Technical Overview

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Ion Exchange Chromatography for Biomolecules: Method Development and Troubleshooting Tips

Abstract

Ion exchange (IEX) chromatography is a powerful analytical tool, particularly for separating closely related biomolecules, but it requires particular attention to method details and column selection because it is sensitive to differences in pH and salt concentration. This technical overview provides guidelines to assist users with column selection, method development, and solutions to problems commonly encountered in IEX chromatography methods.

Introduction

Ion exchange (IEX) chromatography is a powerful separation technique used to analyze or purify proteins, peptides, nucleic acids, and other charged biomolecules based on their net surface charge. It works by exploiting the electrostatic interactions between charged analytes and oppositely charged functional groups on a resin or stationary phase.

IEX chromatography is ideal for separating closely related biomolecules and is fully scalable and cost-effective, particularly compared to preparative-scale reversed-phase chromatography. Nevertheless, IEX chromatography method development and troubleshooting can present challenges because careful control of pH and ionic strength are required to achieve optimal separation. This technical overview introduces IEX chromatography principles, method development, column selection guidelines, and troubleshooting tips.

Understanding IEX chromatography

IEX chromatography is widely used to separate biomolecules based on differences in ionic charge. Generally, ions with a greater charge will bind more strongly to the stationary phase and elute later than ions with less charge. It is a mild, non-denaturing technique that does not require organic solvents. Common IEX chromatography applications include charge variant analysis for proteins, as well as peptide, protein, and oligonucleotide purification.

The overall charge of the molecule is dependent on the pH of the surrounding solution, which in turn will affect the ion exchange method that can be used. IEX methods commonly use a salt gradient in which the mobile phase must maintain a controlled pH throughout the separation. Therefore, aqueous buffers are used as eluents. During salt gradients, the ionic strength of the mobile phase is increased to elute proteins in order of increasing binding strength to the column (Figure 1). In a pH gradient, salt may be used to wash the column after the pH gradient is complete, but the gradient itself does not contain salt. In this case, proteins elute at the pH where their net charge is zero — at their isoelectric points.

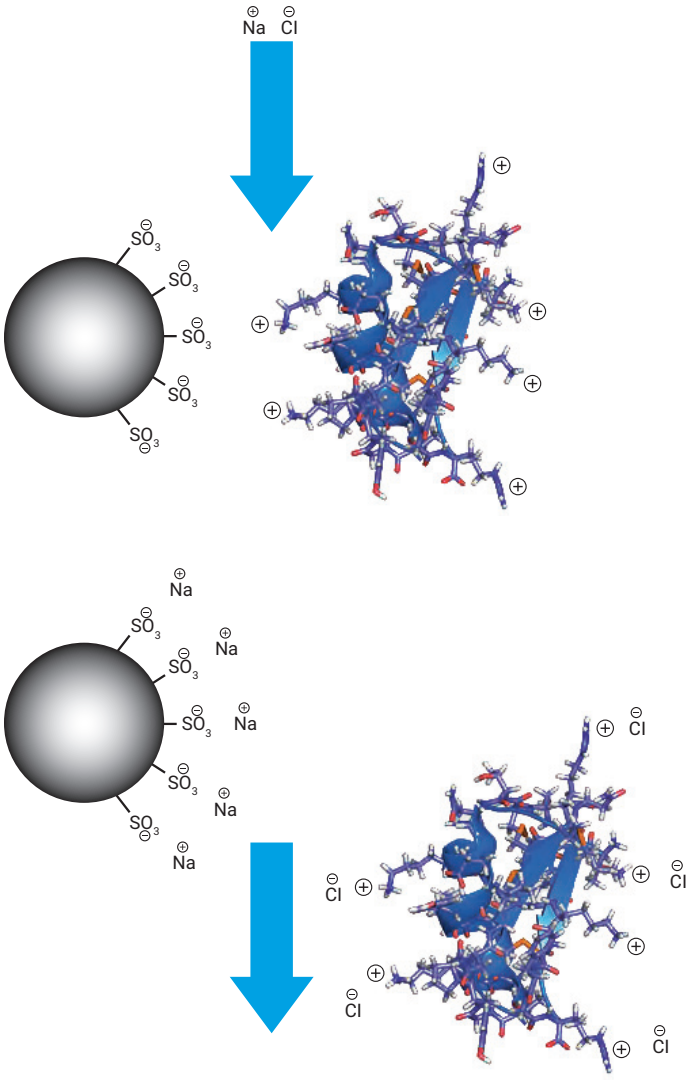

Figure 1. IEX separation mechanism.

Selecting columns

Cation versus anion and strong versus weak

As with most chromatographic techniques, there is a range of columns from which to choose. For IEX chromatography, the first consideration is selecting either an anion or cation exchange column. The functional group in a strong cation exchange column is sulfonic acid, where the stationary phase is negatively charged in all but the strongest acidic mobile phases. Cation exchange chromatography is commonly employed for basic peptides and proteins (Figure 2). Conversely, the functional group in a strong anion exchange column is a quaternary amine group, which is positively charged in all but the most basic mobile phases. Anion exchange chromatography is typically used for acidic peptides, proteins, and oligonucleotides (Figure 2).

The second consideration is choosing a strong or weak ion exchange column. Strong ion exchange columns are a good first choice, because they provide the widest operating range. Weak ion exchange columns are more sensitive to mobile phase conditions, but offer distinct selectivity and can be valuable for fine-tuning separations. These columns are thus complementary to strong ion exchange columns, allowing resolution to be adjusted through careful optimization of operating parameters.

Pore size

The non-porous Agilent Bio IEX HPLC columns are recommended when method resolution is more important than capacity. For the analysis of exceptionally large biomolecules, or where maximum speed is sought, the Agilent Bio-Monolith ion exchange columns provide optimum results. The Agilent PL-SCX and Agilent PL-SAX column particles are fully porous with 1,000 or 4,000 Å pores. It is important to ensure the pores are sufficiently large enough to allow the biomolecule to fully enter the particle unhindered. Fully porous particles provide greater surface area and greater loading capacity, which is better suited to preparative separations.

Particle size

Particle size is an important consideration in column selection. Smaller particle sizes provide more efficient separation, but at the cost of higher operating pressure. Because biomolecules are relatively large and have slower rates of diffusion, smaller particle sizes do not necessarily provide the same level of improvement in resolution that is observed with small molecules. Agilent Bio MAb and Bio IEX columns have different pressure ratings based on particle size, but all of these columns should be operated at or below 200 bar for optimal results.

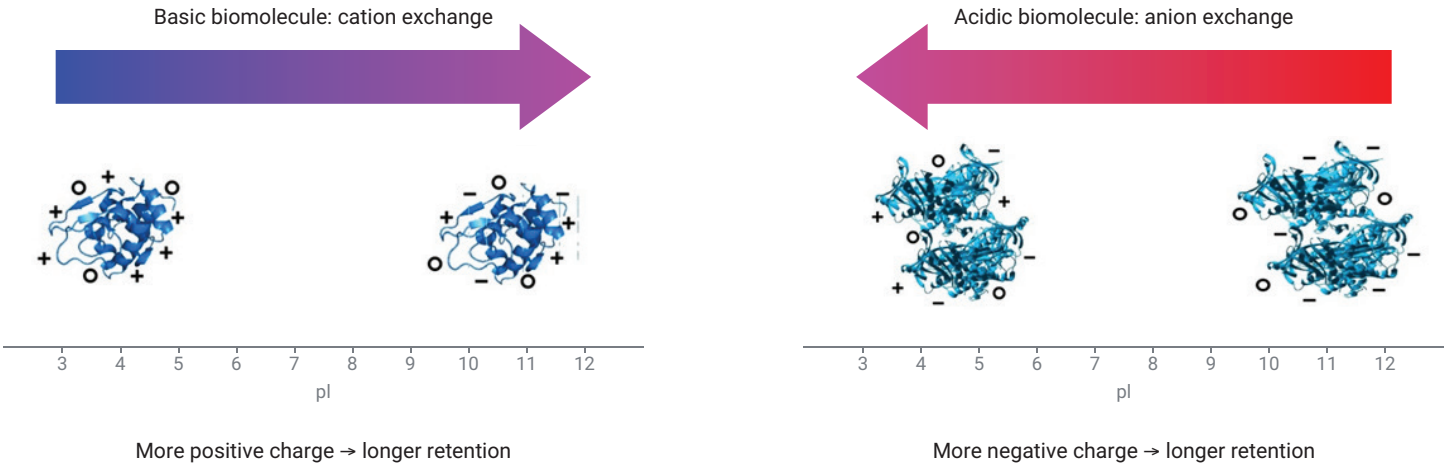

Figure 2. Comparison of cation and anion exchange chromatography.

Column hardware

Bio MAb and Bio IEX columns are available in PEEK or stainless-steel hardware. Salt gradients can be corrosive if left in contact with stainless-steel for too long. Although PEEK columns must be operated at lower backpressures, they do not suffer from salt corrosion and are beneficial for the analysis of metal-sensitive molecules. For a metal-free sample flow path, a PEEK column should be coupled with a bioinert or biocompatible liquid chromatography system such as the Agilent 1260 Infinity III Bio-Inert LC or Agilent 1290 Infinity III Bio LC.

Column diameter and length

Columns with 4.6 mm internal diameter (id) are commonly used for IEX chromatography methods. If only limited amounts of material are available, 2.1 mm id columns are useful. To prevent excessive dispersion and loss of resolution when using smaller id columns, it is important to minimize system volumes between the column and detector. For ion exchange separation of biomolecules, it is not always necessary to use a 250 mm length column. Nevertheless, there are occasions when additional column length can improve resolution. However, longer columns result in higher backpressure, so larger particle sizes may be required. To improve speed and throughput, shorter columns packed with smaller particle sizes will provide increased resolution and reduce separation time by up to five-fold. Table 1 shows a summary of suggested columns for common applications.

Method development tips: starting conditions and mobile phases

Sample preparation

Ideally, samples should be dissolved in the starting mobile phase conditions. Samples should also be freshly made and analyzed as soon as possible, as bacterial growth can develop quickly in buffered solutions. If the sample is cloudy, it may be necessary to adjust the mobile phase conditions or to filter the sample. If a smaller-sized particle (1.7 or 3 µm) column will be used, filtering the sample is critical.

Flow rate

Typical flow rate ranges for 2.1 and 4.6 mm id columns are 0.2 to 0.4 mL/min and 0.5 to 1.0 mL/min, respectively. For some applications, the speed of analysis is essential. Shorter columns, for example 50 mm instead of 150 or 250 mm, can be used to reduce the analysis time. Analysis time can also be shortened by increasing the flow rate, taking care not to exceed column pressure limitations.

Mobile phase selection and buffer preparation

Agilent Buffer Advisor software is an effective and efficient starting point for mobile phase selection because it provides a fast and easy way to create pH or salt gradients, supporting quality by design experiments. Buffers commonly used in IEX chromatography are shown in Figure 3.

Table 1. Suggested columns per application.

Application	Agilent Column	Notes
Monoclonal Antibodies	Bio MAb	Bio MAb HPLC weak cation exchange columns feature a unique resin specifically designed for high-resolution charge-based mAb separations.
Peptides and Proteins	Bio IEX	Bio IEX columns contain polymeric, nonporous, ion exchange particles, designed for high resolution, high recovery, and efficient separations.
Proteins, Peptides and Deprotected Synthetic Oligonucleotides	PL-SAX	PL-SAX material provides a fully porous polystyrene divinylbenzene (PS/DVB) particle with strong anion exchange functionality.
– Globular Proteins and Peptides	1000 Å pore sizes	
– Very Large Biomolecules/ High Speed Separations	4000 Å pore sizes	
Small Peptides to Large Proteins	PL-SCX	PL-SCX material provides a fully porous PS/DVB particle with a very hydrophilic coating and strong cation exchange functionality.
– Globular Proteins	1000 Å pore sizes	
– Very Large Biomolecules/ High Speed Separations	4000 Å pore sizes	
Antibodies (IgG, IgM), Plasmid DNA, Viruses, Phages, and Other Macro Biomolecules	Bio-Monolith	Bio-Monolith HPLC columns are well suited for high-speed separations.
– Viruses, DNA, Large Proteins	Bio-Monolith QA	Strong anion exchange phase
– Plasmid DNA, Bacteriophages	Bio-Monolith DEAE	Weak anion exchange phase
– Proteins, Antibodies	Bio-Monolith SO3	Strong cation exchange phase

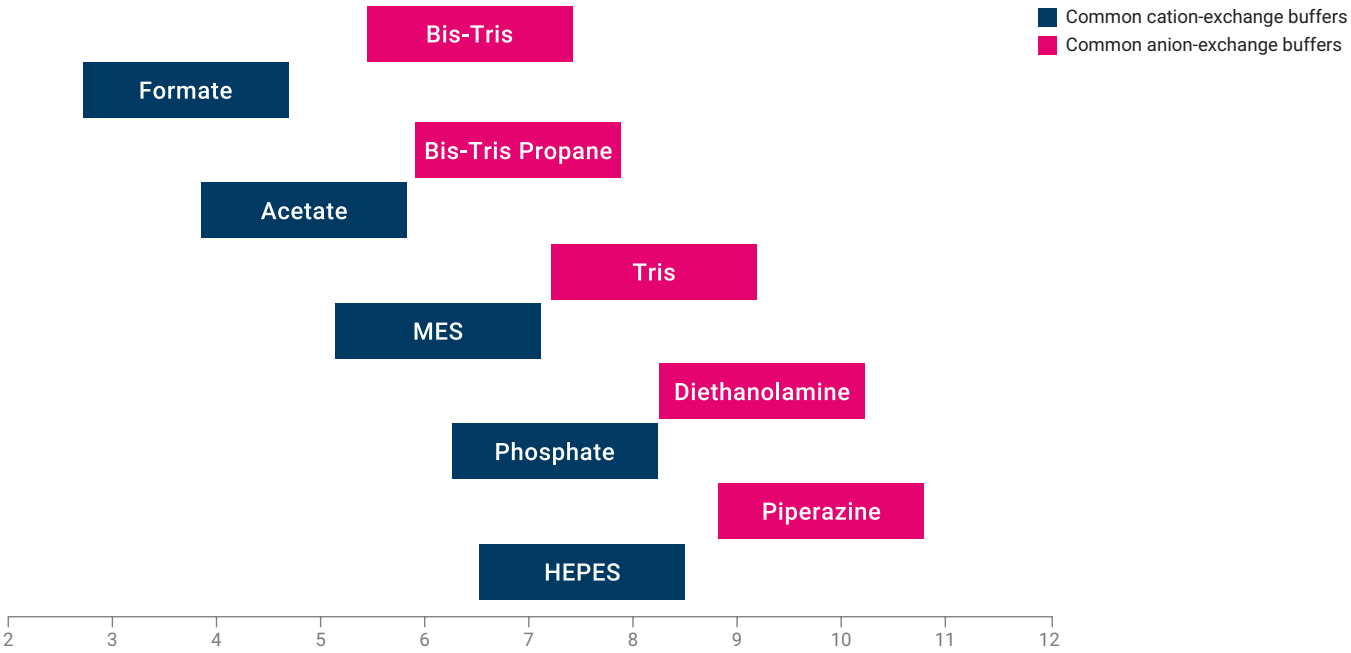

Figure 3. Common buffers used in ion exchange chromatography.

The mobile phase should contain buffer to maintain the desired operating pH, typically 20 mM. Fresh mobile phase should be made and used promptly because bacterial growth is rapid in dilute buffer stored at room temperature. Mobile phase shelf life is less than seven days unless refrigerated. Mobile phase should not be "topped up," and instead only replaced with freshly prepared solutions. Mobile phase must be filtered before use because particulates can be present, particularly in buffer salts. Before first use, new columns should be conditioned carefully according to the user guide.

In salt gradients, the role of the buffer is to control the change in pH during the separation to maintain a consistent charge on the compounds being analyzed. Mobile phase B contains the same concentration of buffer as mobile phase A with a higher concentration of salt, such as sodium chloride. Addition of sodium chloride to the mobile phase will alter the pH, so the pH should be adjusted as necessary. Because temperature also affects pH, the pH value should be adjusted after the buffers have been equilibrated to the desired running temperature.

Tip: It is important to make up buffers systematically and accurately, because minor differences in ionic strength or pH can affect the retention time of analytes, resulting in poor resolution and variability in the chromatographic profile.

The isoelectric point (pI) of the analyte is a strong consideration when choosing the pH of the mobile phase. For basic peptides and proteins, if pH < pI, the protein will have a net positive charge. The pH of the starting buffer should be 0.5 to 1 pH unit below the pI. If the pI is unknown, start with pH 6. Typical buffers for cation exchange include formate, acetate, MES, phosphate, and HEPES between pH 4 to 7.

Note: When preparing phosphate buffer, be aware that sodium dihydrogen phosphate and disodium hydrogen phosphate are available in different hydrated forms. These variations affect their formula weights, so each must be prepared accordingly.

For acidic peptides, proteins and oligonucleotides, also consider the pI of your analyte when choosing the pH of the mobile phase. If pH > pI, your protein will have a net negative charge. The pH of the starting buffer should be 0.5 to 1 pH unit above the pI. If the pI is unknown, start with pH 8. Typical buffers for anion exchange include Bis-Tris, Tris, diethanolamine, triethylammonium acetate, and piperazine between pH 7 to 10.

Note: Tris is available in two forms: Tris HCl and Tris base which have different formula weights and must be prepared accordingly. Tris HCl must be titrated to the desired pH with NaOH, while Tris base must be titrated to the desired pH with HCl.

Method development tips – gradients

Salt gradients

Salt gradients are typically linear, ending between 500 mM and 1 M salt. Shallow gradients can help to optimize challenging separations but require precise solvent mixing, and accurately and precisely delivered gradients; therefore, a binary pump is preferred. However, running too shallow of a gradient will result in peak broadening. In the application note "How Shallow Can You Go?", a gradient of 0.66% mobile phase B per minute optimized resolution without sacrificing peak shape (Figure 4).

pH gradients

pH gradients are typically used less often than salt gradients, but are more compatible for mass spectrometry (MS) detection and can prevent corrosion of the column and LC system. A pH gradient consists of a starting buffer and an elution buffer. In the starting buffer, the analyte must have a net surface charge that allows it to interact effectively with the ion exchange resin's functional groups. For example, in cation exchange, the starting pH of the buffer system is low. The pH is then increased linearly, resulting in deprotonation of the protein's amino acids, and subsequently elutes once the pI of the protein and the pH of the mobile phase match. At the same time, the buffer forms a pH gradient across the column. In contrast, in anion exchange chromatography, the starting conditions are at high pH, and the pH decreases towards acidic conditions during the chromatographic run. An example of a method that uses a pH gradient is in application note 5994-3257EN.

Table 2. Summary of advantages and disadvantages of salt and pH gradients.

	Advantages	Disadvantages
Salt Gradient	Easier to run a scouting method/generic gradient	Can cause corrosion to stainless-steel components in the LC and column
pH Gradient	– Enables MS detection with a volatile mobile phase – Can fine-tune the method – Can provide higher resolution	Can be challenging to buffer mobile phases to a consistent pH and ionic strength

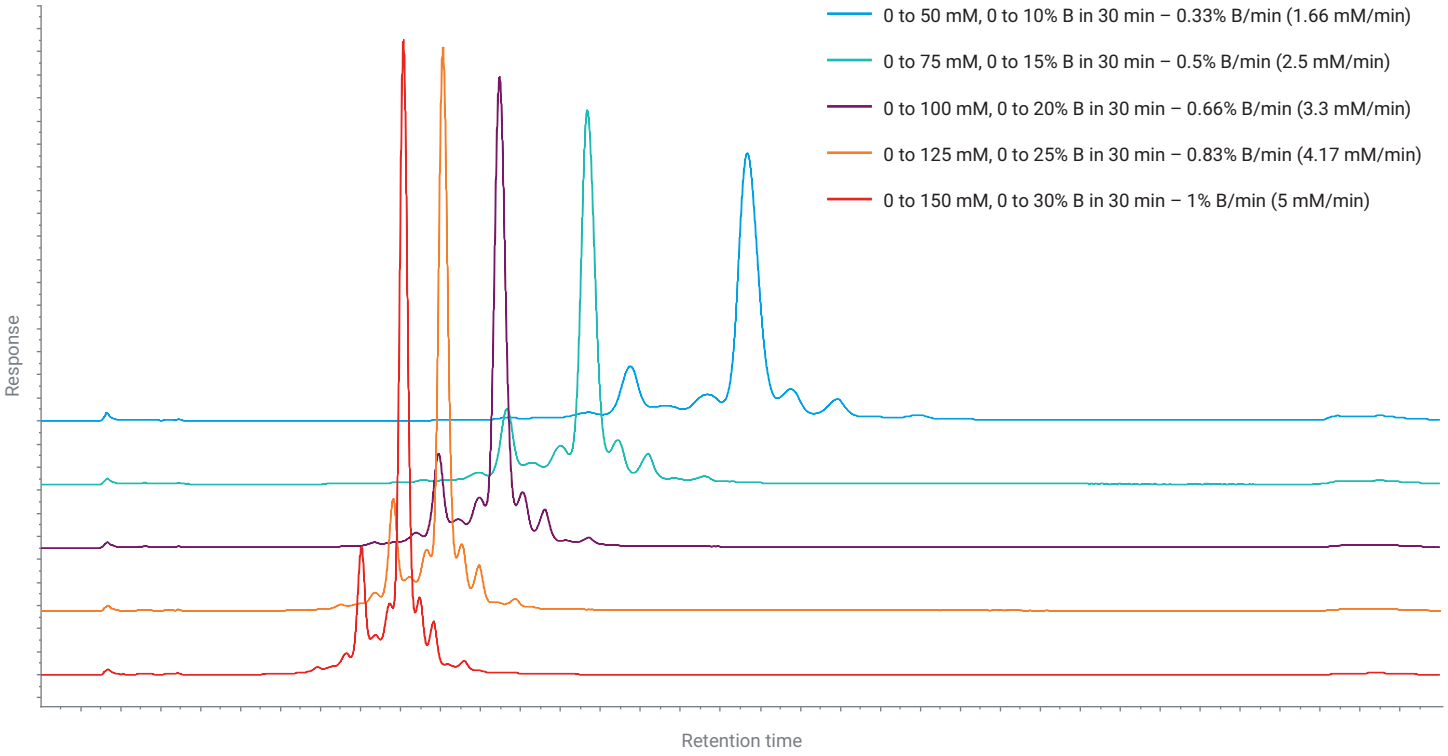

Figure 4. A gradient increasing by 0.66% mobile phase B per minute optimized charge variant resolution while maintaining sharp peaks.

Method development tips – column conditioning and equilibration

Tip: For reproducible ion exchange separations, the column equilibration and cleanup phases of the gradient are critical. Analyte elution is achieved by increasing the ionic strength or changing the eluent pH, or both. At the end of each analysis, the column must be fully equilibrated back to the starting conditions, including ionic strength, and pH, using 5 to 10 column volumes of mobile phase. For reference, Table 3 lists column dimensions and corresponding column volume. If equilibration is not done carefully, the next column run will have a different profile because the analyte will interact differently with the column.

Table 3. List of column dimensions and their corresponding column volume. To equilibrate an ion exchange column, all of the volumes in the table must be multiplied by 5 to 10 to achieve full equilibration.

Column Dimension (mm)	Column Volume (mL)
2.1 × 50	0.17
2.1 × 150	0.52
2.1 × 250	0.87
4.0 × 10	0.13
4.95 × 5.2	0.10
4.6 × 50	0.83
4.6 × 150	2.49
4.6 × 250	4.2
7.5 × 50	2.2
7.5 × 150	6.6
10 × 250	19.6
21.2 × 250	88.3
25 × 50	24.5
25 × 150	73.6
50 × 150	294.5
100 × 300	2,356.2

Considerations for oligos

- Adding 0.1% Agilent InfinityLab Deactivator Additive to mobile phases A and B can increase abundance and sharpen peaks by eliminating nonspecific binding in metal flow paths.
- Ion exchange separations are influenced by oligo length, sequence composition (especially GC content), and secondary structure.
- Secondary structures such as hairpins or duplexes can shield or expose phosphate groups, which changes the effective charge density of the oligo, which in turn can affect retention and resolution. Higher temperatures or a low amount (~10%) of organic can help to reduce secondary structures.

Detection options

UV detection: UV detection is most commonly used with IEX chromatography because it has a higher tolerance for salt than MS detection and its wavelengths are tunable to detect different analytes.

Note: some buffers absorb at low UV wavelengths and may prevent use of wavelengths of 210 to 230 nm for detection.

Recommended wavelengths by analyte type

- Peptides: 214 or 280 nm
- Proteins: 220 or 280 nm
- Oligonucleotides: 260 or 270 nm

Mass spectrometry detection: MS detectors are specific and sensitive but less tolerant of salts. If MS detection is preferred, a pH gradient can be employed. If a salt gradient is used, an offline clean-up or 2D-LC method must be used to remove non-volatile salts from entering the mass spectrometer.

Troubleshooting

This section identifies the problems commonly encountered in IEX chromatography and provides practical solutions.

Issue: Poor reproducibility with shifting retention times or variable resolution

Root Cause	– Inconsistent buffer preparation – Not enough column wash or equilibration time
Resolution	– Prepare buffers with consistent salt concentration and pH to ensure reproducible results. The buffering agent should have a pKa within ± 0.5 pH units of the operating pH. Otherwise, there is a risk of dramatic pH fluctuation due to the limited buffering capacity of the buffering agent. – To ensure reproducible separations, the gradient should have an adequate wash at high salt and a long equilibration time, at least 5 to 10 column volumes.

Issue: High pressure

Root Cause	– Low interstitial space between particles – Viscous (methanol/water or high salt) solvents at high flow rates – Analyte adsorption to packing material or frit – Algal growth
Resolution	– Bio IEX/Bio MAb 1.7 µm particles only have a 0.263 µm interstitial diameter and are easily clogged, so it is important to filter mobile phase and samples with a 0.22 µm filter. – Aqueous buffers can be quite viscous. Flush the column at a low flow rate (approximately 0.1 mL/min) with high %A and then high %B until the pressures have stabilized. Slowly ramp the flow rate and adjust the method flow rate if needed. – Clean the column to remove any protein or oligonucleotide that may be stuck to it. See pages 3 and 4 of this user manual for instructions. – Algae contamination can easily occur in aqueous solvents. Refer to the cleaning protocol in the technical note: Best Practices for Using an Agilent LC System. – If cleaning and flushing the column and LC system does not fix the issue, the column may need to be replaced.

Issue: Ghost peaks

Root Cause	Buffer contaminants
Resolution	Use high-purity buffer salts in the mobile phase, and ensure that bottles are clean and detergent-free.

Issue: Broad or split peaks

Root Cause	Particle compression or column void due to application of too much pressure to the column. Column void can also be caused by improper connections from the column to the capillary.
Resolution	– Always slowly ramp the flow rate when using IEX columns. Do not increase the flow rate until the pressure has stabilized. – Bio IEX and BioMAb columns have varying pressure limits, but regardless of their particle size, all columns should operate under 200 bar. – PL-SCX, PL-SAX, and Bio-Monolith columns have low pressure limits, 207 and 150 bar respectively, so carefully monitor backpressure. – Look at the column's certificate of performance to understand its flow rate and backpressure operating range. – The column may need to be replaced.

Issue: Loss of resolution and peak efficiency over time

Root Cause	– LC issues – Dirty column – Particle compression or column void
Resolution	– Ensure there have not been changes to the LC system, including capillary changes or leaks. Also, check to see if the LC pump is working properly. – If the column is dirty, follow the instructions in the ion exchange column user manual to clean it. – If there is a column void or the particles have been compressed, assess the method to ensure back pressure does not go too high. – The column should be replaced. – See the "Issue: Broad or split peaks" section of the troubleshooting area for more troubleshooting tips.

Conclusion

IEX chromatography is a powerful tool, particularly for separating closely related biomolecules, but it can be tricky to deploy because it is sensitive to differences in pH and salt concentration.

Key takeaways

- Buffers should be made carefully with consistent pH and salt concentration. pH meters should be calibrated regularly, and electrodes should be replaced annually. Buffers should be refrigerated when not in use and changed every few days if running analyses at room temperature.
- Flush the LC weekly to ensure there is no salt or algae build-up. Run water through the system first, because organic solvents may precipitate salts.
- The starting pH of mobile phase A is critical to ensure adequate separation, and should be 0.5 to 1 unit from the pI of the analyte. Cation exchange buffers should have a lower pH than the analyte. Anion exchange buffers should have a higher pH than the analyte's pI.
- To ensure reproducible separations, the gradient should have an adequate wash at high salt and a long equilibration time with at least 5 to 10 column volumes of mobile phase.

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