.

Column: Dionex CarboPac MA1 guard, 4 × 50 mm
Dionex CarboPac MA1, 4 × 250 mm
Eluent: Multi-step NaOH gradient (see AN73009)
Temperature: 30 °C
Flow rate: 0.4 mL/min
Detection: PAD, Au (Disposable)

Peaks:
1. Erythritol
2. Xylitol
3. Levoglucosan
4. Mannosan
5. Mannitol
6. Galactosan
7. Mannose
8. Glucose
9. Galactose
10. Fructose
11. Sucrose

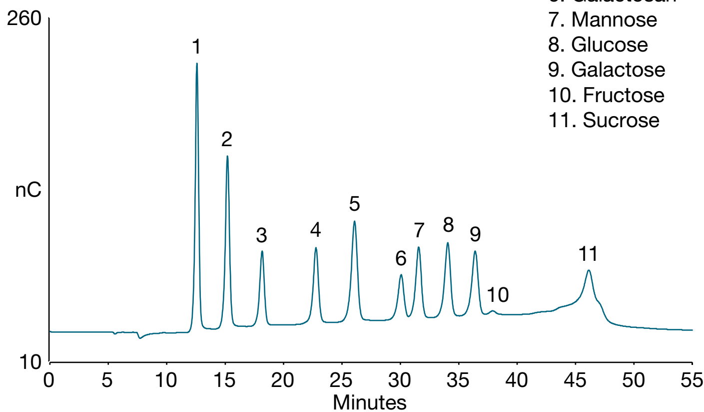

Figure 10. Separation of 11 carbohydrates found in air standard

Determination of mono- through tetrasaccharides

Carbohydrate analysis of food samples often requires only a determination of mono- through tetrasaccharides. While many Dionex CarboPac columns can be used for this analysis, the Dionex CarboPac PA210-4µm column was developed specifically for this analysis. This column is ideal for a carbohydrate analysis of honey. Honey contains a large quantity of fructose and glucose and small quantities of many di-, tri-, and tetrasaccharides. These saccharides can indicate the honey's authenticity as well as its origin. The sensitivity and resolution of HPAE-PAD established it as a technique for determining the authenticity of honey. Figure 11 shows the separation of carbohydrates from honey on a Dionex CarboPac PA210-4µm column. This separation only requires hydroxide eluent and thus can be done with an eluent generator. Earlier honey analysis applications required sodium acetate eluents. While turanose and palatinose are only partially resolved, they coelute with other Dionex CarboPac columns, or if resolved, turanose coelutes with 1-kestose. More details on this analysis can be found in Application Note 1158.

Column: Dionex CarboPac PA210-4µm and guard
Eluent (EGC-KOH): 0–25 min: 30 mM KOH
25–30 min: 100 mM KOH
30–45 min: 30 mM KOH
Temp. 30 °C
Flow rate: 0.8 mL/min
Inj. volume: 10 µL
Detection: PAD (Au) Disposable, Waveform A (TN21)

Peaks:
1. Trehalose
2. Glucose
3. Fructose
4. Sucrose
5. Isomaltose
6. Melezitose
7. Kojibiose
8. Raffinose
9. Gentiobiose
10. 1-Kestose
11. Turanose
12. Palatinose
13. Nigerose
14. Maltose
15. Erlose

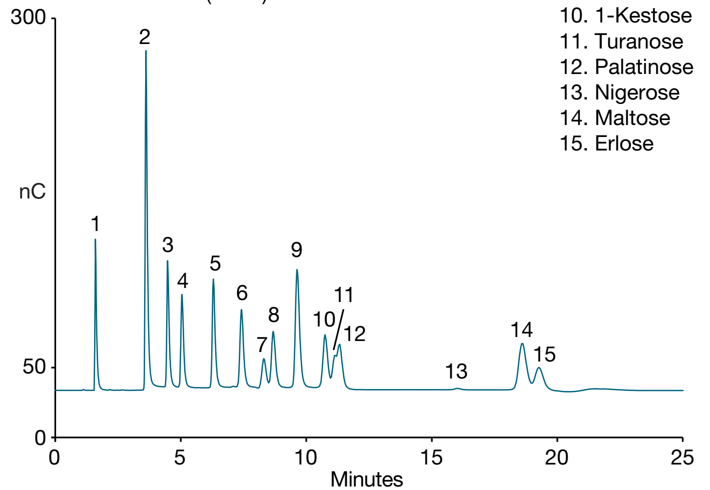

Figure 11. Chromatogram of 15 honey sugar standards

Profiling oligosaccharides released from glycoproteins

This application begins with the release of oligosaccharides (glycans) from the glycoprotein. If the oligosaccharides are attached to an asparagine (*N*-linked), they are typically released with the amidase PNGase F. After either a simple dilution or a short sample clean-up, the oligosaccharides are separated on a Dionex CarboPac PA200 column with a sodium acetate gradient in the presence of a fixed concentration of sodium hydroxide (typically 100 or 150 mM). Figure 12 shows a separation of the *N*-linked oligosaccharides from human α_1-acid glycoprotein. The majority of the separated glycans, from 40 min on, contain multiple sialic acids. More details on this analysis can be found in Application Update 72829. Other examples of profiling *N*-linked oligosaccharides can be found by searching AppsLab.

Column: Dionex CarboPac PA200 (1 × 250 mm) with guard (1 × 50 mm)
Flow rate: 0.063 mL/min
Column temp.: 25 °C
Eluent: 0–70 min 150 mM NaOH 6–190 mM sodium acetate, 70–75 min 150 mM NaOH 200 mM sodium acetate, 75–90 min 150 mM NaOH 6 mM sodium acetate
Detection: PAD

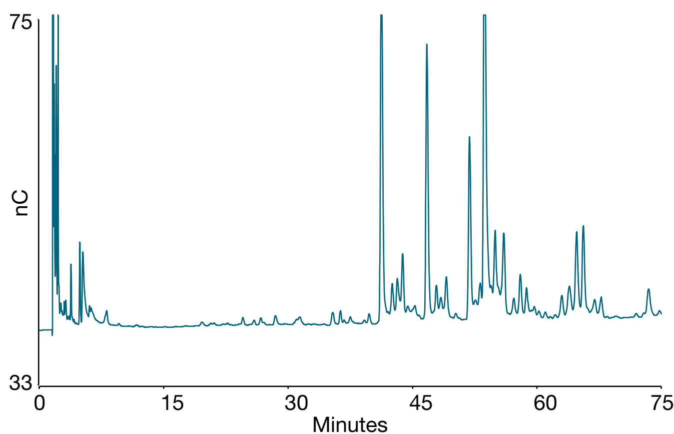

Figure 12. Separation of human α_1-acid glycoprotein *N*-linked oligosaccharides

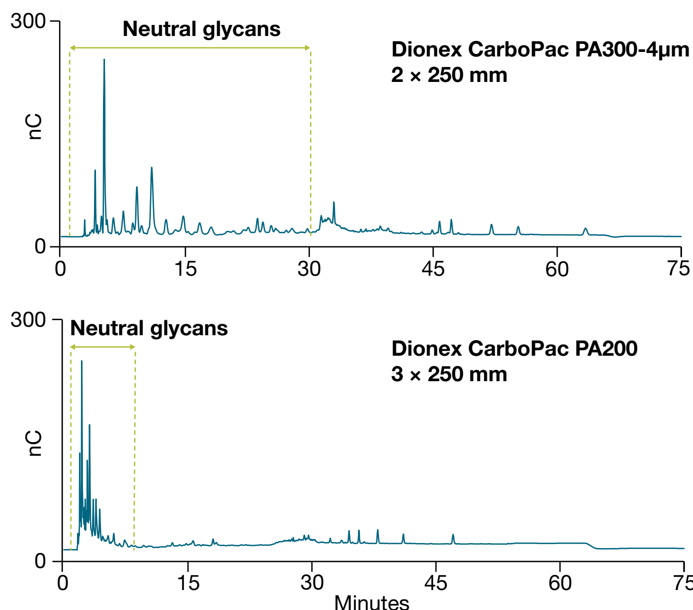

Figure 13. Comparison of the separation of porcine gastric mucin type III *O*-glycans on the Dionex CarboPac PA300 and PA200 columns

The other common linkage type for an oligosaccharide to glycoprotein is through a serine or threonine residue. Oligosaccharides linked in this manner are referred to as *O*-linked. While the Dionex CarboPac PA200 column is a good column for *N*-linked oligosaccharides, it is often not adequate for *O*-linked oligosaccharide separations. This is due to many *O*-linked oligosaccharides being both small and uncharged (i.e., no attached sialic acids or sulfate groups). To address this need, the Dionex CarboPac PA300-4µm column was developed. Figure 13 shows a separation of the *O*-linked oligosaccharides from porcine gastric mucin type III using a Dionex CarboPac PA300-4µm column with comparison to a separation using the Dionex CarboPac PA200 column. This figure (Figure 5 in Application Note 74042) shows the improved separation of early eluting oligosaccharides with the Dionex CarboPac PA300-4µm column. An enzyme is not available for releasing *O*-linked oligosaccharides, so they are released by reductive alkaline β-elimination. More details on this release procedure and the separation of the *O*-linked oligosaccharides can be found in Application Note 74042.

HPAE-PAD separations of *N*- and *O*-linked glycans can be directly interfaced to a mass spectrometer through an electrolytic desalter (Thermo Scientific™ Dionex™ ERD™ 500 Electrolytically Regenerated Desalter). After separation, the eluent is either split with one half going to the electrochemical cell and the other half going to the Dionex ERD500 device and then the mass spectrometer, or the eluent goes to the electrochemical cell, which is followed by the Dionex ERD500 device then the mass spectrometer. The Dionex ERD500 device exchanges the sodium in the eluent with hydronium ion produced electrolytically. This yields water and acetic acid, which are acceptable for the mass spectrometer's electrospray ionization interface. Details on the parallel and sequential setups for HPAE-PAD/MS can be found in Technical Note 72478 and Application Note 74042.

Profiling oligosaccharides derived from plants

Oligosaccharides and small polysaccharides derived from plants are commonly analyzed by HPAE-PAD in the same manner as oligosaccharides derived from glycoproteins, though higher acetate concentrations are typically needed to elute the larger oligosaccharides. Figure 14 shows a separation of fructooligosaccharides (FOS) and fructans (small polysaccharides) on a Dionex CarboPac PA200 column. More details on this analysis can be found in Application Note 1149. Other examples of profiling oligosaccharides derived from plants can be found by searching AppsLab.

Column: Dionex CarboPac PA200 and guard
Eluent: -5 min: 100 mM NaOH/20 mM NaOAc,
0–15 min: 100 mM NaOH/20 mM NaOAc,
15–70 min 100 mM NaOH/20–450 mM NaOAc,
70–70.1 min: 100 mM NaOH/20 mM NaOAc,
70.1–75 min: 100 mM NaOH/20 mM NaOAc,
curve 5
Temperature: 30 °C
Flow rate: 0.5 mL/min
Inj volume: 10 µL
Detection: PAD(Au), Waveform A (TN 21)

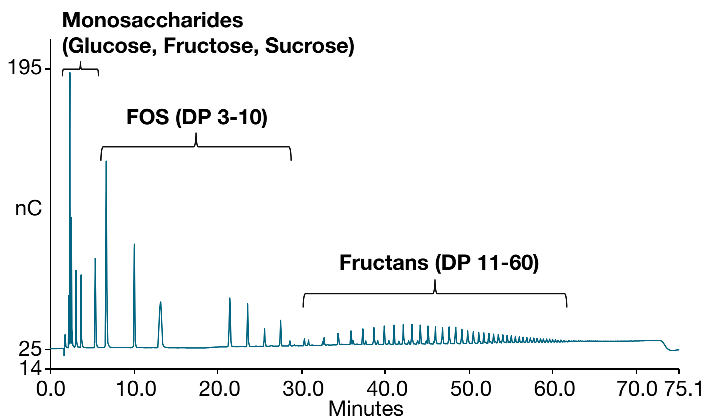

Figure 14. Separation of fructooligosaccharides (FOS) and fructans

Oligosaccharides derived from either glycoproteins or plants can also be separated using Dual EGC Mode with potassium hydroxide and potassium methanesulfonate eluents. Methanesulfonate is a stronger eluting anion than acetate, so some method development will be required if converting an application from acetate eluents. A good starting point is to consider methanesulfonate to be three times stronger than acetate. For example, if a separation has a final acetate concentration of 180 mM, use 60 mM methanesulfonate for the final concentration. Figure 15 shows separations of the *N*-linked oligosaccharides from two human α_1-acid glycoprotein preparations (different vendors). Compare these separations to Figure 12 that used a manually prepared acetate eluent. More details on this analysis can be found in Application Note 72914.

Column: Dionex CarboPac PA200, analytical, 1 × 250 mm
Dionex CarboPac PA200, guard, 1 × 50 mm
Eluent source: Dionex EGC 400 MSA in series
with Dionex EGC 400 KOH
Eluent: 0-70 min: 5-80 mM KMSA in 70 mM KOH
70-75 min: 80 mM KMSA in 70 mM KOH
75-90 min: 5 mM KMSA in 70 mM KOH
Temperature: 30 °C (detector) 25 °C (column)
Flow rate: 0.063 mL/min
Inj. volume: 0.4 µL
Detection: PAD, Au on PTFE (Disposable),
1 mil gasket, Ag/AgCl ref. electrode

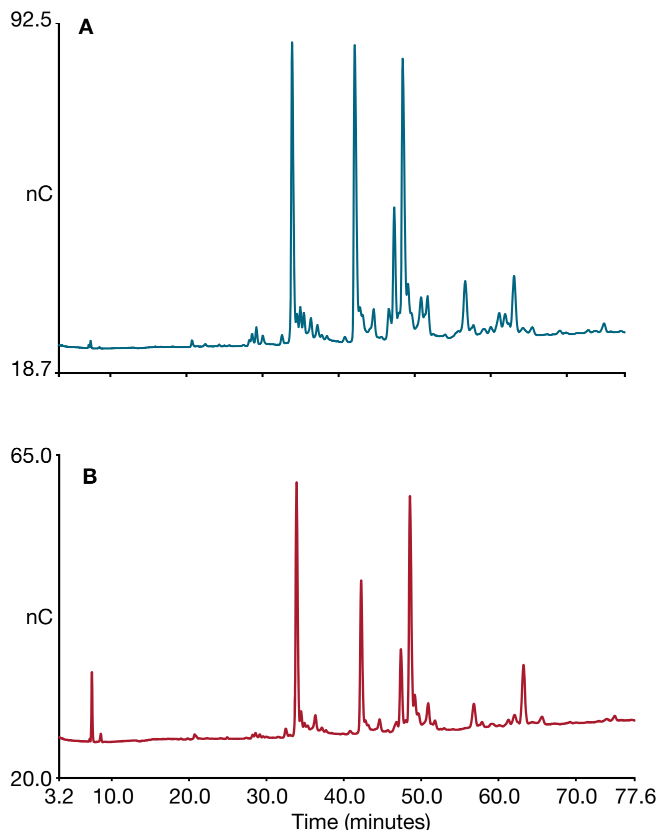

Figure 15. Separation of human α_1-acid glycoprotein *N*-linked oligosaccharides from two commercial samples using Dual EGC Mode

Conclusion

Over the past 40 years, HPAE-PAD has become an established analytical technique for determining a broad range of carbohydrates in a wide variety of samples. This success results from high-resolution separations and sensitive detection of native carbohydrates (i.e., no analyte derivatization for either separation or detection). This Technical Note reviewed the basics of HPAE-PAD, the columns available for carbohydrate separations, and the major applications of HPAE-PAD.

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