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Monosaccharide and Sialic Acid Determinations in Biosimilars Using HPAE-PAD

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A large number of innovator biotherapeutics are currently losing patent protection, resulting in an increased interest to develop biosimilar therapeutic proteins. A key step to proving biosimilarity is to ensure proper N-glycosylations, because variations in glycosylation can affect the efficacy and safety of therapeutic proteins, influencing their biological activity, serum half-life, and immunogenicity. Monosaccharides, including sialic acid composition analysis can detect variations in glycosylation, and support quality control for process development and manufacturing procedures. As a result, proteins are routinely analyzed to determine their monosaccharides and sialic acids amount and identity.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for carbohydrates analysis. HPAE-PAD separates carbohydrates with specific interactions between the hydroxyl and carboxyl groups of carbohydrates based on charge, size, composition, isomers, and linkages. Therefore, HPAE-PAD is the best method for monosaccharides, sialic acids, and other carbohydrates analysis.

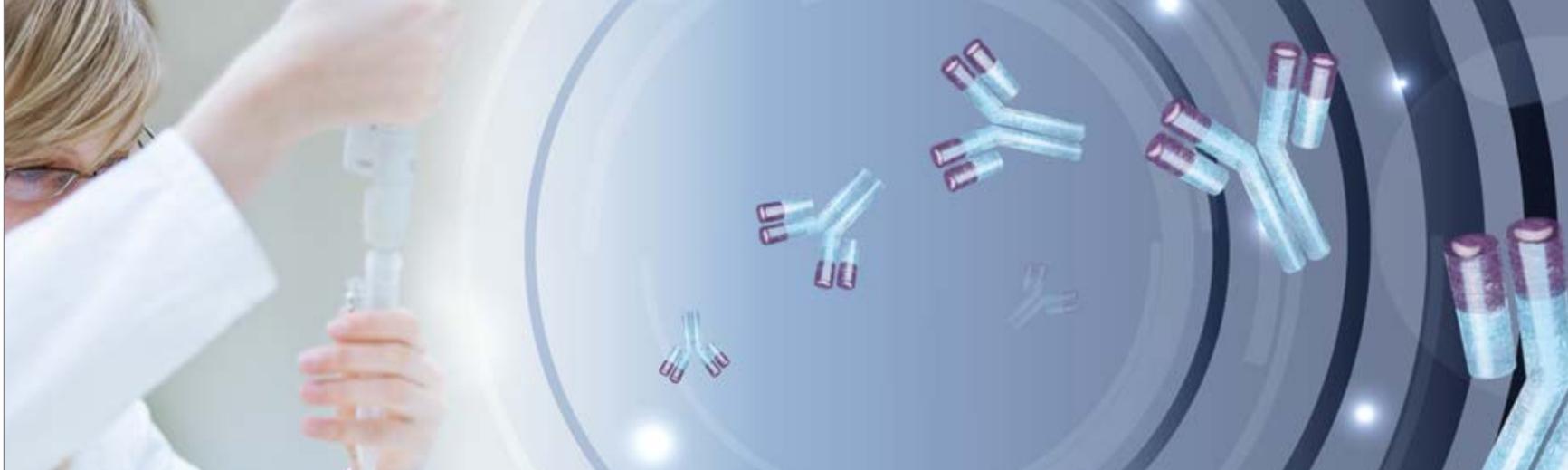
- Direct detection - no sample derivatization required saving analyst time, expense and exposure to hazardous chemicals
- Ultra-selective, sensitive detection
- Fast separations without loss of resolution using Thermo Scientific™ Dionex™ CarboPac™ PA20 Fast columns

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- Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation
- Rapid Screening of Sialic Acids in Glycoproteins by HPAE-PAD
- Evaluating Protein Glycosylation in Limited-Quantity Samples by HPAE-PAD
- Sialic Acids in Glycoprotein Hydrolyzates by HPAE-PAD

Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation

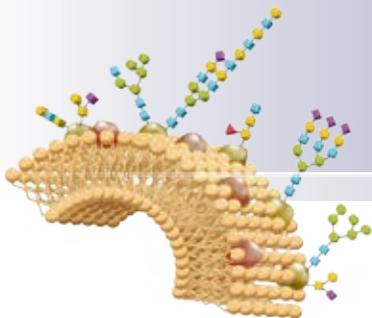


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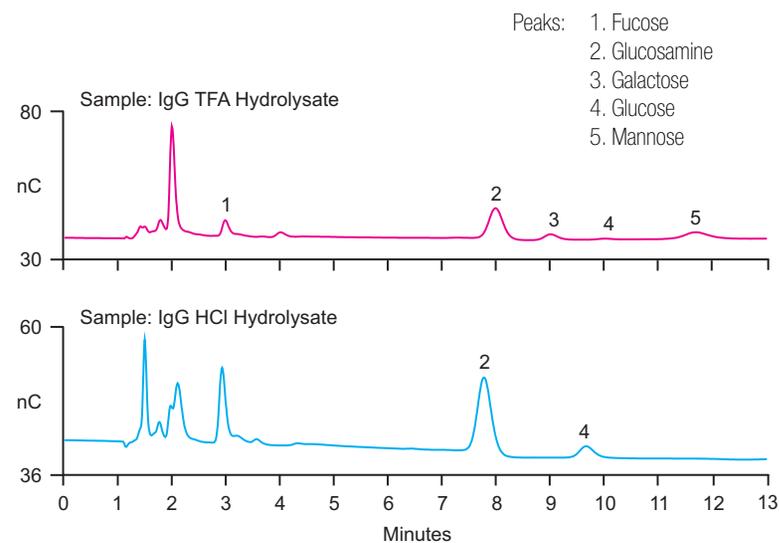
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Characterization of glycoproteins routinely involves carbohydrate analysis. Minor variations in glycosylation can affect the efficacy of protein therapeutics. Monosaccharide composition analysis can detect variations in glycosylation, and support quality control for process development and manufacturing procedures. There are over 30 approved glycoprotein-based biodrugs on the market and the number is increasing rapidly. Agencies such as the U.S. FDA and the European Medicines Agency have increased pressure on biopharmaceutical manufacturers to demonstrate satisfactory programs for understanding, measuring, and controlling glycosylation in glycoprotein-based drugs.

Conditions

Columns:	Dionex CarboPac PA20 Analytical, 3 × 150 mm Thermo Scientific™ Dionex™ AminoTrap™, 3 × 30 mm
Eluent:	10 mM KOH
Eluent Source:	Thermo Scientific Dionex EGC III KOH Eluent Generation Cartridge Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL (partial loop injection mode with a 4 µL cut volume)
Column Temp.:	30 °C
Cell Temp.:	30 °C
Backpressure:	2200 psi
Detection:	PAD
Background:	30–50 nC
Working Electrode:	Carbohydrate PTFE Disposable Au Working Electrodes
Reference Electrode:	Mode: Ag/AgCl mode Noise: 10–30 pC

In this technical note, the HPAE-PAD-based method on a Thermo Scientific Dionex ICS-5000 chromatography system for monosaccharide composition analysis is fast and capable of providing reproducible retention time and detector response for hundreds of samples over several days. The disposable Au working electrode contributes to the reproducibility of PAD between electrodes and between laboratories. The method has high sample throughput, high precision and performance ruggedness for glycoprotein monosaccharide analysis.



Monosaccharide composition analysis of human serum IgG.

Rapid Screening of Sialic Acid in Glycoproteins by HPAE-PAD



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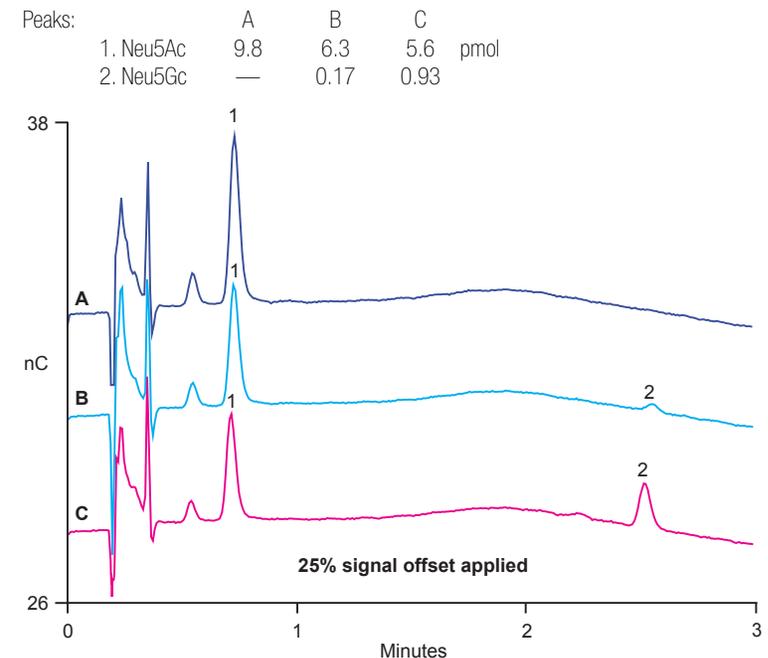
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Glycoprotein sialylation has been shown to be critical to bioavailability, stability, metabolism, and immunogenicity of therapeutic proteins. As a result, such proteins are routinely analyzed to determine sialylation amount and identity. Although over 50 forms of sialic acid have been identified, two forms of this carbohydrate are routinely determined, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Of these, Neu5Gc is generally not found in human proteins. Due to this lack of Neu5Gc in healthy human tissue and the natural occurrence of antibodies against Neu5Gc, this sialic acid has the potential to cause an immune response in patients when present in a glycoprotein therapeutic.

Conditions

Column:	Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent Gradient:	70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Eluents:	A: 100 mM NaOH, B: 1.0 M sodium acetate in 100 mM NaOH
Flow Rate:	0.5 mL/min
Injection Volume:	4.5 µL (full loop)
Temperature:	30 °C
Detection:	Pulsed amperometric, disposable Au on PTFE working electrode
Background:	18–25 nC (using the carbohydrate waveform)
Noise:	~15–30 pC
Backpressure:	~750 psi

In this application update, sialic acids are determined in five representative glycoproteins by acid hydrolysis release followed by HPAE-PAD. The method is both specific and direct, eliminating the need for sample derivatization common in other chromatographic methods. Good recoveries, precision, and linear detection for Neu5Ac and Neu5Gc are demonstrated, indicating the method is appropriate for glycoprotein analysis.



Separation of fetuin, h. AGP, s. AGP hydrolyzates (1:100 dilution) on the Dionex CarboPac PA20 Fast Sialic Acid column.

Evaluating Protein Glycosylation in Limited-Quantity Samples by HPAE-PAD

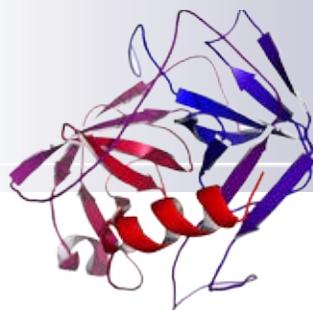


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Changes in protein glycosylation are frequently studied in cancer research to identify potential biomarkers. Factors investigated include differences in overall oligosaccharide (glycan) content, monosaccharide content changes, differences in sialylation amount and sialylation linkages, the degree of fucosylation, and differences in glycan branching. PSA is an example of a well known glycoprotein cancer marker. The glycosylation of this protein is of importance as researchers seek to understand the changes that occur in this protein during carcinogenesis and tumor growth to provide a greater understanding the of the disease.

In this study, the glycosylation of human transferrin (as a model of PSA) and PSA are investigated by HPAE-PAD using $<10 \mu\text{g}$ of protein. Two methods are utilized to evaluate protein glycosylation. One method is used to investigate the *N*-linked oligosaccharides and glycan sialylation. A second method evaluates the potential presence of *O*-linked glycans by monitoring monosaccharide composition. By using a combination of acid hydrolysis and enzymatic digestion steps prior to HPAE-PAD analysis, information about the glycosylation, as well as linkages within the glycans, is determined.

Conditions Monosaccharides¹³

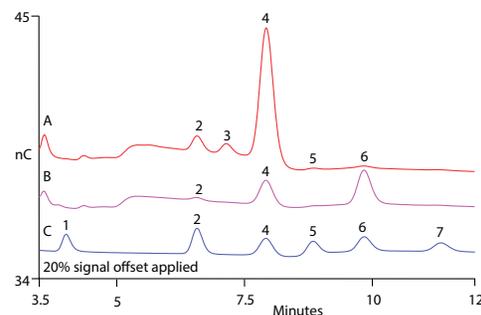
Columns:	Thermo Scientific™ Dionex™ AminoTrap™, 3 x 30 mm Dionex CarboPac PA20 Analytical, 3 x 150 mm
Eluent:	10 mM potassium hydroxide (KOH)
Eluent Source:	Dionex EGC III KOH Cartridge with Dionex CR-ATC Continuously Regenerated Anion Trap Column
Flow Rate:	0.5 mL/min
Inj. Volume:	5 μL (partial loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	~30 nC
Noise:	~20 pC
System Backpressure:	~2200 psi

* Installed before the Dionex CarboPac PA20 column

Samples: Hydrochloric acid hydrolyzates of A) 0.67 μg PSA,
B) PSA glycans after PNGase F release (equivalent to 0.09 μg
of protein), and C) monosaccharide standards

Peaks:	A	B	C
1. Fucose	ND	ND	5 pmol
2. Galactosamine	1.6	0.3	5
3. Unknown	—	—	—
4. Glucosamine	22	4.5	5
5. Galactose	N/A	N/A	5
6. Glucose	N/A	N/A	5
7. Mannose	ND	ND	5

ND = Not Determined



Separation of PSA acid hydrolysates on a Dionex CarboPac PA20 column. Both the total protein hydrolysis and the PSA-N-linked glycans have a similar ratio of galactosamine/glucosamine of 0.07. The hydrolysis conditions here do not allow reliable quantification of galactose and glucose.

Sialic Acids in Glycoprotein Hydrolyzates by HPAE-PAD

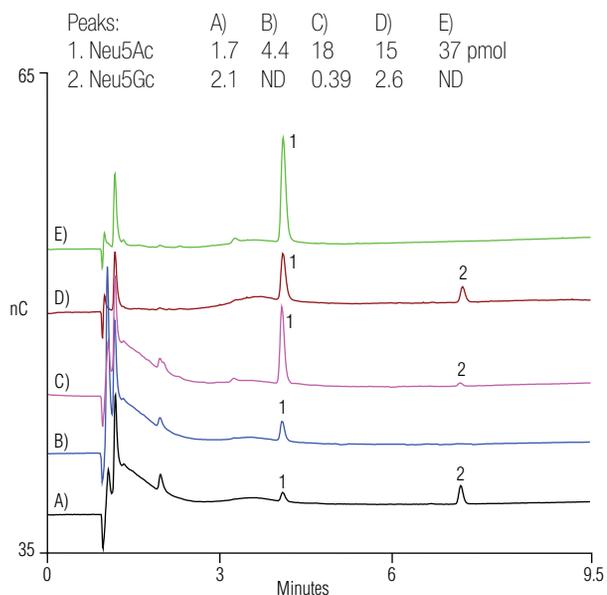
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Sialic acid determination can be performed by many methods. Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are many options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can cause overestimation of the concentration of sialic acids in many samples. Therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred. Among the chromatographic methods, there are those that require further sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC), and direct detection methods such as HPAE-PAD. Of these methods, HPAE-PAD offers the advantage of direct analysis without sample derivatization.



Sialic acid determination of five glycoprotein acid hydrolyzates. A 10% signal offset has been applied

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis release and HPAE-PAD. Determination of the sialic acids Neu5Ac and Neu5Gc by HPAE-PAD on a Dionex CarboPac PA20 column is specific and direct. After sample hydrolysis or enzymatic treatment, there is no need for further sample derivatization. Disposable Au on PTFE working electrodes with a four-week lifetime simplify system maintenance compared to conventional gold electrodes. The gradient method discussed separates Neu5Ac and Neu5Gc with a total analysis time of 16.5 min, which is faster than previous methods, allowing greater sample throughput.

Conditions

Columns:	Dionex CarboPac PA20, 3 × 150 mm Dionex CarboPac PA20 Guard, 3 × 30 mm
Eluent Gradient:	70–300 mM acetate in 100 mM NaOH from 0–7.5 min, 300 mM acetate in 100 mM NaOH from 7.5–9.0 min, 70 mM acetate in 100 mM NaOH from 9.0–9.5 min, 7 min of equilibration at 70 mM acetate in 100 mM NaOH
Eluents:	A: NaOH, 100 mM B: Sodium acetate, 1.0 M, in 100 mM NaOH
Flow Rate:	0.5 mL/min
Temperature:	30°C (column and detector compartments)
Inj. Volume:	10 µL
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	18–25 nC (using the carbohydrate waveform)
Noise:	~15–30 pC
System Backpressure:	~3000 psi

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- Technology Overview
- Electrochemical Detection and Dionex CarboPac Columns

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High-Performance Anion-Exchange Chromatography

HPAE chromatography can be used to separate analytes that can be ionized under high pH conditions. Carbohydrates typically have pKas in the range of 12–13. Once the pH rises above the pKa of the analyte, it becomes ionized in solution. This is accomplished using hydroxide-based eluents. And with the development of highly cross-linked, ethylvinyl benzene-divinyl benzene pellicular resins that have broad pH stability (0 to 14), separations at high pH conditions are feasible. The columns' nonporous resins have small anion-exchange microbeads carrying the anion-exchange functional groups which are permanently attached electrostatically to a larger cation-exchange resin particle. The nonporous nature of the resin minimizes band-broadening and imparts highly effective separation of a wide variety of carbohydrates, including branched oligosaccharides.

Pulsed Amperometric Detection

The detection of underivatized analytes can be achieved using pulsed amperometric detection. The potential variations are known as a waveform. The variations result in oxidizing and reducing conditions on the electrode surface, which in turn causes the oxidation of analytes bound to the working electrode surface. Pulsed amperometry detects only those compounds that contain functional groups which become oxidized at the detection voltage employed. Detection is sensitive and highly selective for electroactive species, since many potentially interfering species cannot be oxidized or reduced, and are not detected. It is also important to note that neutral or cationic sample components in the matrix elute in, or close to, the void volume of the column. As a result, the carbohydrate components of interest are not impacted even if the neutral or cationic sample components are oxidized.

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Dionex ICS-5000⁺ HPIC and Dionex ICS-4000 Capillary HPIC Systems

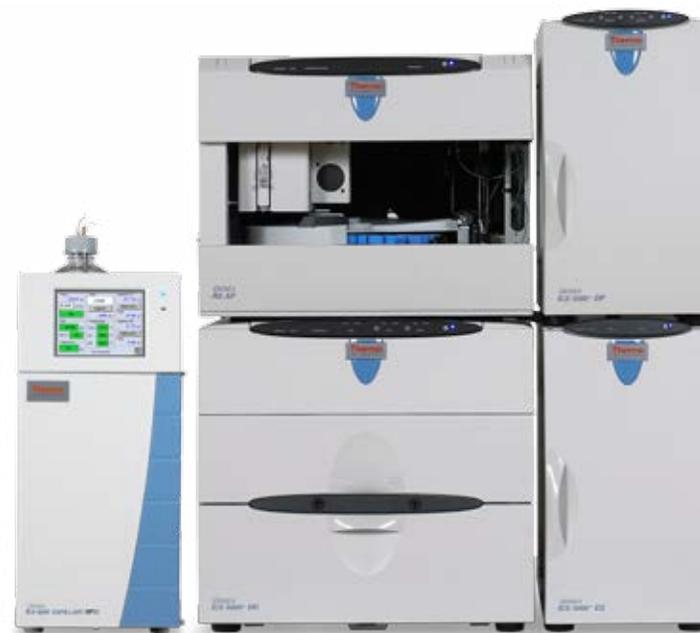
The Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC™ and Thermo Scientific Dionex ICS-4000 Capillary HPIC systems are ideal for ion-exchange chromatography of carbohydrates using electrochemical detection. With completely metal-free, all PEEK flowpaths, ion chromatography (IC) systems eliminate the possibility of metal contamination and improve robustness.

The analysis of mono- and disaccharides can be performed using Reagent-Free™ IC (RFIC™) systems with eluent generation that only requires deionized water to electrolytically generate the eluent. This technology provides consistent results and the highest reproducibility, day-to-day, user-to-user, and lab-to-lab. The all-PEEK pump of the Dionex ICS-5000⁺ system is capable of performing quaternary gradients at high flow rates, for demanding application needs.

The Dionex ICS-5000⁺ HPIC and the Dionex ICS-4000 Capillary HPIC systems use 0.4 mm i.d. columns with 1/100th the cross-sectional volume of 4 mm columns, requiring only 1/100th the sample size and using only 1/100th the eluent. Reduced eluent usage means reduced labor, reduced waste, and reduced operating costs.

Capillary HPAE-PAD

Capillary HPAE-PAD is a technique that uses capillary columns (e.g., 0.4 mm i.d. columns) packed with ion-exchange resin and a capillary amperometric cell with a gold working electrode and a palladium hydrogen (PdH) reference electrode. An electrolytic eluent generator optimized for operation at capillary flow rates is also included in the system. The system requires only very small injection volumes (0.4 µL).



Dionex ICS-4000 Capillary HPIC and Dionex ICS-5000⁺ HPIC systems.

Electrochemical Detection and Dionex CarboPac Columns

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Dionex ICS-5000+ and Dionex ICS-4000 Electrochemical Detector (ED)

The electrochemical detector cells have been redesigned to be flow and volume optimized for capillary, microbore and standard bore formats. The optional palladium hydrogen reference electrode is robust and calibration-free.

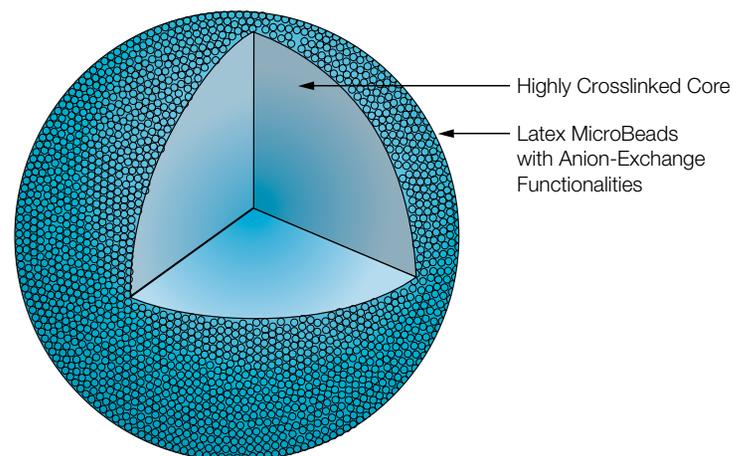
Gold working electrodes for carbohydrate analysis are available in both conventional and disposable formats. Conventional working electrodes will last for extended periods of time. However, these electrodes require periodic polishing to refinish the surface and have longer equilibration times when newly installed in a system. Disposable electrodes provide efficient, sensitive, reproducible analyses, electrode-to-electrode and lot-to-lot. After installation, rapid system re-equilibration (less than 30 min) supports quick system start-up.



Electrochemical detector cell for applications on standard bore and microbore size columns.

Dionex CarboPac Columns - Innovative Column Technology

The Dionex CarboPac family of columns offers a selection of columns, each optimized for a different class of compounds. They enable high resolution separations of closely related glycoprotein oligosaccharides, monosaccharides, and sialic acids, and a wide variety of other carbohydrates. The Dionex CarboPac columns use pellicular resin technology for improved chromatographic resolution, peak shape, and efficiency. The Thermo Scientific™ Dionex™ MicroBead™ latex particle is optimized to further improve column performance by imparting a unique chromatographic selectivity. This selectivity results in a significantly improved resolution between previously problematic analytes.



Dionex MicroBead particle.

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