

Chapter

BIST – Bridge Ion Separation Technique – A New Mode of Separation for Charged Molecules

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Abstract

General review of recently reported novel technique for separating charged molecules using a doubly charged ionic modifier in the mobile phase, and the use of anion-exchange column for separation of cations and a cation-exchange column for separation of anions. This new separation technique, termed Bridge Ion Separation Technique (BIST), is universal for both positively and negatively charged species, including organic and inorganic ions. Additionally, it is suitable for analytes ranging from small molecules to macromolecules. It is particularly effective for the separation and analysis of multicharged compounds, which are often difficult to resolve using traditional ion-exchange systems due to excessively strong interactions. It can be universally used to separate both hydrophobic and hydrophilic charged molecules. The selectivity of BIST is very often different from any other separation mode. Representative examples include small ions as well as biopolymers such as peptides and oligonucleotides. The proposed retention mechanism of this technique will be discussed and compared with other modes of liquid chromatography separation. Since this is a very new technique, understanding of its mechanism and scope of application is still developing.

Keywords: bridge ion separation, ion separation, multicharged ion analysis, oligonucleotides analysis, polymer analysis, proteins separation, peptide separation

1. Introduction

The separation of molecules by liquid chromatography (LC) depends primarily on three fundamental characteristics: molecular size, polarity, and electric charge. All LC separation modes use at least one of these properties to analyze and/or separate target compounds. Size-exclusion chromatography (SEC) is based on the molecular size, whereas normal-phase (NP) and reversed-phase (RP) are governed by differences in polarity. Hydrophilic Interaction Liquid Chromatography (HILIC) is generally considered a variant of normal-phase LC and is likewise polarity-driven. Ion-exclusion (IEC) and ion-exchange (IEX) chromatography are utilized for the separation of charged species.

One significant limitation of IEX chromatography is that multicharged analytes are generally difficult to analyze [1]. In IEX mode, where the stationary phase and analytes carry opposite charges, species with more than two charges often become difficult to elute without the use of high concentrations of ionic modifiers in the mobile phase. This challenge becomes even more pronounced when dealing with multicharged polymers, which in many cases interact irreversibly with the oppositely charged stationary phase.

In some instances, elution can be achieved using molar concentrations of ionic modifiers in the mobile phase, as demonstrated in heparin analysis [2]. However, the use of such high ionic strength conditions introduces additional complications. Increased salt concentration raises the viscosity of the mobile phase, promotes salt precipitation – particularly in the presence of organic solvents – and may lead to salt crystallization within HPLC system components. In addition, it restricts the usable mobile phase composition and negatively affects detector performance. It increases background noise in ultraviolet (UV) detection, suppresses signal in mass spectrometry (MS), evaporative light-scattering detection (ELSD), and charged aerosol detection (CAD), and causes instability in refractive index detection.

Another issue associated with multicharged analytes is peak asymmetry, which can result in reduced resolution, poor quantitation accuracy, and decreased method sensitivity. Common workarounds include switching to reversed-phase separation with ion-pairing reagents; however, this approach introduces its own limitations. Among those are complex gradient optimization, long equilibration times, and incompatibility with MS in most cases. Derivatization is another alternative, but it increases procedural complexity and often results in analyte loss during sample handling and recovery.

IEC chromatography can also be used for the analysis of multicharged ions, but it suffers from limited selectivity and specificity. It is applicable only to a narrow range of cases [3]. Separation typically occurs in the pre-void region with a very limited separation window. Extending this window generally requires longer columns and reduced flow rates.

During investigations of IEC chromatography, it was observed that when the mobile phase contains a low concentration of a doubly charged ionic modifier with a charge opposite to both the stationary phase and analytes, ion exclusion is suppressed, and noticeable retention of analytes is observed.

This chapter describes a new separation mode that was discovered only a few years ago by the research team at SIELC Technologies, Inc. Owing to its relatively recent discovery, the number of publications describing this technique in scientific literature remains limited; however, the growing availability and adoption of columns specifically designed for this approach suggest that it is gaining recognition among chromatography practitioners. It is anticipated that interest in this technique will continue to expand within the broader chromatography research community. The technique also relies on the charge of molecules in solution but differs significantly from traditional IEC and IEX approaches. The Bridge Ion Separation Technique (BIST) represents an additional mode of separation of charged analytes.

2. A brief overview of BIST

The first observation of BIST was made during the analysis of protonated dopamine on an anion-exchange column in the presence of sulfuric acid as an ionic modifier [4].

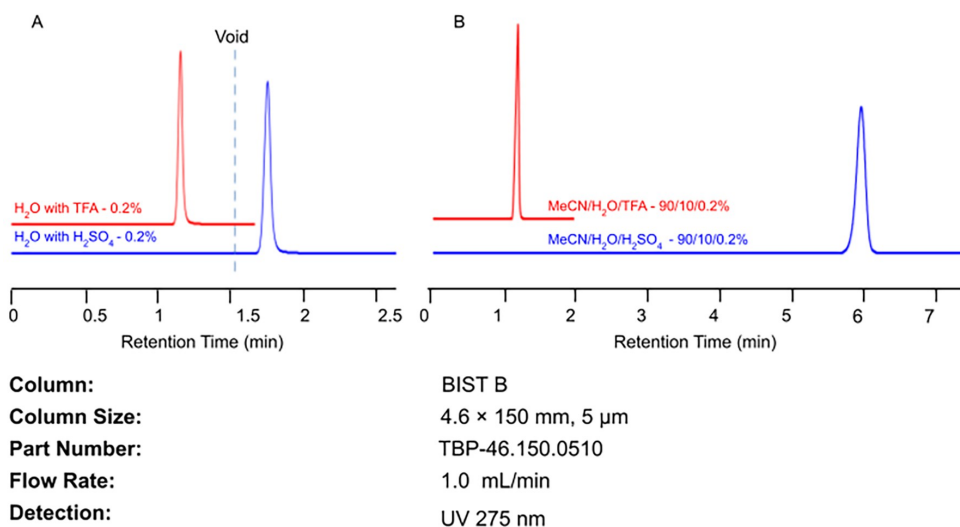


Figure 1. (A) slight retention and pre-void elution of dopamine with sulfuric acid and TFA. (B) the increase in retention when the mobile phase has high organic concentration with sulfuric acid, while no such effect is observed when TFA is used.

A small retention of the dopamine peak was observed. This observation was particularly noteworthy, as prior experimental results and established theory predict ion-exclusion of positively charged analytes from pores with a positively charged surface.

When sulfuric acid was replaced with trifluoroacetic acid (TFA), the retention disappeared, and dopamine eluted in the pre-void region, as expected (**Figure 1A**). Furthermore, classical IEC theory does not predict a significant change in retention or exclusion of ions with increased concentration of organic modifiers in the mobile phase.

In contrast, when sulfuric acid was used, increasing the organic content of the mobile phase led to increased retention of dopamine. Upon reverting to TFA under the same elevated organic conditions, the retention again decreased to pre-void elution (**Figure 1B**).

A similar experiment was conducted using the doubly charged analyte m-xylylenediamine, with sulfuric acid and perchloric acid as mobile phase additives (**Figure 2**). Consistent with previous observations, retention was observed in the presence of sulfuric acid but not with perchloric acid. In addition, a significant increase in retention was observed with increasing organic content in the mobile phase compared to purely aqueous conditions.

A new explanation of this behavior was needed, as it had not been previously observed in classical IEX or IEC chromatography. One plausible explanation for this phenomenon is the formation of a negatively charged “bridge” composed of sulfate ions, connecting the positively charged stationary phase and positively charged analytes when the stationary phase is equilibrated with doubly charged ions from sulfuric acid. The “bridge” is not formed between the positively charged stationary phase and a positively charged dopamine molecule when the ionic modifier carries only a single charge (**Figure 3**).

In mobile phases where water is the dominant solvent, it forms solvation layers around ions, limiting their interactions and keeping them dispersed. As the organic

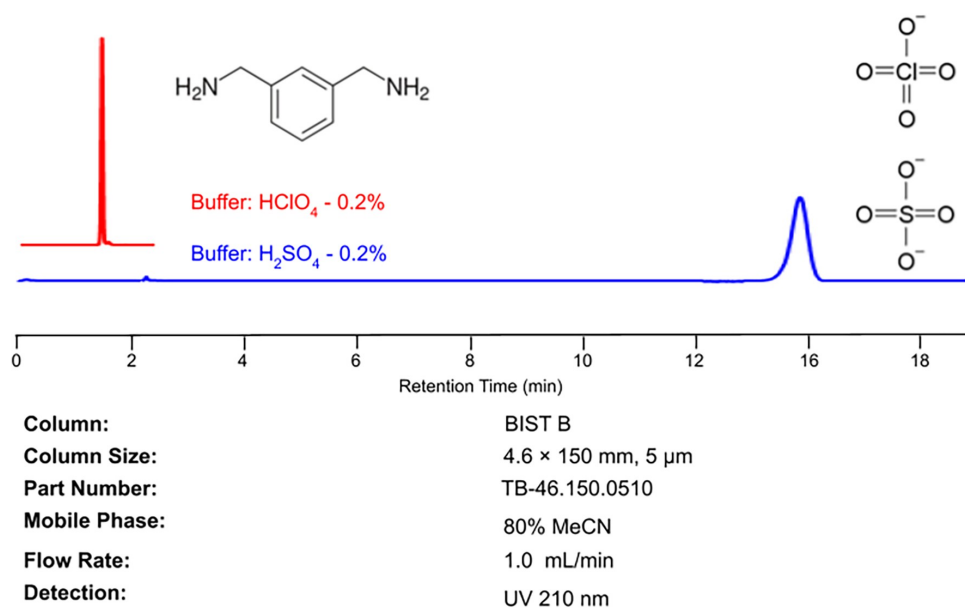


Figure 2.

Drastic increase in retention of xylylenediamine with sulfate ion in the mobile phase compared with perchlorate ion at otherwise identical conditions.

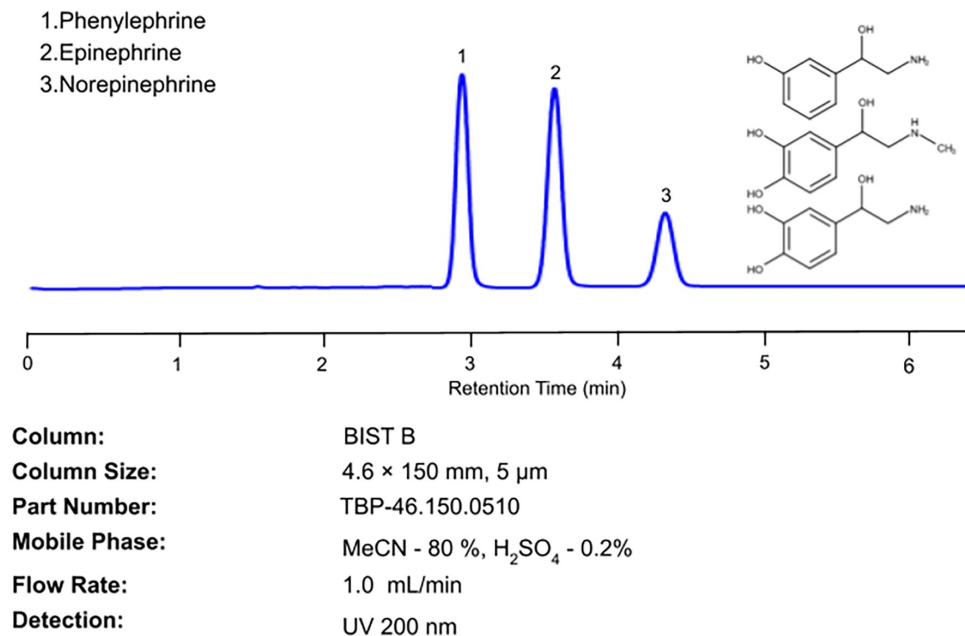


Figure 3.

Example of efficient separation of three neurotransmitters with high organic concentration and with sulfuric acid as an ionic modifier.

content increases and the water content decreases, these solvation layers become thinner, bringing oppositely charged species into closer proximity and thereby increasing the probability of bridge formation. This results in increased analyte retention.

Furthermore, loading the stationary phase with doubly charged ions effectively alters the surface properties, leading to a transition from a net positive charge to a net negative charge.

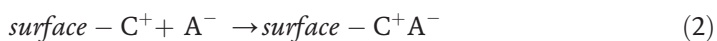
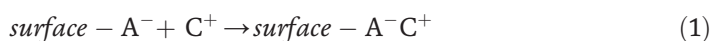
3. BIST as a new separation mode in more details

BIST was observed for positively charged analytes in the presence of sulfuric acid as an ionic modifier. A deeper understanding of this phenomenon requires examining the interaction of ions with the charged surface of the stationary phase.

Ion-exchange columns are designed with stationary phase surfaces functionalized to carry fixed charges. In anion-exchange columns, the surface contains positively charged functional groups, whereas in cation-exchange columns, it bears negatively charged groups. These fixed charges are balanced by an equivalent number of counterions of opposite charges. Both the surface functional groups and their associated counterions are solvated by water molecules, forming structured solvation layers that spatially separate oppositely charged species.

When the water content of the mobile phase is reduced through the addition of an organic solvent, such as acetonitrile (MeCN), the extent of solvation decreases. As a result, the thickness of the solvation layers diminishes, and the distance between oppositely charged ions is reduced [5]. At sufficiently high organic solvent concentration, oppositely charged ions are no longer effectively separated by solvation layers, and the remaining separation becomes negligible.

Under these conditions, the stationary phase surface can be described as a system in which each charged site is closely associated with a single counterion. This state can be represented by the equations below for cation-exchange (Eq. 1) and anion-exchange (Eq. 2) columns, where each surface ion is in a bound state with one counterion.

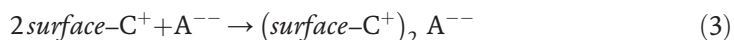


Where:

A^- denotes an anion in solution or a negatively charged functional group on the stationary phase surface;

C^+ denotes a cation in solution or a positively charged functional group on the stationary phase surface.

The situation becomes more complex when dissolved doubly charged ions are in equilibrium with the charged stationary phase. Under these conditions, two distinct states can be defined, as represented by the equation below for an anion-exchange column.



The proton (H^+) in Eq. 4 can be supplied by water under acidic conditions. Eq. 3 indicates that the ionic capacity of the column to hold doubly charged ions is half of that described in Eq. 4. We propose, supported by observed evidence (see below), that both states coexist to some degree.

At high organic solvent concentration in the mobile phase, the equilibrium described by Eq. 4 becomes increasingly significant. If ionization occurs to any degree as described in Eq. 5, the overall surface will exhibit an excess of negative charge, while the same surface in an aqueous mobile phase (Eq. 6) is positively charged.

Predominantly organic MP:

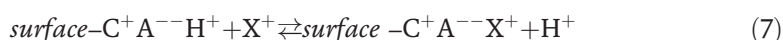


Predominantly aqueous MP:



Upon introduction of an analyte to the surface, the following equilibrium is established:

Predominantly organic MP:



Where: X^+ denotes a positively charged analyte

This phenomena is illustrated in **Figure 4**, where a doubly charged cationic analyte forms a strong association with the positively charged stationary phase surface via a doubly charged sulfate anion.

Consistent and reproducible performance of this technology required the development of a dedicated line of columns specifically tailored for BIST and evaluated during production using appropriate analytical and physical tests. The BIST applicable columns were defined as BIST A, BIST A +, BIST B, and BIST B +.

BIST A columns provide retention and separation of negatively charged acidic compounds, while BIST B columns are designed for positively charged basic compounds. Both “plus” (+) variants offer increased retention compared to the corresponding none-plus BIST columns.

A key question is whether BIST represents a variation of HILIC phenomena. A clear distinction between these two separation mechanisms is shown in **Figure 5**. The BIST A column demonstrates strong retention and selectivity for two quaternary ammonium compounds only in the presence of a doubly charged ion in the MP. Replacing the doubly charged sulfate with a singly charged perchlorate results in a complete loss of retention, despite otherwise identical HILIC-like conditions.

This retention phenomenon was further evaluated using several other doubly charged organic and inorganic ions. Similar behavior was observed with phosphate ions and hexafluoroglutaric acid (HFGA). HFGA is a volatile compound with a boiling point of 134–138 °C at 3 mmHg [6]. At low concentrations, it can be used as a volatile ionic modifier compatible with MS and other detectors based on the liquid-gas phase transition principle, as shown in **Figure 6** for the analysis of Diquat.

The next logical question is how general this phenomenon is and whether negatively charged analytes can be retained in an analogous manner on

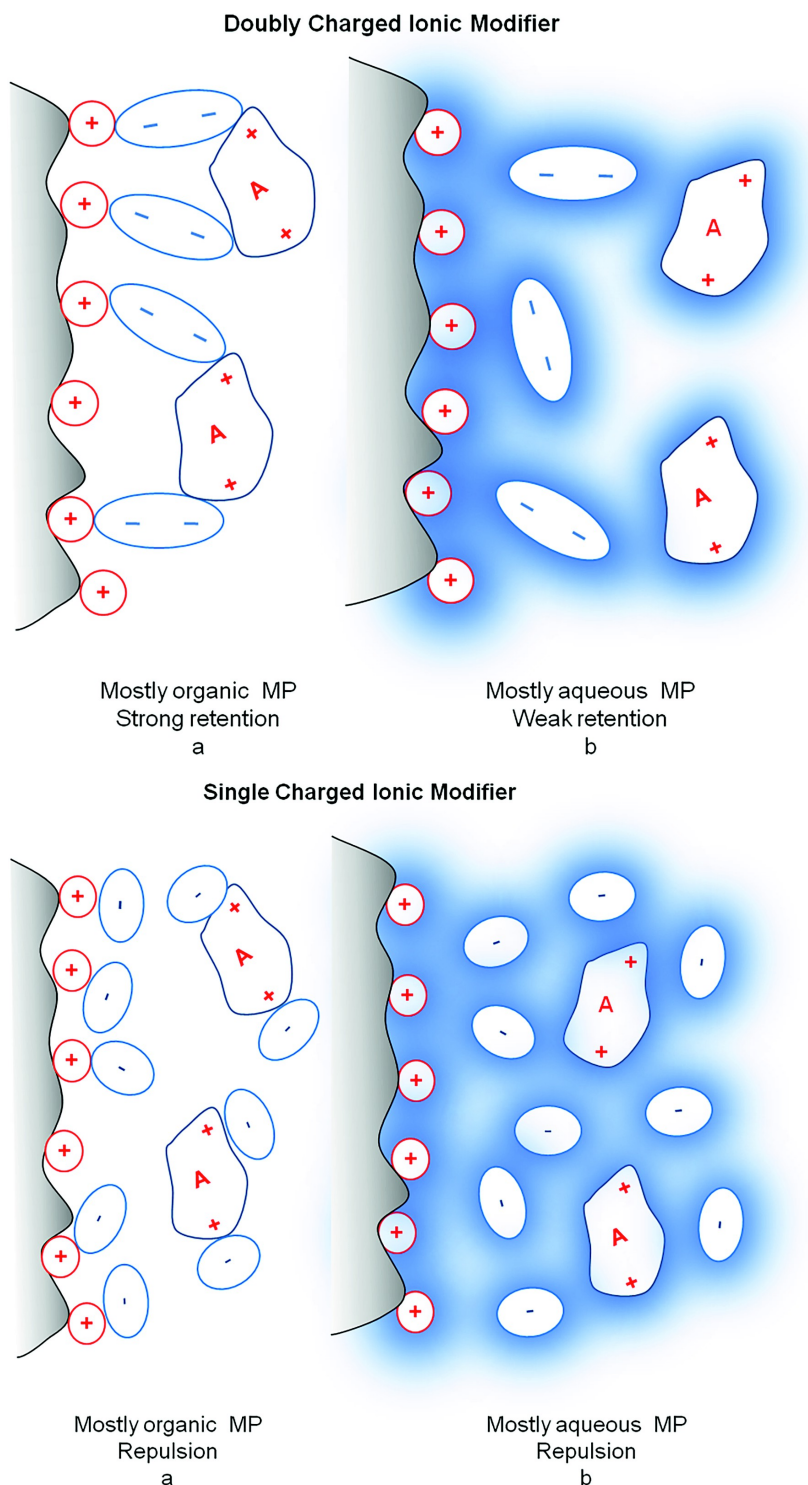


Figure 4.
 Schematic illustration of a bridge formed by doubly charged sulfate ions under high organic solvent conditions, leading to analytes retention. No such retention is observed with singly charged TFA ions.

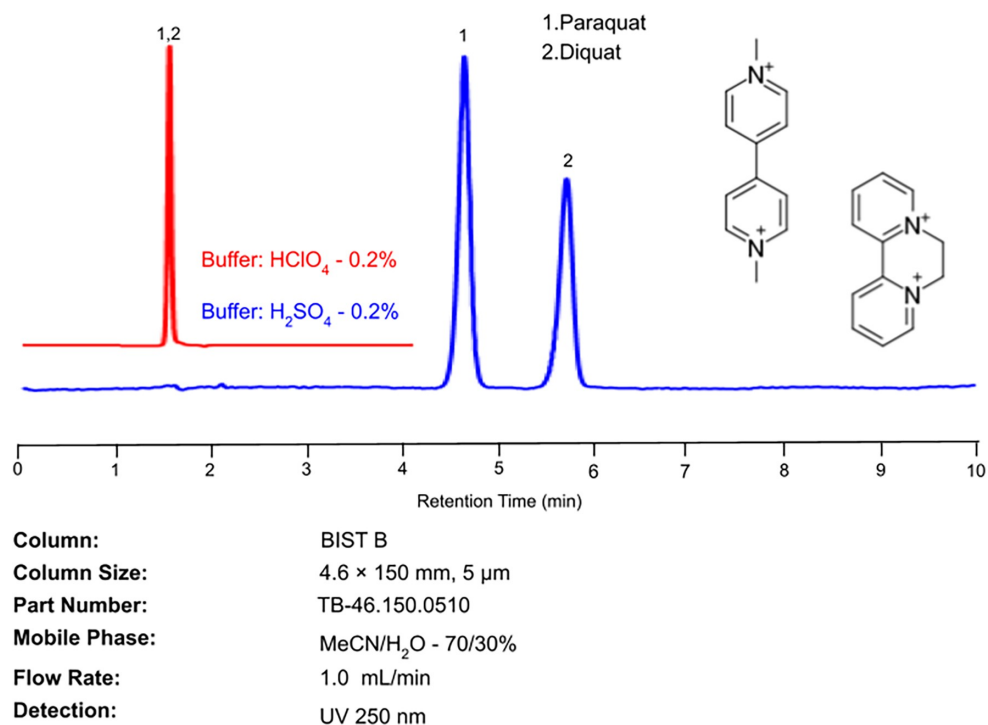


Figure 5.

Comparison of the separation of two basic analytes in a high organic mobile phase using singly charged (perchloric acid) versus doubly charged (sulfuric acid) ionic modifiers.

a cation-exchange column. In this case, the ionic modifier in the mobile phase would need to be a doubly charged cation, which can be readily generated from diamino compounds (**Table 1**).

Primary and secondary amines are less stable in air. Therefore, tertiary amines were selected as ionic mobile phase modifiers. Some of these molecules are shown in **Figure 7**. TMEDA, TMDAP, and DMP are among the most suitable candidates, although many structurally related compounds can also be used in a similar manner.

Tetramethyl-1,2-ethylenediamine (TMEDA) (a); Dimethylpiperazine (DMP) (b); Tetramethyl-1,3-diaminopropane (TMDAP) (c).

In order to protonate these diamino compounds, an acidic component must be present in the MP. Suitable options include formic acid to adjust the pH to 3-4 or acetic acid to adjust the pH to 4-5. These mobile phase conditions are compatible with evaporative detection techniques such as MS, ELSD, and CAD, but are less appropriate for low-wavelength UV detection below 230 nm.

Figure 8 shows an example of the BIST mechanism for analyzing negatively charged analytes on a negatively charged stationary phase using diamines as doubly charged ionic modifiers. A silica-based cation exchange column was used as the stationary phase, with protonated TMDAP serving as the ionic modifier in the mobile phase. This system enabled the separation of sulfated cyclodextrins based on the number of sulfated groups in the molecule, i.e., the number of negative charges. Retention increased significantly with increasing negative charges of the molecule.

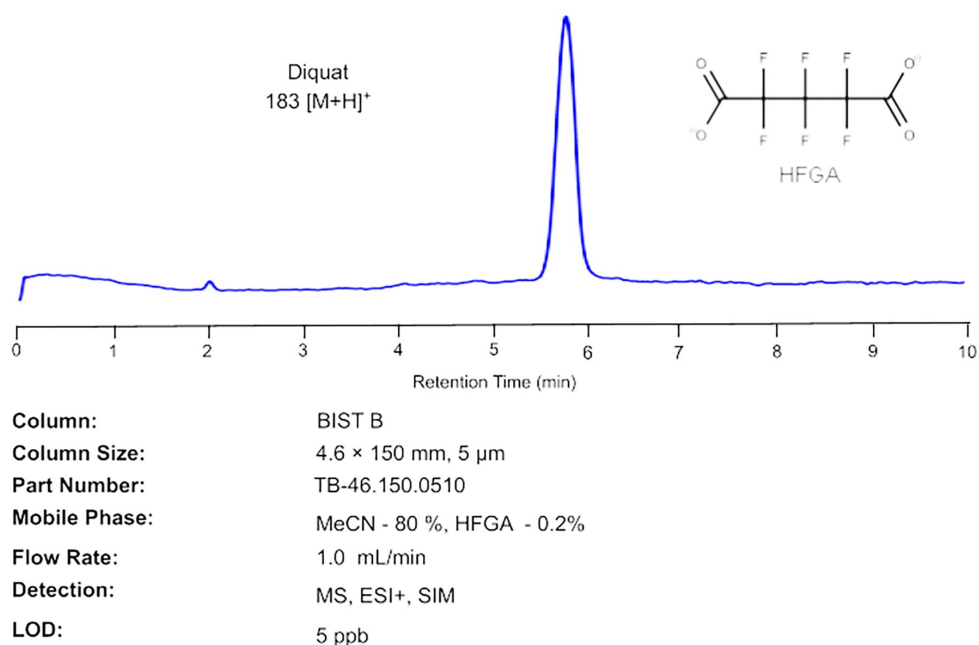


Figure 6.
 Example of diquat analysis in a high organic mobile phase using doubly charged hexafluoroglutaric acid (HFGA) as the ionic modifier. The conditions are compatible with MS, ELSD, and CAD detection techniques.

Amines	Short	MW	BP, °C
Ethylenediamine		60.1	116
N, N, N', N'-Tetramethylethylenediamine	TMEDA	116.2	122
N, N-Dimethylethylenediamine		88.2	119
N, N'-Dimethylethylenediamine		88.2	107
1,2-diaminopropane		74.1	120
N, N, N', N'-Tetramethyl-1,3-diaminopropane	TMDAP	130.2	140
Piperazine		86.1	146
1,3-Diaminopropane		74.1	133
trans-2,5-Dimethylpiperazine		114.2	162
N, N'-Dimethylpiperazine	DMP	114.2	133
1,4-Diazabicyclo[2.2.2]octane		112.2	174
1,4-Diaminobutane		88.2	159
1,2-Diaminopropane		74.1	120

Table 1.
 Diamines suitable as ionic modifiers for the BIST separation method.

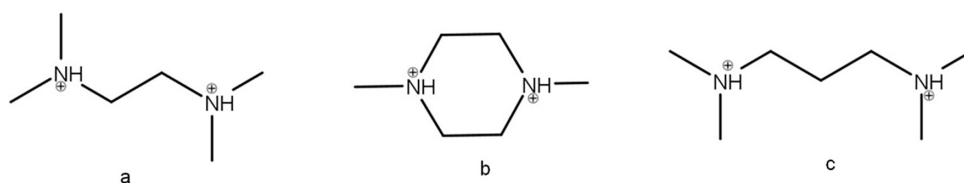


Figure 7.
Doubly charged diamines commonly used in the BIST separation method.

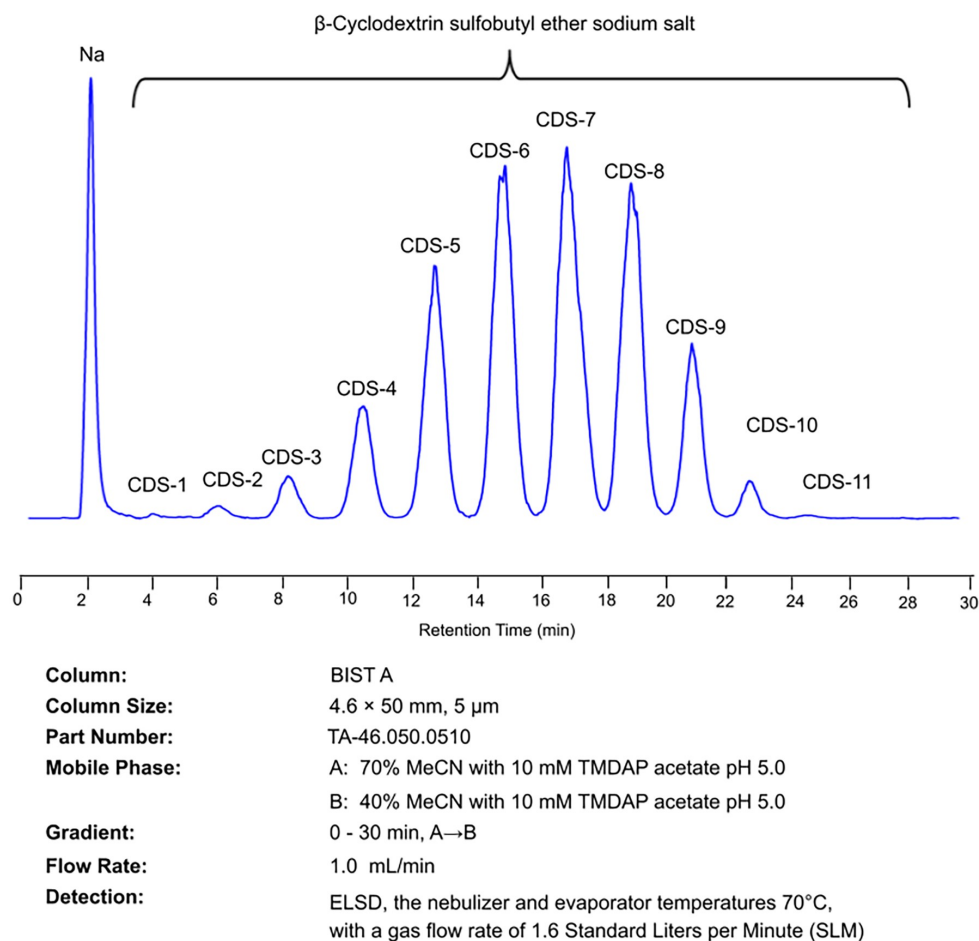


Figure 8.
Example of HPLC separation of sulfated cyclodextrins based on the number of sulfate groups per molecule (degree of sulfation).

In contrast to classical IEX chromatography, where elution of multicharged analytes typically requires an increase in buffer concentration, BIST shows the opposite behavior. In BIST, increasing the water content of the mobile phase produces a similar or even stronger effect without any change in the buffer concentration.

For methods requiring low-wavelength UV detection, alternative ionic modifiers must be used. One such system utilizes TMDAP protonated with phosphoric acid to pH 4. **Figure 9** shows an example of this approach, where a mixture of carboxylic acids were separated and detected at 200 nm by UV.

In BIST, relatively low concentrations of ionic modifiers are sufficient, as the retention factor plateaus above approximately 10 mM (**Figure 10**) [7]. Lower concentrations can still be used, although they result in reduced analyte retention. Notably, in the absence of TMDAP, retention shifts to repulsion, yielding negative K' values.

The effect of the limited concentration of ionic modifiers required to achieve the BIST mechanism can be explained by the underlying mechanism of this separation mode.

Unlike traditional IEX, reversed-phase chromatography, or HILIC, BIST does not rely on competitive interaction between analytes and mobile phase components. In IEX chromatography, buffer ions compete with and displace bound analytes from the stationary phase due to their similar interaction mechanism or binding properties.

In RP chromatography, organic components of the MP compete with analytes for hydrophobic interactions with the stationary phase. In HILIC, water acts as a competitive component of the MP for the polar interaction sites.

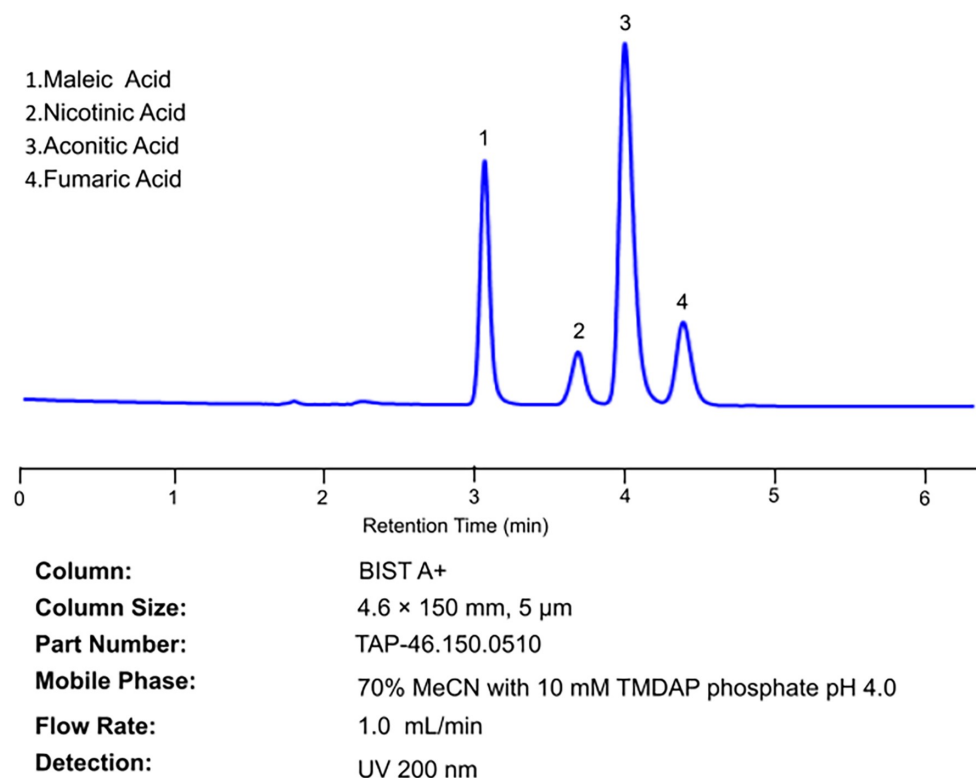


Figure 9.
Example of HPLC separation of a mixture of carboxylic acids using a mobile phase compatible with low-wavelength UV detection.

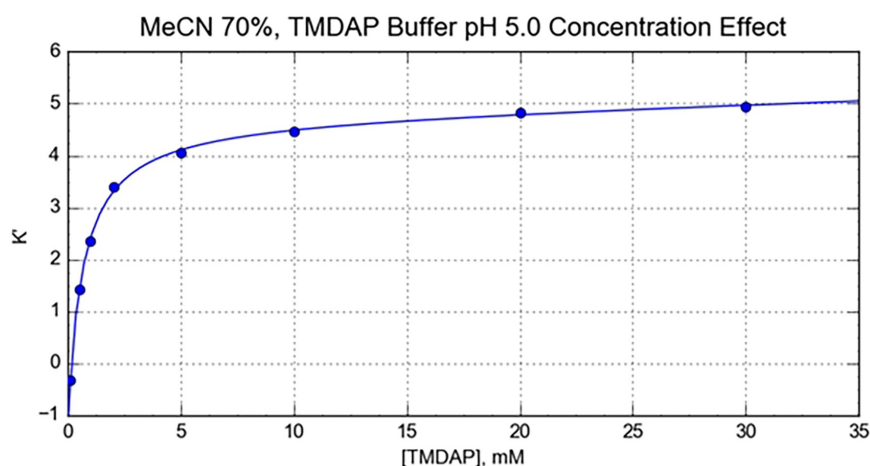


Figure 10.

Effect of TMDAP concentration as an ionic modifier on the retention factor of tartrazine. The retention factor begins to plateau above approximately 10 mM.

BIST operates without such competition. The amount of bound doubly charged ions on the stationary phase remains largely constant and is not significantly affected by variations in water content [7] or buffer concentration in the mobile phase. Once the stationary phase is saturated with counterions, it behaves as a relatively stationary system, with analyte retention governed primarily by the water content of the MP.

However, when the concentration of doubly charged ions supplied by the MP becomes too low, the stationary phase begins to lose these bound species. They are gradually replaced by singly charged ions originating from water dissociation, H^+ (cation-exchange column) or OH^- (anion-exchange column). This substitution reduces the ability of the stationary phase to form bridging interactions responsible for BIST retention.

4. Representative applications of BIST

4.1 Oligonucleotides

Oligonucleotides are short sequences of nucleotides – the fundamental building blocks of DNA and RNA – that play a vital role in modern biotechnology, life sciences, and medical research. Typically consisting of 2 to 50 bases, these synthetic molecules are indispensable tools in a variety of applications, including genetic testing, molecular diagnostics, gene editing, and therapeutic development. Their precise ability to target specific genetic sequences makes them invaluable in areas such as antisense therapy, RNA interference (RNAi), and CRISPR-based gene editing. They represent an important group of negatively charged biomolecules with broad relevance in both medicine and biotechnology.

Analyzing oligonucleotides presents unique analytical challenges due to their complex structure and physicochemical properties. Their highly charged nature, stemming from multiple phosphate groups, often results in strong interactions with stationary phases, complicating chromatographic separation. Additionally, variations in sequence length, base composition, and chemical modifications can impact retention and resolution, making it difficult to achieve consistent separation. The presence of impurities such as truncated or extended sequences, as well as modified byproducts, further necessitates high-resolution methods to ensure accurate differentiation and quantification. Moreover, reproducibility can be challenging, as even minor fluctuations in analytical conditions can significantly influence retention times and peak shapes.

BIST mode offers a simplified approach to oligonucleotide analysis compared to traditional modes. The method requires a small amount of double-charged positive ionic modifiers, such as TMEDA in the mobile phase, to act as a “bridge” between the negatively charged stationary phase and the oligonucleotide. A key advantage of BIST is improved selectivity, enabling better differentiation between closely related species, particularly those of the same length.

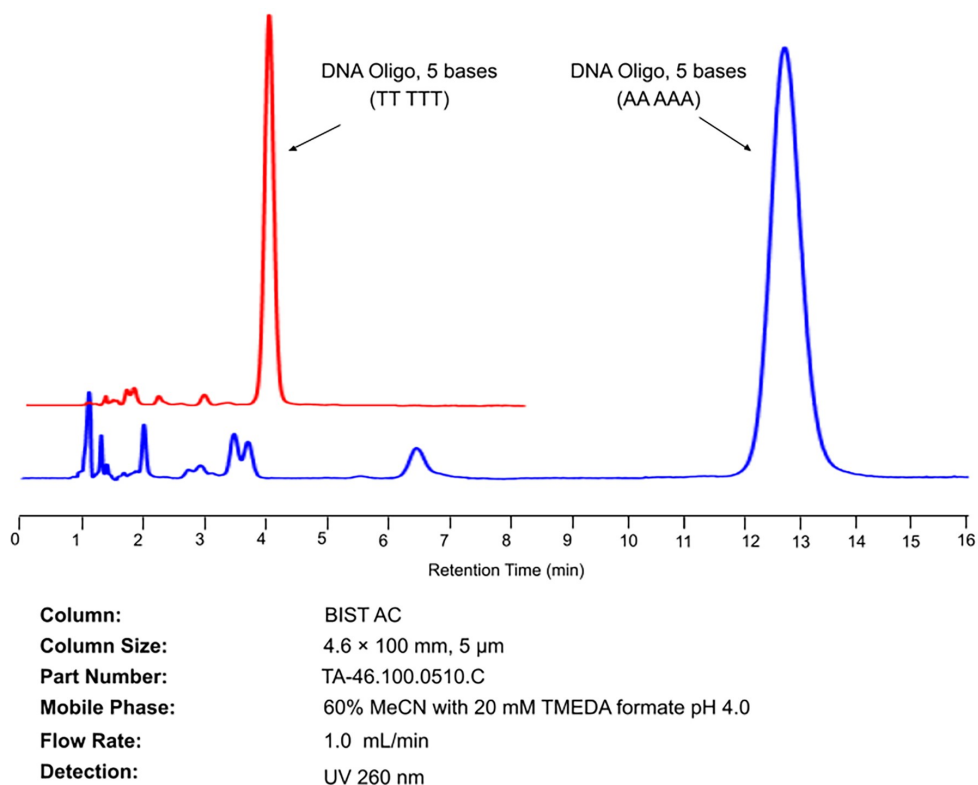


Figure 11.
Effect of nucleobase type on the retention of oligonucleotides.

The approach initially developed requires a small amount of doubly charged positive ionic modifiers, such as TMEDA, which act as a “bridge” between the negatively charged stationary phase and the negatively charged oligonucleotide. In this system, 20 mM TMEDA was added to the mobile phase. To ensure protonation of both amino groups of TMEDA, the pH of the solution was adjusted to 4.0 using formic acid. The degree of retention can then be modulated by the ratio of MeCN and water in the MP.

Separation and retention of different oligonucleotides with symmetrical peak shape and high efficiency can be readily achieved under these conditions. The degree of retention is not only related to the number of nucleotides but also to their type. A significant difference in retention is observed between adenine-based and thymine-based oligonucleotides (**Figure 11**). However, within a single base type, the retention follows a strict dependence on oligonucleotide length (**Figure 12**).

Changing the amount of organic modifier changes analyte retention in the BIST system. As the water content increases, solvation of ions increases, reducing the

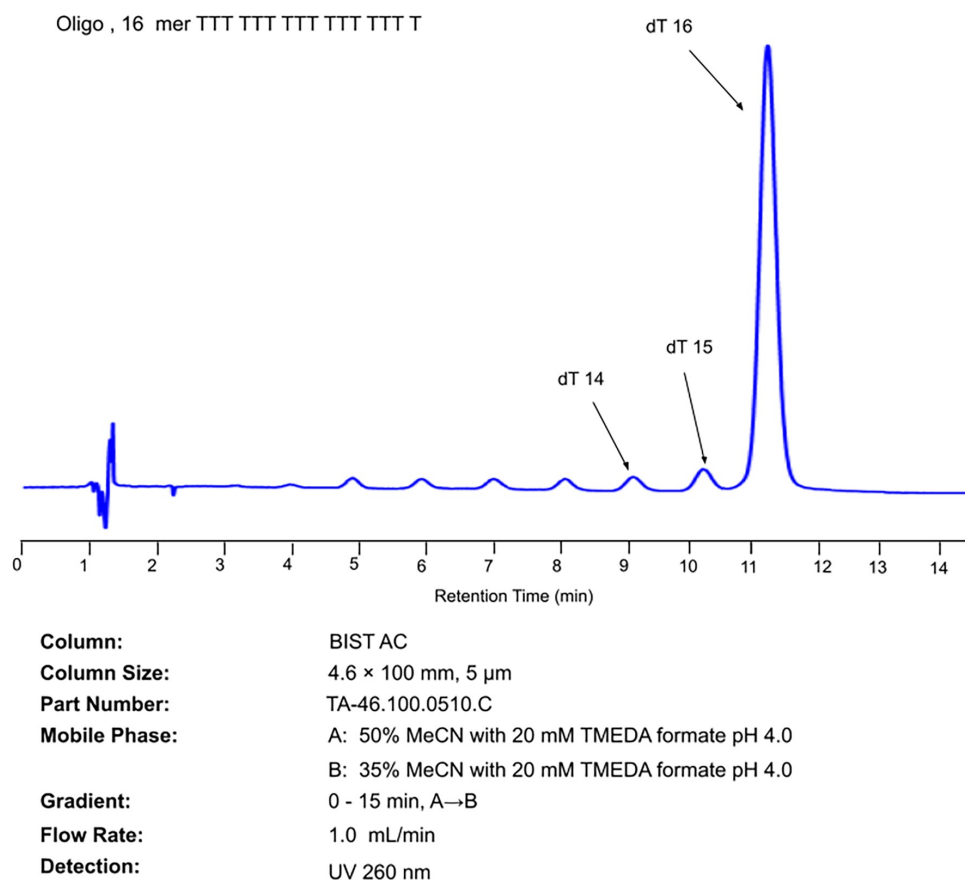


Figure 12.
Separation of poly(T) oligonucleotides according to sequence length.

interaction between the bridging ion and the oligonucleotide and leading to lower retention (**Figure 13**).

Thus, the amount of water (rather than buffer concentration) is an important factor in this separation mode, even though the dominant retention force is electrostatic interaction.

As previously mentioned, when ionization proceeds according to Eqs. 5 or 7, the stationary phase does not lose doubly charged ions, but instead hydroxyl anions are exchanged with analyte anions. In order to further investigate this behavior, it was decided to evaluate the separation in the absence of an ionic modifier in the MP, while instead preloading the maximum amount of counterions onto the cation-exchange column.

Ion-exchange columns can exist in the H^+ form in the case of cation-exchange phases or in the OH^- form in the case of anion-exchange phases. In addition, the charged stationary phase can be loaded with different ions beside H^+ and OH^- .

To examine this, a 20 mM $MgAc_2$ solution in water/MeCN (30/70, v/v) was prepared and used to saturate the column with magnesium ions. Approximately 40 column volumes of this solution were passed through the column using a separate pump to ensure full saturation. The saturated column was then transferred to a standard HPLC system and evaluated for oligonucleotide retention using a water-MeCN mobile phase without any ionic additives (**Figure 14**).

It is expected that some magnesium ions would gradually leach from the stationary phase even in the absence of competing ionic species in the mobile phase. However, since water is the only medium available for ionization, magnesium ions are most likely to leave the column in the form of $Mg(OH)_2$. Given the very low solubility of $Mg(OH)_2$ in water (0.00064 g/100 mL at 25 °C, according to literature[8]), which is

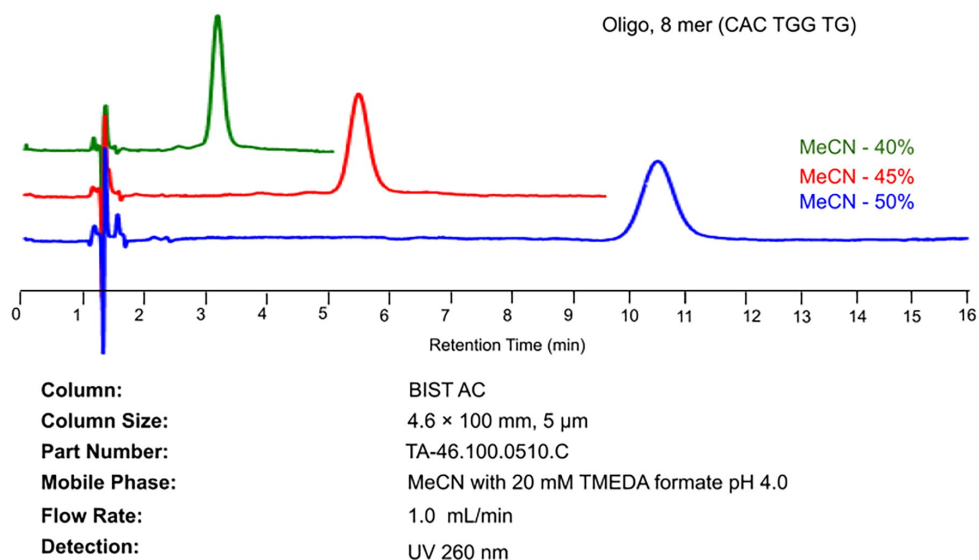


Figure 13.
Effect of the organic modifier concentration on oligonucleotide retention.

expected to be even lower in organic-water mixtures, the mobility of magnesium species is limited. As a result, the column remains effectively in a loaded state over extended operation.

This assumption was evaluated using an oligonucleotide mixture and was confirmed experimentally. Once loaded, the column could be operated for hundreds of injections without significant loss of retention (**Figure 14**).

4.2 Small ions

Different small positively and negatively charged ions can be analysed using BIST.

Retention characteristics generally correlate with the number of charges carried by the ions. Due to the low buffer concentration, detectors such as conductivity can be used to measure analyte signals against a relatively low mobile phase conductivity background (**Figure 15**).

When a volatile mobile phase is required, TMDAP-formate serves as a convenient ionic modifier for BIST-type separations. The low buffer concentration is advantageous in this case, as it reduces ion suppression in MS

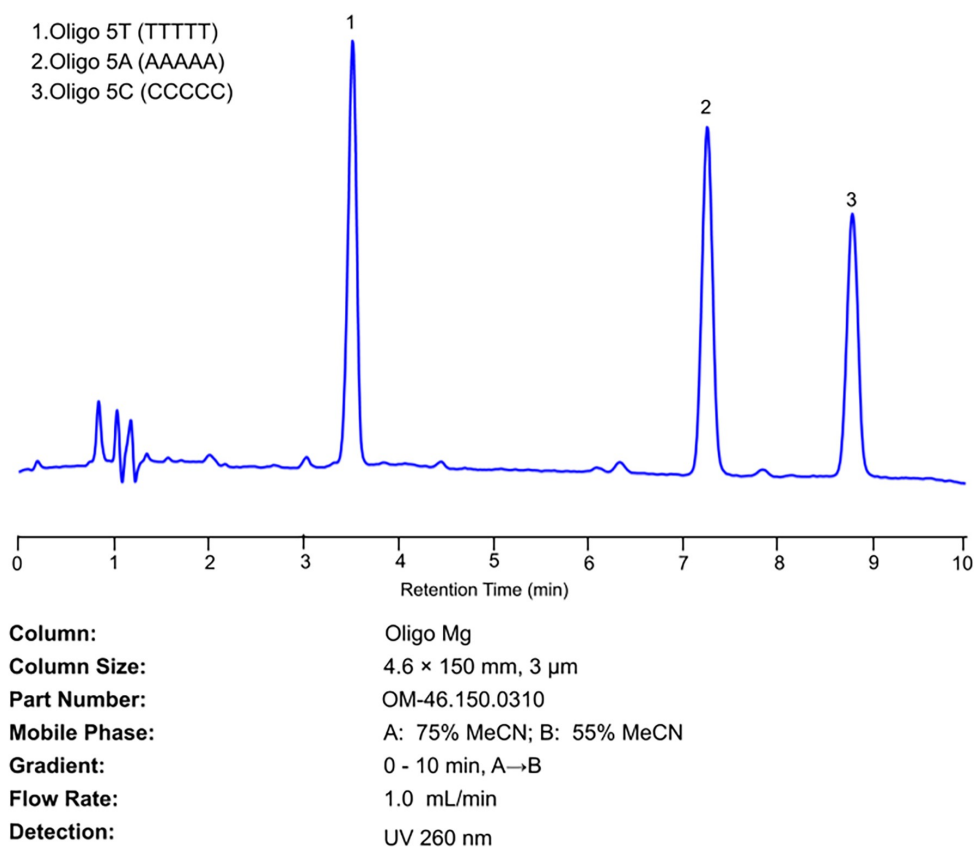


Figure 14.

Example of separation of synthetic oligonucleotides in the absence of buffer in the mobile phase.

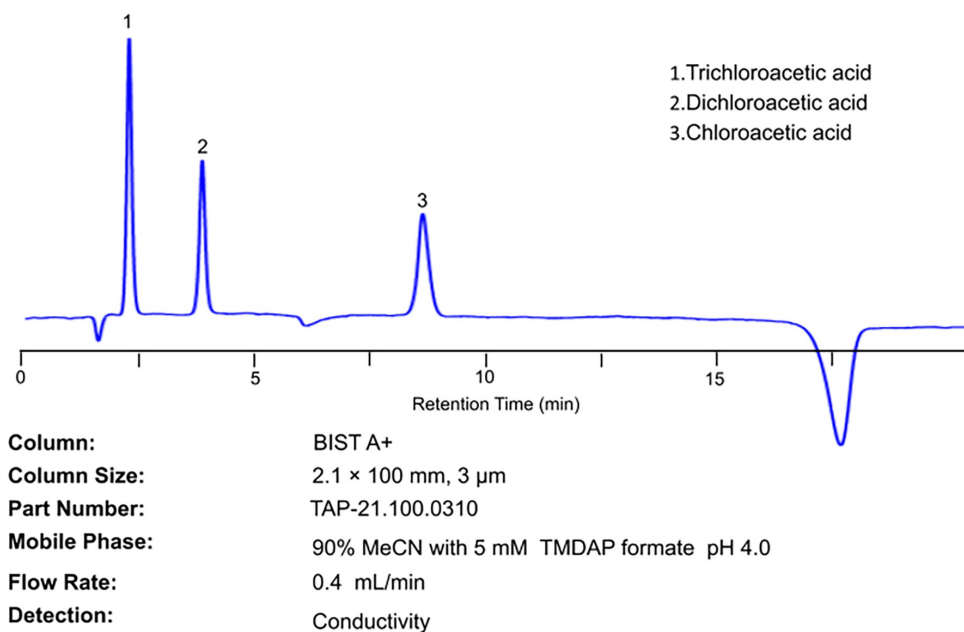


Figure 15.
 Example of separation of three chloroacetic acids on a cation-exchange column under BIST conditions using conductivity detection.

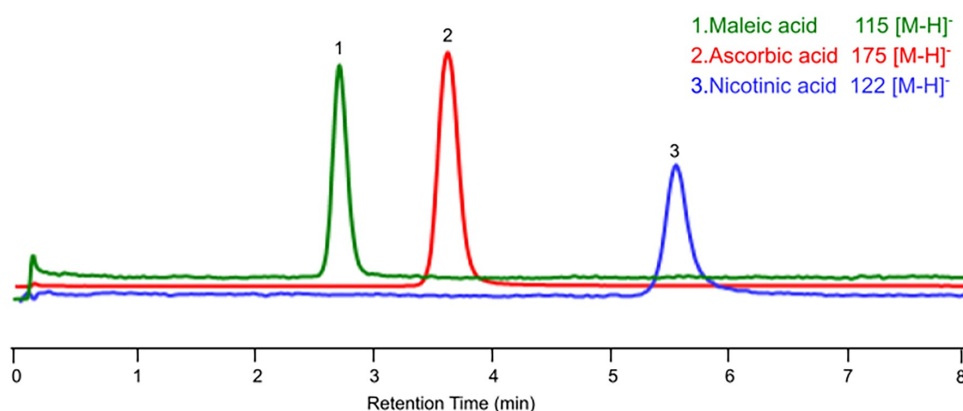
detection. Furthermore, the opposite charge of the doubly charged ionic modifier relative to the analyte ions reduces interference under negative ionization conditions (**Figure 16**).

Another important factor that significantly affects retention is the hydrophobicity of the analytes in their ionic form. More hydrophobic ions exhibit lower retention in BIST mode, similarly to HILIC separation mode. This behavior can be attributed to the inherently polar nature of ion-exchange stationary phases. Hydrophobic ions have lower affinity for such surfaces, resulting in less effective bridge formation and, consequently, reduced retention (**Figure 17**).

Small inorganic ions, as shown in **Figure 18**, elute in a different order under classical ion-exchange LC mode, where the stationary phase is oppositely charged to the ions (**Figure 18B**), compared to BIST conditions, where both the column and analytes carry the same charge (**Figure 18A**).

4.3 Charged polymers

Polymers are large molecules with a wide range of properties and diverse applications across industries. The complexity of their structural and chemical properties makes them highly difficult to analyze using conventional ion-exchange chromatography. Strong interactions between charged monomeric units and oppositely charged stationary phases often result in excessively strong retention, making elution challenging. In many cases, an extremely high concentration of ionic modifier is required to achieve elution. Such conditions can compromise performance



Column: BIST A
Column Size: 2.1 × 100 mm, 3 μm
Part Number: TA-21.100.0310
Mobile Phase: 70% MeCN with 10 mM TMDAP formate pH 4.0
Flow Rate: 0.2 mL/min
Detection: MS, ESI⁻, SIM

Figure 16.

Example of retention of three carboxylic acids on a cation-exchange column under BIST conditions using MS detection.

due to associated operational limitations, including salt deposition in instrument components, increased mobile phase viscosity, and reduced detector sensitivity.

An alternative approach for polymer analysis involves the use of ion-pairing reagents with hydrophobic chains in combination with RP chromatography. This enables separation based on both molecular size and charge. However, this technique has several limitations. Column equilibration after a run can be time-consuming to restore initial conditions, and solubility issues may arise for higher-molecular-weight polymers.

In contrast, within the BIST separation mode, water is crucial in disrupting the “bridge” interactions, allowing elution to be effectively controlled by increasing the water content of the mobile phase. A step-gradient approach, transitioning from a retentive state to a fully solvated, weak interaction state, helps resolve issues related to broad signal and molecular weight distribution [9].

Polylysine is an example of a positively charged polymer available in a range of molecular weight distributions. Low-molecular-weight samples can be resolved into individual oligomers using sulfuric acid as the ionic modifier under BIST separation mode conditions (**Figure 19**).

Higher-molecular-weight fractions can be separated as a group under retentive conditions (**Figure 20**). Typically, size-exclusion chromatography (SEC) is used for such analysis.

There are cases where fractionation of a polymer is not required, and the polymer only needs to be separated as a whole from other interfering compounds. In such cases, a step-gradient method can be used to generate a single polymer peak (**Figure 21**).

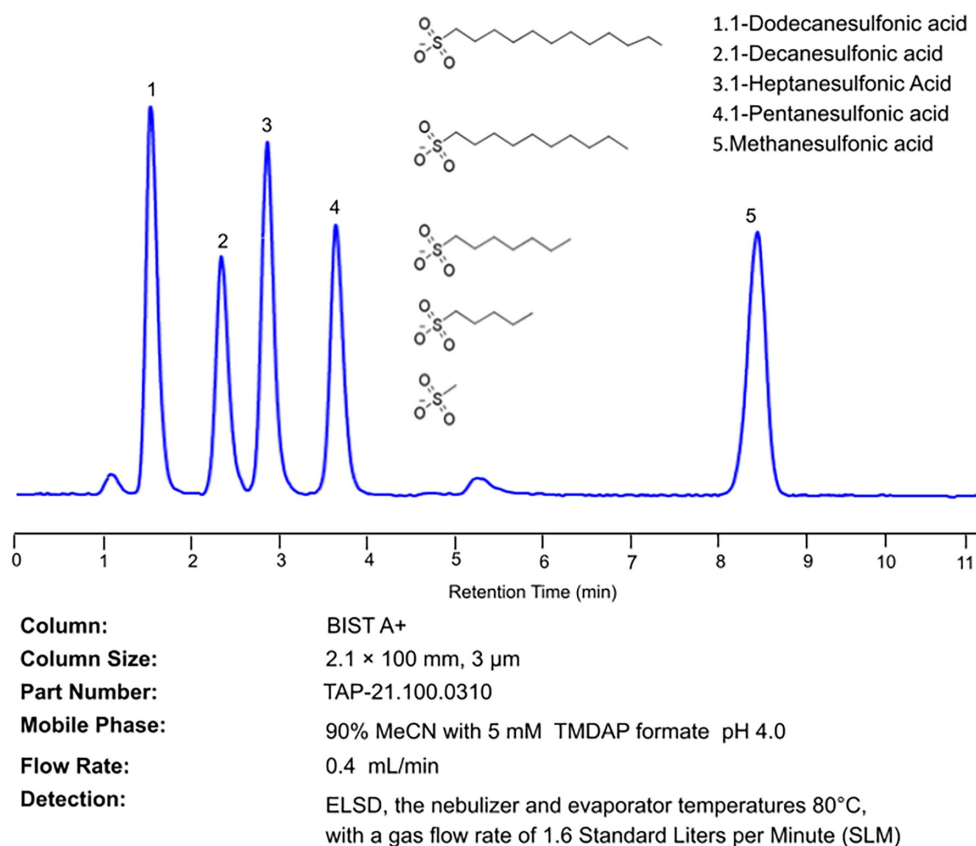


Figure 17.
 Example of retention of five alkyl sulfates on a BIST column. Less hydrophobic analytes exhibit longer retention under these conditions.

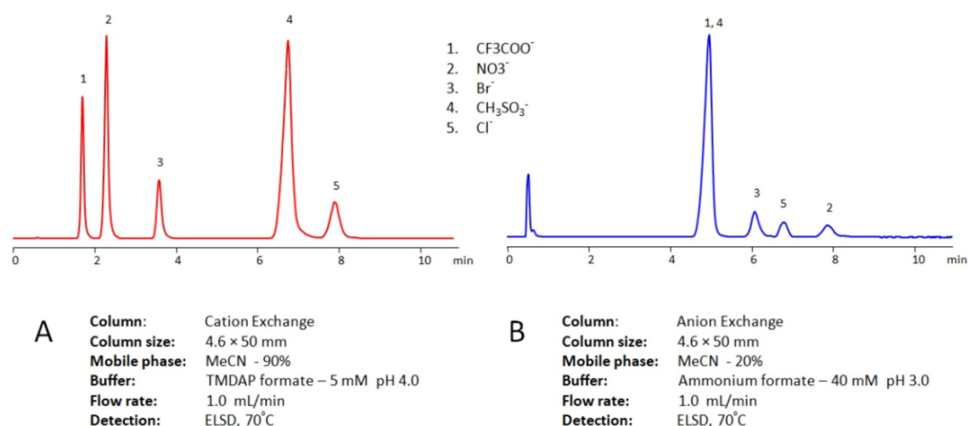


Figure 18.
 (A) separation of small ions using BIST, where both the ions and the stationary phase are negatively charged and TMDAP acts as a bridging agent in a high-organic mobile phase. (B) separation of small ions using classical ion-exchange mode, where the ions and the stationary phase carry opposite charges, with ammonium formate facilitating the ion-exchange process in a low-organic mobile phase.

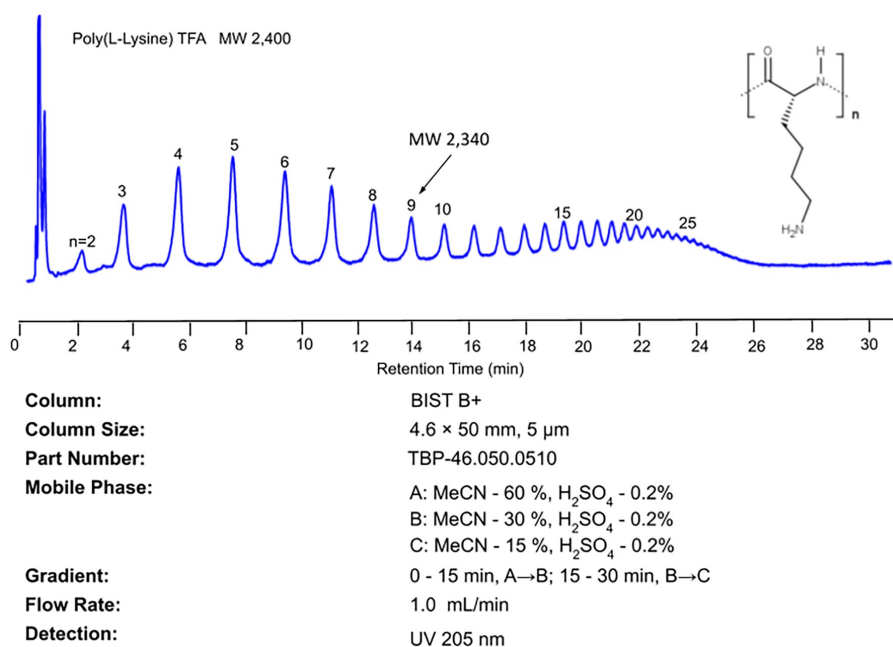


Figure 19.

The separation of polylysine is supplied as a molecular weight fraction of 2,400.

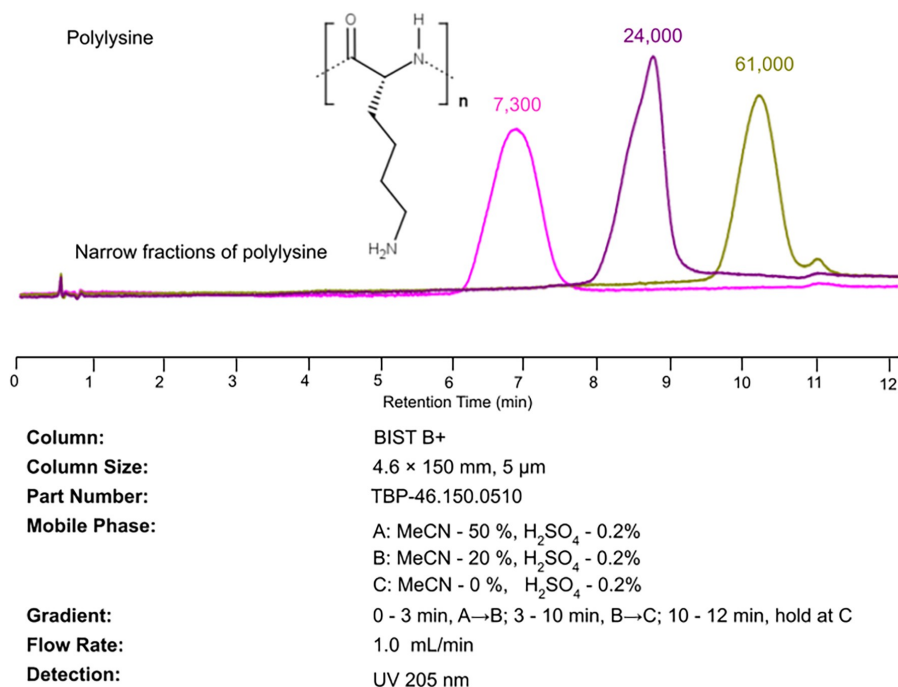


Figure 20.

Separation of polylysine polymers with different molecular weights.

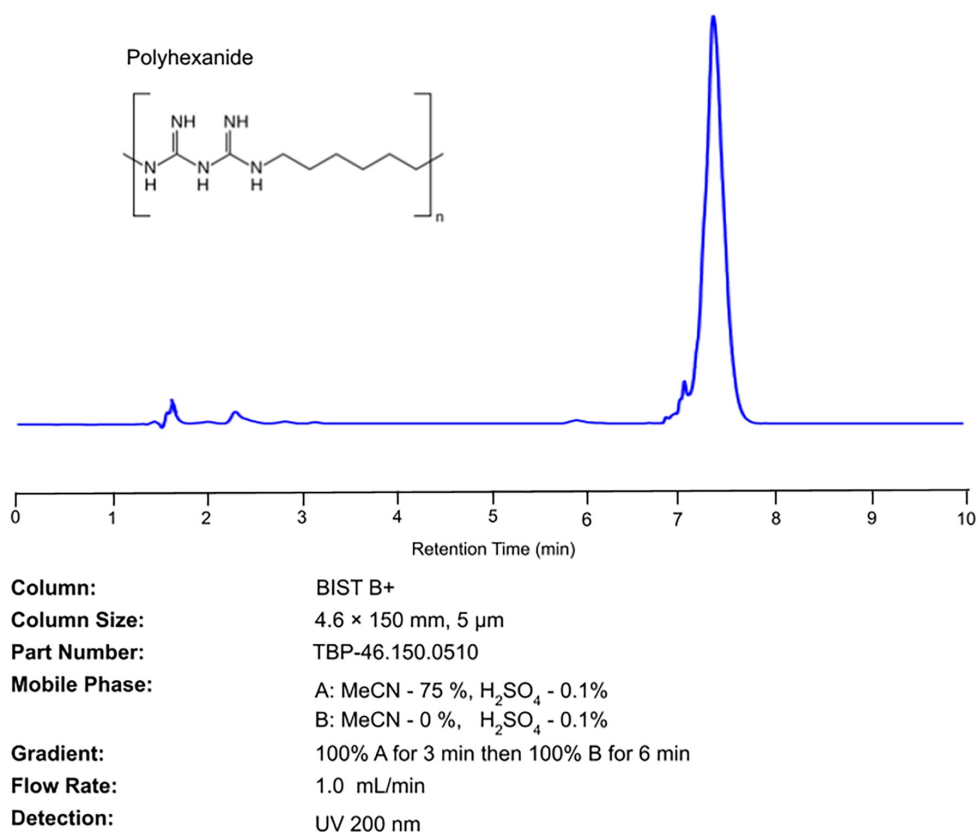


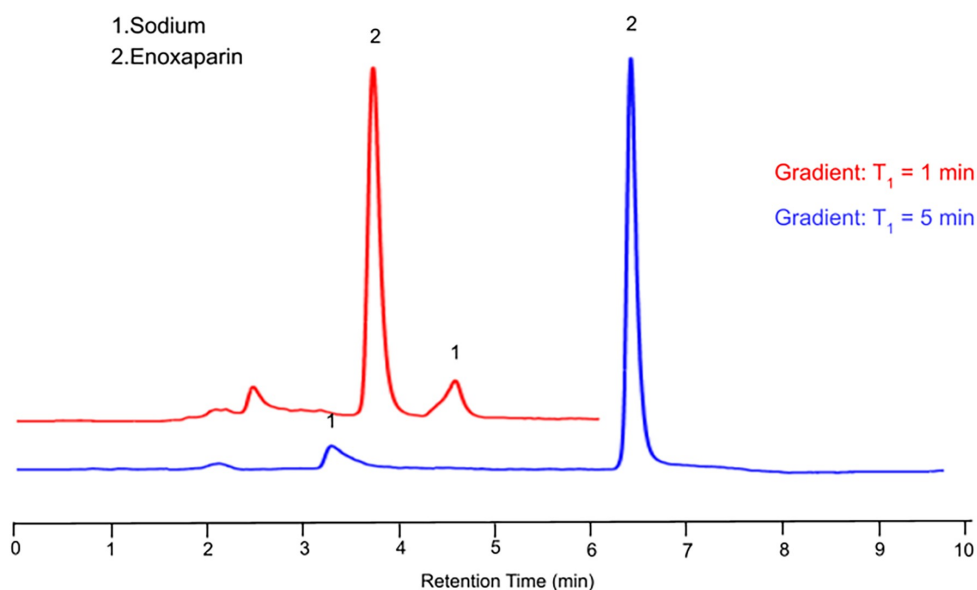
Figure 21.

Elution of polyhexanide (PHMB) as a single peak using a step gradient. Elution time can be controlled by the delay of switching from mobile phase a to b.

If a sample has a matrix of low-molecular-weight or non-charged components, an initial high-organic mobile phase A retains the polymer on the column, while the unwanted matrix compounds elute. Following a step change to mobile phase B (higher water content), the polymer is released and eluted at a controlled retention time determined by the A-to-B switching point.

In cases where full separation of individual components in a polymer sample is not necessary, shorter step-gradients can be utilized for broader, more general analysis. The method for retaining Enoxaparin (**Figure 22**), demonstrates how the step-gradient delay influences the retention time of the polymer. A single polymer peak is observed as a result of this approach, independent of the step time, similar to the behavior observed for PHMB in the previous example.

This technique is effective for both positively charged analytes on an anion-exchange column (PHMB, **Figure 21**) and negatively charged analytes on a cation-exchange column, including Enoxaparin (**Figure 22**) and Heparin (**Figure 23**).



Column: BIST A
Column Size: 4.6 × 150 mm, 5 μm
Part Number: TA-46.150.0510
Mobile Phase: A: 60% MeCN with 10 mM N,N'-Dimethylpiperazine pH 4.0 with AcOH
 B: 10% MeCN with 10 mM N,N'-Dimethylpiperazine pH 4.0 with AcOH
Gradient: 100% A for T₁ min then 100% B for 6 min
Flow Rate: 1.0 mL/min
Detection: ELSD, the nebulizer and evaporator temperatures 70°C, with a gas flow rate of 1.6 Standard Liters per Minute (SLM)

Figure 22.

Elution of enoxaparin as a single peak using a step gradient. Elution time can be controlled by the delay of switching from mobile phase a to b.

5. Conclusion

Many biologically and medically important compounds are charged. These include small drug molecules, neurotransmitters, amino acids, energy-transfer molecules, lipids, vitamins, and many others. Larger charged species include peptides, proteins, oligonucleotides, anticoagulants, and related macromolecules.

The BIST separation mode provides a new approach for the analysis of charged compounds. In BIST, there is no limitation with respect to the polarity or number of charges carried by ionic analytes. In contrast to classical ion-exchange chromatography, which relies on gradients of increasing buffer concentration, BIST employs a gradient of organic modifier from high to low to accommodate ions with varying interaction strengths toward the stationary phase.

BIST offers alternative selectivity compared with well-established LC techniques on other interaction mechanisms. It is compatible with a wide range of detection modes, including UV, MS, ELSD, CAD, and conductivity.

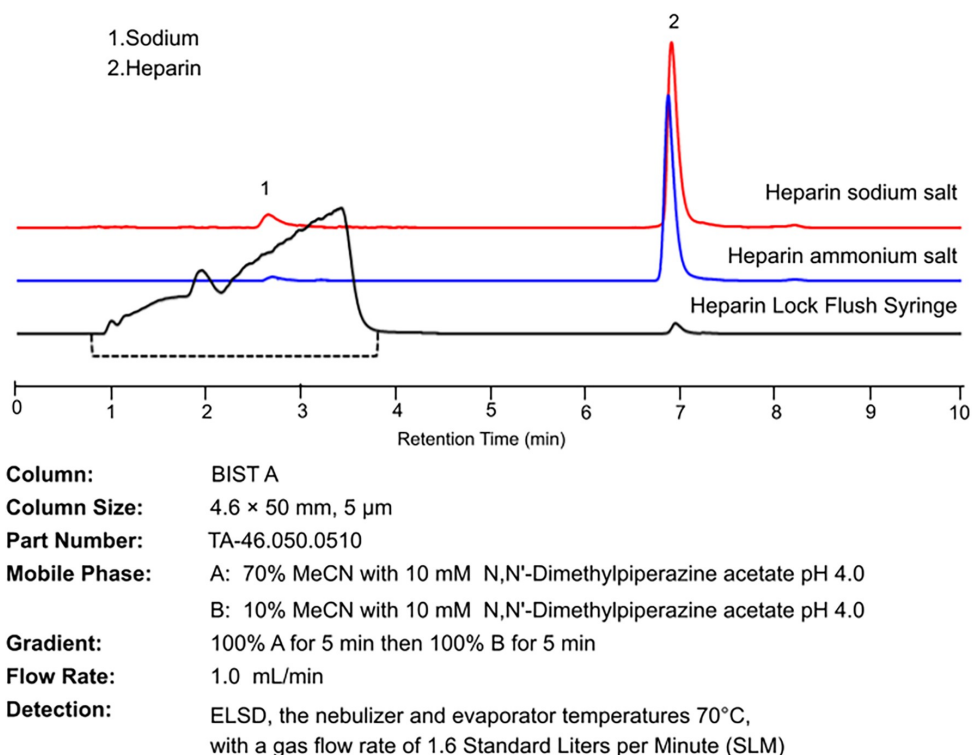


Figure 23.
 Elution of heparin as a single peak using a step gradient. Elution time can be controlled by the delay of switching from mobile phase a to b.

Importantly, BIST is applicable to all charged molecules, regardless of their overall hydrophobicity. Although still a relatively new technique requiring further investigation, it already represents a powerful tool in modern chromatography, particularly for the analysis of charged polymers where conventional alternatives are limited.

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