

Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

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Introduction

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a widely used technique for determining an extensive set of carbohydrates including, but not limited to, monosaccharides, disaccharides, oligosaccharides, smaller polysaccharides, sialic acids and other sugar acids, sugar alcohols, sugar phosphates, and sugar nucleotides. HPAE-PAD has replaced many carbohydrate analysis techniques because, unlike most of those techniques, it can determine more than one class of carbohydrates. It also uses direct detection—thereby reducing the labor and cost of analysis, and eliminating the errors associated with analyte derivatization. HPAE-PAD provides high-resolution separations of both small carbohydrates (e.g., monosaccharides) and larger carbohydrates (e.g., oligosaccharides).

Dionex, now part of Thermo Scientific, first commercialized HPAE-PAD more than twenty years ago with the high-resolution Thermo Scientific™ Dionex™ CarboPac™ PA1 column, a metal-free polymeric liquid chromatography system designed to withstand the caustic eluents used with HPAE-PAD, and the first commercial PAD detector for liquid chromatography. We have continuously improved HPAE-PAD technology by adding five new Dionex CarboPac columns to improve separations of individual carbohydrate classes, and by improving the reproducibility and ruggedness of PAD.

As we have observed over the years, the most common barrier to success for analysts using this technique is the proper preparation and use of HPAE-PAD eluents. The majority of HPAE-PAD applications use an eluent consisting of at least two of these three components: water, sodium hydroxide, and sodium acetate. This technical note describes purity criteria for these three components, proper preparation of eluents, proper eluent storage, proper eluent use, identification of contaminated eluents, and the consequences of using contaminated eluents.

Water for HPAE-PAD Eluent Preparation

Ideally, water used to prepare HPAE-PAD eluents should be high quality deionized water of low resistivity (18 MΩ-cm or better) that contains as little dissolved carbon dioxide as possible. The water must be free of biological contamination (e.g., bacteria and molds) and particulate matter. Borate should also be absent from this water. Some of these contaminants, and others that will be discussed below, will not necessarily influence other techniques, such as HPLC and ion chromatography, but have been found to affect HPAE-PAD results.

Carbonate Contamination

Carbon dioxide dissolved in water becomes carbonate at $\text{pH} \geq 12$, the pH range of nearly all HPAE-PAD eluents. Carbonate is a divalent anion that is a stronger eluent than hydroxide, the anion commonly used for HPAE-PAD monosaccharide separations. Excessive carbonate in HPAE-PAD eluents causes a loss of carbohydrate retention and consequently reduces resolution.

While it is not feasible to eliminate all carbon dioxide from water, steps should be taken to minimize it. Prior to installing water on the HPAE-PAD system or using it for eluent preparation, vacuum degas while sonicating the water for 15 min. Alternatively, water may be degassed by vacuum filtration using a commercially available filter apparatus connected to a vacuum pump operated at a vacuum greater than 500 mm Hg (20 in. Hg), as described below. Do not vacuum degas the water in a polypropylene Thermo Scientific Dionex eluent bottle as these bottles are not designed for use under vacuum and may collapse. After degassing, immediately install the water on the HPAE-PAD system and blanket it under helium or nitrogen at 34 to 55 kPa (5 to 8 psi).

Water that is first deionized and then distilled may still be warm if freshly distilled. This will increase the amount of dissolved carbon dioxide in the water. Allow the water to reach room temperature before degassing and installing it on the system. Consequences of carbonate contamination will be discussed in more detail under Preparation of Sodium Hydroxide Eluents, below.

Borate Contamination

When the deionization cartridges of a deionized water system start to fail, one of the first ions to break through is borate. There are no other obvious signs of cartridge depletion. Borate can pair with the vicinal hydroxyls of some carbohydrates. This leads to peak tailing of the affected carbohydrate. Mannose is a common carbohydrate affected by borate contamination. The top chromatogram in Figure 1 shows how eluent contaminated with borate leads to tailing of the mannose peak (i.e., increased peak asymmetry). With 10 µg/L borate in the eluent the mannose peak has a peak asymmetry value of 1.53. To prevent this peak tailing, a Thermo Scientific™ Dionex™ IonPac™ ATC-HC Borate Form Anion Trap Column cartridge can be installed between the pump and the injection valve. After the Dionex IonPac ATC-HC Borate Form Anion Trap Column is installed the peak asymmetry value of mannose drops to 1.11 (bottom chromatogram in Figure 1). Monitor mannose asymmetry to detect and correct this problem before running additional samples. An increase in mannose peak asymmetry indicates that a deionization cartridge and/or the Dionex IonPac ATC-HC Borate Form Anion Trap Column need to be replaced. This is a problem that will not be observed by other LC techniques. Deionized water system problems that may be observed by other LC techniques, such as more extensive cartridge depletion or a reversed osmosis system failure, will also cause HPAE-PAD problems.

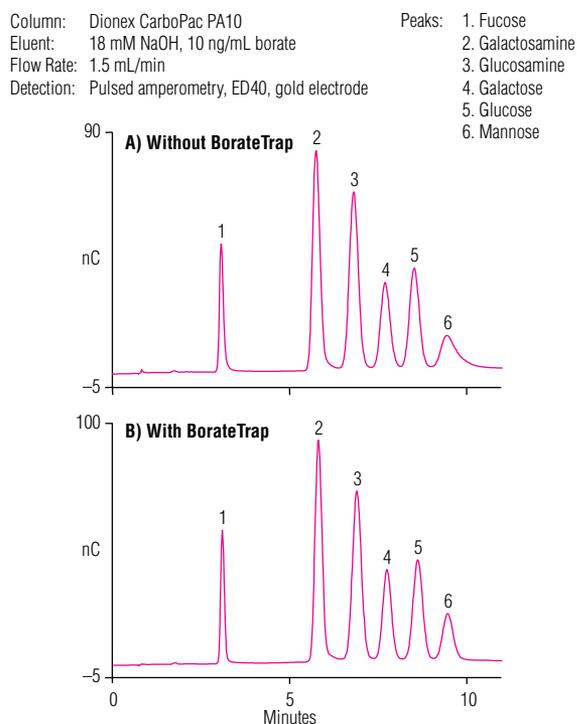


Figure 1. Tailing of the mannose peak (peak 6), seen in chromatogram A, is reduced by use of the Dionex IonPac ATC-HC Borate Form Anion Trap Column chromatogram B.

Biological Contamination

With a well-maintained and properly functioning deionized water system, biological contamination is not a concern. Most systems have both a UV light and a filter to kill and remove microorganisms and any particles that can damage the pump's check valves or piston seals. Proper maintenance of a deionized water system includes a yearly hydrogen peroxide treatment to eliminate microorganisms. If these steps are not taken to eliminate microorganisms from the deionized water system, water must be vacuum-filter degassed through a 0.2-µm nylon filter (e.g., P/N 66602, Pall Corporation) at a vacuum greater than 500 mm Hg (20 in. Hg). This can be accomplished using either a glass filter apparatus that clamps to the top of a filter flask or a sterile disposable filter apparatus (e.g., Nalgene™ 90-mm Media-Plus, P/N 164-0020; Nalge Nunc International). All glassware should be clean and free of residual surfactants. For best reproducibility, dedicate a set of glassware to HPAE-PAD eluent preparation. It is important to use a nylon filter, as filters made of carbohydrates or other PAD-active materials will contaminate HPAE-PAD eluents.

Biologically contaminated water will exhibit a high background signal (the detector signal with eluent alone flowing through the system) when used to prepare a sodium hydroxide or sodium hydroxide/sodium acetate eluent or when mixed with a sodium hydroxide eluent by the chromatography pump for monosaccharide analysis. Normal background observed when using the recommended 4-potential waveform for HPAE-PAD (Waveform A, Thermo Scientific Technical Note 21) with a 10-20 mM sodium hydroxide eluent is approximately 20 nC. When using sodium acetate eluents containing 100 mM sodium hydroxide, the background is approximately 30 nC. The background will increase a few nC while running a sodium acetate gradient. Biologically contaminated water may only double the expected background. When this occurs it often goes unnoticed. Increased contamination, and hence increased background, reduces the peak area response of the analytes of interest. This is often misinterpreted as a loss of sensitivity, but in reality the normal response is masked by the high background.

Caution: An elevated background is also a symptom of disposable gold electrode failure.

If using a disposable electrode, replace it before investigating a possible contamination issue. Whatever your application, you should record the background when the system is operating normally so that you have a benchmark for future comparisons. If desired, you can disable the autozero function in the Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System Software program so that the actual background level is monitored continuously.

Minor biological contamination of deionized water may not be observed in HPAE-PAD eluents, but may be observed as extra peaks in an HPAE-PAD chromatogram when running a gradient elution or when that water is used to prepare samples. For example, if the water is used to dissolve a sample prior to acid hydrolysis for monosaccharide analysis, one may observe a very large glucose peak. On one occasion, a series of peaks in the middle of a sodium acetate gradient during an oligosaccharide analysis was found to be due to biologically contaminated plastic tubing used to remove water from the still of a deionized-distilled water system.

Peroxide Contamination from Annual Treatment

Annual peroxide treatment can lead to a short-term problem for water used for HPAE-PAD eluents. If the deionized water system is not adequately flushed after peroxide treatment, residual peroxide in the deionized water will produce a high background. This contamination with peroxide will be transparent to most LC detection methods, but not PAD. Figure 2 shows the background produced by eluents made with deionized water from a water system recently treated with hydrogen peroxide. When the high background was noticed, the eluents were replaced with eluents prepared using a different source of deionized water. When the fresh eluents reached the detector, the background returned to normal. If the lab is located in an area with water use restrictions that do not allow adequate flushing of the deionized water system, reserve some water in clean containers prior to the peroxide treatment to ensure continued chromatographic operations and to provide a means to troubleshoot unusual background levels should they be observed.

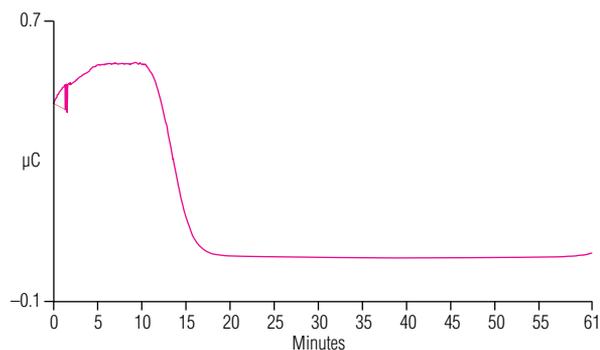


Figure 2. Effect of residual peroxide on HPAE-PAD response. The initial high background caused by peroxide contamination of the eluent decreases once uncontaminated eluent reaches the detector (after approximately 15 min).

Glycerol Contamination

Glycerol is another deionized water system contaminant that can be transparent to other LC techniques. For example, we found that when water purification filters were replaced, the water could be temporarily contaminated with glycerol. After two occurrences, we requested and now receive filters that are not manufactured with glycerol. We do not know the prevalence of this problem. When glycerol or other electrochemical contaminants, such as electrochemically active surfactants used by filter manufacturers, are present in either the NaOH eluent or the NaOH proportioned eluent, high background levels are observed. Figure 3 shows two chromatograms of a blank run (water injection) of a monosaccharide analysis method, one prepared with glycerol-containing water (chromatogram A) and one prepared with water from another deionized water system (chromatogram B). Notice the reduction in background between chromatograms A and B. Also note the large peak in the column cleanup step in chromatogram A and the large dip in the baseline at the void volume. Large dips are often a sign of a high background due to eluent contamination. Like the problem with residual peroxide, this contaminant will eventually subside in the source water with water system use.

Column: Dionex CarboPac PA1 and guard column
 Eluent: 0–12 min 150 mM Sodium Hydroxide, 150 mM Sodium Acetate;
 12–17 min 150 mM Sodium Hydroxide, 500 mM Sodium Acetate;
 17–25 min 150 mM Sodium Hydroxide, 150 mM Sodium Acetate
 Flow Rate: 1.0 mL/min
 Inj. Volume: 25 µL
 Temperature: 30 °C
 Detection: PAD waveform B (TN 21)
 Sample: Water
 Chromatograms: A. Water with glycerol from DI cartridges.
 B. Water from alternate DI system.

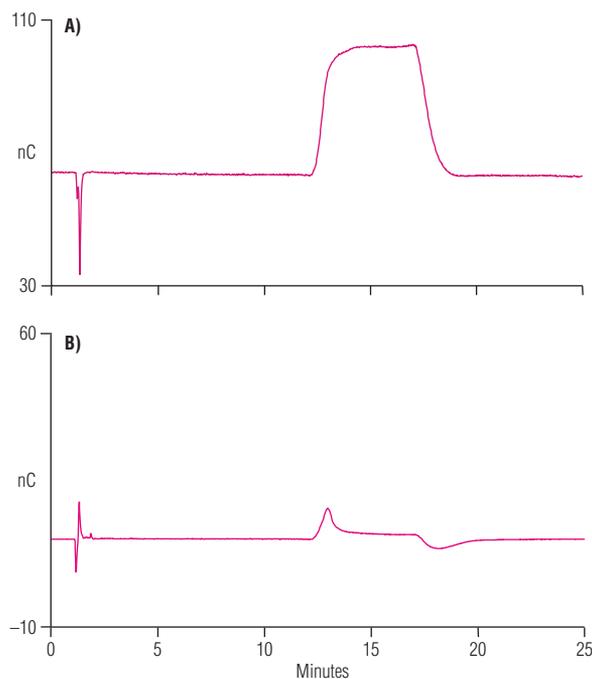


Figure 3. Effect of glycerol in the eluent on HPAE-PAD response. The normal rise and fall in background caused by the gradient steps between 150 mM and 500 mM sodium acetate (chromatogram B) are exaggerated by glycerol contamination (chromatogram A).

When your HPAE-PAD system is yielding the expected backgrounds, it is a good time to qualify bottled HPLC water for use in eluent preparation (i.e., use it in the water eluent bottle and prepare a hydroxide eluent with the same water). While bottled HPLC water is not 18 M Ω -cm, a previously unopened bottle can offer a temporary solution when there is a problem with the deionized water, or can be used to prepare fresh eluents when there is a suspected problem with deionized water.

Preparation of Sodium Hydroxide Eluents

Use of an eluent generator is the most reliable method for producing high-purity hydroxide eluents for monosaccharide analysis. Because the eluent is generated electrolytically, carbonate is not introduced from the NaOH solution or from the atmosphere during mixing. Thermo Scientific Technical Note 40 details the operation and value of using an eluent generator for monosaccharide analysis of mammalian glycoproteins.

Sodium hydroxide eluent contamination comes primarily from either the water used for eluent preparation or from carbonate. As discussed above, carbonate can not be completely eliminated from manually prepared eluents for HPAE-PAD, but contamination can be minimized.

The water used to prepare sodium hydroxide eluents must meet purity requirements and should be degassed, for best results, as discussed under Carbonate Contamination above. A 50% (w/w) sodium hydroxide solution (Fisher Scientific P/N 080389) should be used as the source of sodium hydroxide. *Sodium hydroxide pellets should never be used as they are covered with a thin layer of sodium carbonate.* Also, commercially available 1 N sodium hydroxide solutions should not be used as these solutions have high carbonate concentrations.

To prepare 1 L of 200 mM sodium hydroxide, either pipette 10.4 mL or weigh 16.0 g 50% (w/w) sodium hydroxide into a plastic 1-L volumetric flask containing approximately 800 mL degassed deionized water. If pipetting, use a plastic 5 or 10 mL sterile serological pipette with 0.1 mL gradations. Briefly stir this solution (15–30 s) and then bring to volume. Immediately transfer this solution to the plastic eluent bottle on the HPAE-PAD system and blanket it with helium or nitrogen at 34 to 55 kPa (5 to 8 psi). Gently swirl the bottle to complete mixing. Plastic labware and eluent bottles must be used for all eluents containing NaOH. NaOH dissolves borosilicate glass, releasing borate into the solution.

Fresh eluent should be prepared every week or any time the helium or nitrogen headspace is lost for more than 15 min. Eluent stirring must be brief. Excessive stirring, especially without an inert gas headspace, will result in the introduction of too much carbonate to the eluent.

Figure 4 shows an example of monosaccharide chromatography with a properly prepared sodium hydroxide eluent (chromatogram A) and a sodium hydroxide eluent that was stirred for 30 min (chromatogram B). Note the loss of retention time and loss of monosaccharide resolution in chromatogram B. This is an example of significant carbonate contamination. A lower level of carbonate contamination would cause a smaller loss of retention time and therefore less loss of resolution.

Chromatography similar to the top chromatogram would also be observed if the unavoidable amount of carbonate from properly prepared eluents and samples was allowed to build up in the column, thus consuming column capacity. To prevent accumulation, wash the column with 200 mM sodium hydroxide after each injection.

Column: Dionex CarboPac PA20 and Thermo Scientific™ Dionex™ AminoTrap™
 Eluent: 10 mM NaOH
 Flow Rate: 0.5 mL/min
 Inj. Volume: 10 μ L
 Detection: PAD (Au) disposable Waveform A (TN 21)
 Temperature: 30 °C
 Sample: Mix of six standard (50 μ M)

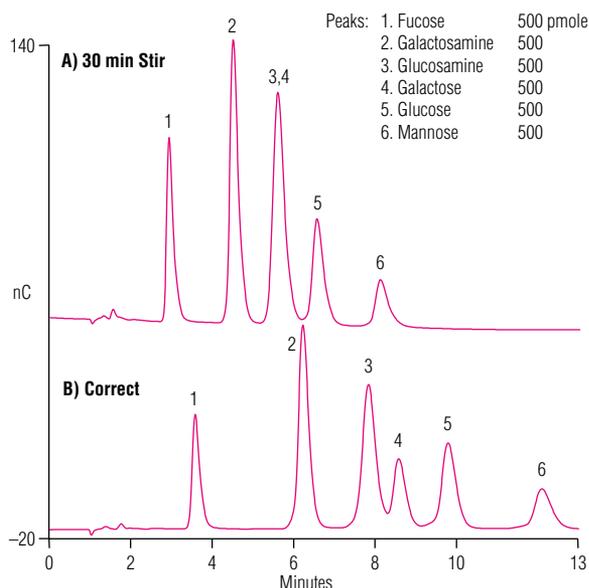


Figure 4. Effect of carbonate contamination on HPAE-PAD monosaccharide analysis. Carbonate, a stronger eluent than hydroxide, reduces resolution of monosaccharides (chromatogram B).

It is also important that the 50% sodium hydroxide solution is handled correctly. This solution will trap carbon dioxide from the atmosphere, forming sodium carbonate that will settle to the bottom of the bottle. Therefore, do not shake the bottle of 50% sodium hydroxide before using, only remove solution from the middle of the bottle, and do not use the last third of the bottle. The remainder of the bottle can be used for other applications in the lab. Dedicated bottles of 50% sodium hydroxide solution for HPAE-PAD help to reduce improper handling by other lab personnel or inadvertent chemical contamination. Failure to follow these instructions will lead to excessive carbonate in the sodium hydroxide eluent.

Contaminated sodium hydroxide eluents have high backgrounds (see guideline under Water for HPAE-PAD Eluent Preparation, above), noise, and large dips in the baseline. This is usually due to the use of contaminated water for eluent preparation. Sodium hydroxide eluents contaminated by carbonate will yield poor chromatography characterized by reduced retention times. The top chromatogram in Figure 4 shows an example of extreme carbonate contamination.

Preparation of Sodium Acetate/ Sodium Hydroxide Eluents

When separating carbohydrates that are charged at neutral pH (e.g., sialic acids) or large oligosaccharides (e.g., asparagine-linked branched oligosaccharides from glycoproteins, maltodextrins, etc.), a stronger eluent than sodium hydroxide is required. Sodium acetate eluents are used for these separations. These separations still require sodium hydroxide because a strongly basic environment is required for detection. Most oligosaccharide separations use a sodium acetate gradient with a constant concentration of sodium hydroxide, usually 100 or 150 mM sodium hydroxide. (See Dionex, now part of Thermo Scientific, Application Note 67 for a comparison of 100 and 150 mM sodium hydroxide for maltodextrin separations.) These separations should be executed by proportioning two eluents, one eluent that has the desired sodium hydroxide concentration and a second eluent with sodium acetate in the same sodium hydroxide concentration as the first eluent. The most common sodium acetate concentration is 1 M, but weaker gradients may require 500 mM sodium acetate.

The quality of acetate from a variety of vendors has been found to be a source of problems at some customer sites. We have used anhydrous sodium acetate from Fluka Chemika-Biochemika without observing any problems. Thermo Scientific sells electrochemical grade anhydrous sodium acetate (P/N 059326) that is tested for AAA-Direct™ anion-exchange of amino acids with integrated pulsed amperometric detection. This acetate can be used with HPAE-PAD and may be helpful in troubleshooting a possible acetate eluent problem.

Prepare 1 L of 100 mM NaOH using the same procedure described for preparing the 200 mM NaOH, but using only 5.2 mL or 8.0 g of 50% NaOH. To prepare 1 L of 100 mM NaOH/1M sodium acetate, first dissolve 82.04 g of high-purity anhydrous sodium acetate into approximately 800 mL deionized water. Vacuum filter this solution through a 0.2 µm nylon filter to remove particles from the sodium acetate that can damage parts of the pump. This filtration is often slow, as the insolubles in the sodium acetate will gradually clog the filter. Remember to disconnect the vacuum from the solution before turning off the vacuum pump to prevent backwash into the filtered eluent. After filtration, transfer the solution to a plastic 1-L volumetric flask, add 5.2 mL or 8.0 g of 50% NaOH (for 100 mM), and bring to volume. Immediately transfer this solution to the plastic eluent bottle on the HPAE-PAD system and blanket it with helium or nitrogen at 34 to 55 kPa (5 to 8 psi). Gently swirl to complete the mixing. Be careful to prevent the solution from coming into contact with the inert gas tubing while swirling.

It is also acceptable to add the sodium hydroxide and bring the solution to volume before filtering (see Thermo Scientific Technical Note 41) as long as stirring is minimized after hydroxide addition. It is most important that whichever of the two approaches is adopted, the chosen approach should be standard operating procedure for the lab. Fresh eluent should be prepared every week or any time the helium headspace is lost for more than 15 min.

While it may be tempting to prepare three eluents—water, hydroxide, and acetate—and create the gradient profile for oligosaccharide separations using the pump to proportion these three eluents, Dionex strongly advises against this approach. This approach may provide short-term success, but often leads to system contamination. A sodium acetate solution without sodium hydroxide is an excellent medium for mold and bacteria. An eluent with microbial contamination can contaminate the whole system.

To decontaminate the system, replace all tubing, including eluent lines, and wash the system with 2 N sodium hydroxide overnight. Labs that use sodium acetate without hydroxide often experience problems in warmer months, but microbial contamination is possible at all times of the year. If the separation requires acetate near neutral pH where sodium hydroxide is added post-column for PAD (e.g., O-methylated galacturonic acids), prepare the acetate eluent under as sterile conditions as possible (e.g., use the disposable filtration device discussed above) and do not use old acetate eluents.

A contaminated sodium acetate eluent will have a high background (>40 nC), and may yield unexpected peaks during gradient programs. Highly contaminated eluents will exhibit backgrounds in the hundreds of nanocoulombs and there will be large dips in the baseline. If the water has been verified to be clean, the culprit is the sodium acetate.

Proportioning three eluents is not as rugged as proportioning two eluents. Therefore, for best long term performance and best success at transferring a method to another system and/or lab, design a two-eluent method rather than a three-eluent method. Dionex also advises that when possible the initial conditions should include a small amount of acetate in the eluent. During transition from no acetate to acetate, a small spike in the baseline will be observed when the column changes from the hydroxide form to the acetate form (the counter ion to the anion-exchange group). As little as 10 mM acetate at the start of the analysis will eliminate this spike. When separating oligosaccharides, it is unlikely that starting with 10 mM rather than no acetate will affect the separation. It will affect the separation of monosaccharides and oligosaccharides (or charged carbohydrates) in the same injection. In these cases, consider analyzing the sample in two injections with two shorter methods, one for monosaccharide analysis and one for the oligosaccharide or charged sugar analysis.

If metal-containing components that are not approved by Dionex (e.g., a pump with stainless steel heads) are used in the flow path, the column and/or working electrode can be contaminated with metal. This is especially problematic when using acetate-containing eluents. Metal-contaminated columns and working electrodes usually require replacement. In the unlikely event that metal contaminates the Dionex pump (e.g., use of a non-approved autosampler), contact the Dionex Technical Support Group for instructions to remove metal contamination from the Dionex pump and other contaminated flow paths.

References

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2. Thermo Fisher Scientific. Dionex Technical Note TN 40: *Glycoprotein Monosaccharide Analysis Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) and Eluent Generation*. TN40_E 05/12S LPN1632-01. Sunnyvale, CA, 2004.
3. Thermo Fisher Scientific. Dionex Application Note 67: *Determination of Plant-Derived Neutral Oligo- and Polysaccharides*. LPN 1474. Sunnyvale, CA, 2003.
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