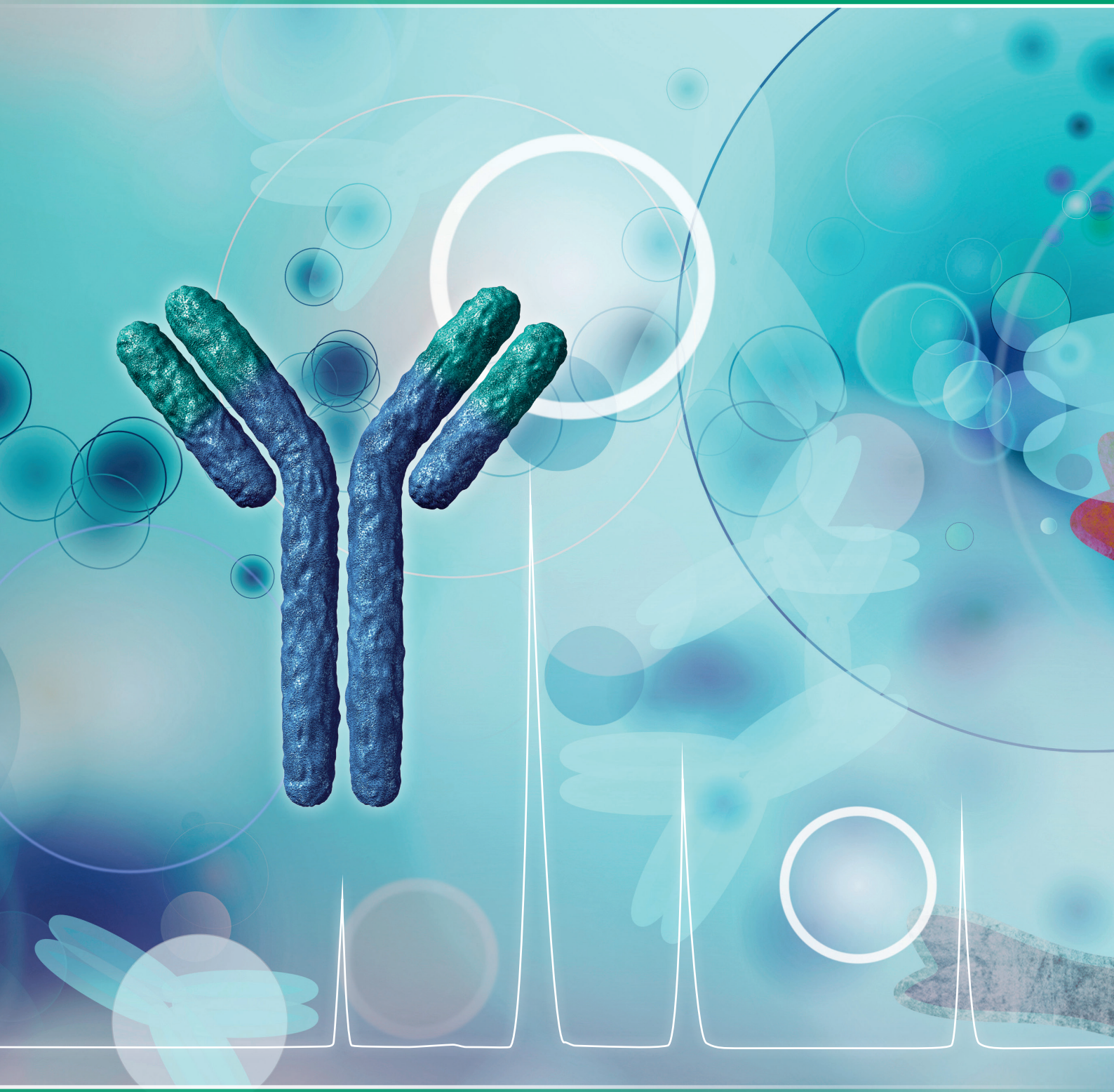
YMC



Purification of Proteins and Antibodies via Ion Exchange Chromatography

THEORETICAL AND PRACTICAL ASPECTS OF METHOD DEVELOPMENT

This Whitepaper ...

... is a practical guide to method development in preparative ion exchange (IEX) chromatography. It describes the most comprehensive and efficient approach for complete process development in preparative LC. This approach consists of method development and loadability studies at the analytical scale.

In addition to important considerations during the method development and loadability studies, many applications are included for the purification of proteins and antibodies. This whitepaper is therefore an excellent resource of knowledge in the laboratory. You are guided through the following aspects:

Content of the Whitepaper

The following icons help to guide through this whitepaper:


Application: Purification of Antibodies


Application: Purification of Proteins

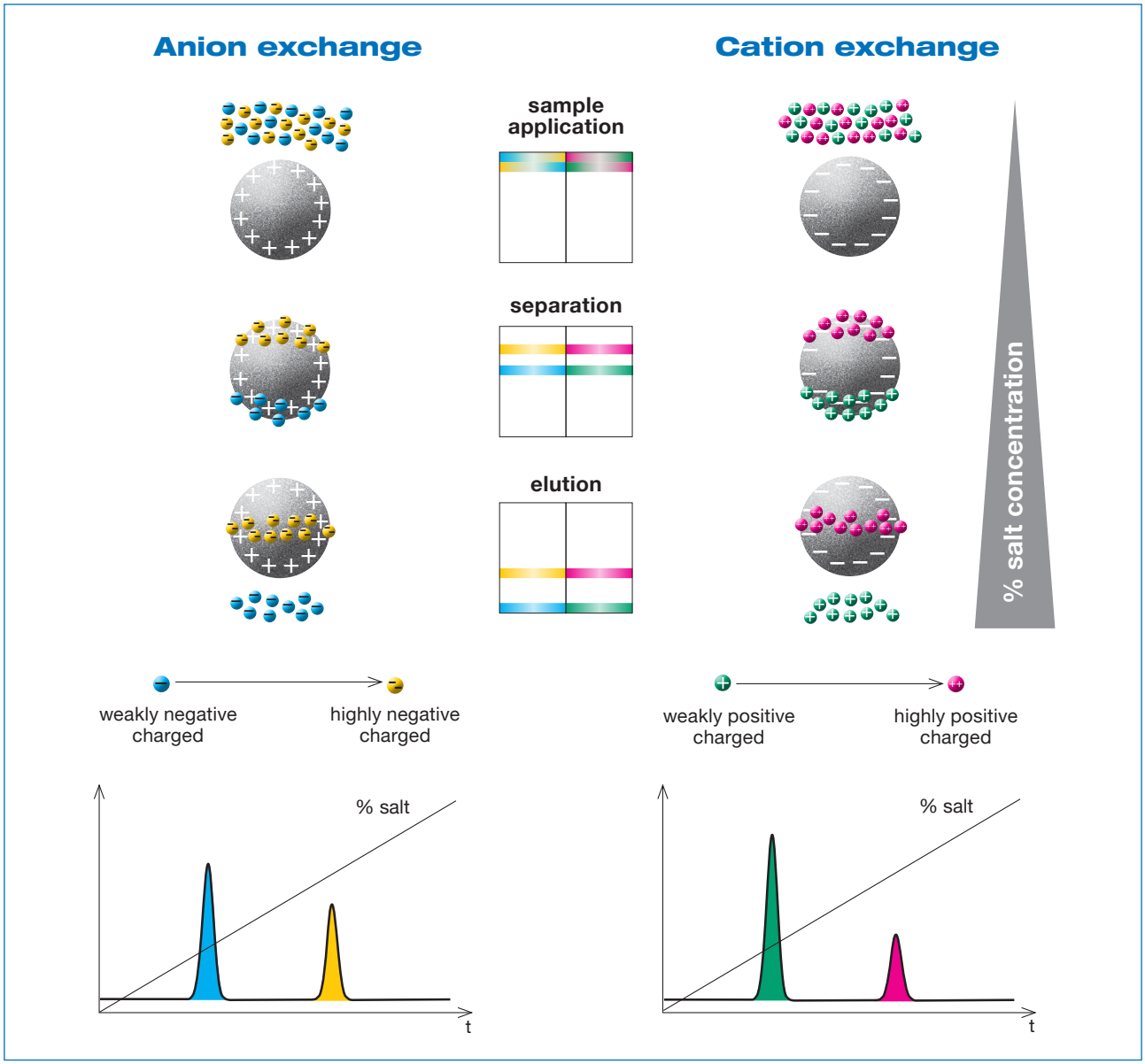

Practical Examples and Expert Tips

1. Introduction

The principles of ion exchange chromatography for the purification of proteins and antibodies

Ion exchange chromatography (IEX) is a well-established method for the purification of biomolecules. The separation technique is based on charge-charge interactions between the target molecules and the positively or negatively charged stationary phase. An anion exchange (AEX) resin has a positively charged modification to bind and interact with negative ions and molecules, whereas cation exchange (CEX) resins have a negatively charged modification and therefore are suitable to bind positive charges. Proteins and antibodies consist of different amino acid side chains and exhibit three-dimensional structures with varying surface charges depending on their amino acid composition and potential modifications. Therefore, they are perfectly suited for the separation via IEX.

Based on their charge profile, they interact with the charged surface of the resin and are eluted by increasing the ionic strength of the mobile phase. As nearly every protein or antibody exhibits a surface charge, IEX is an excellent and versatile chromatographic separation mode for biomolecules.

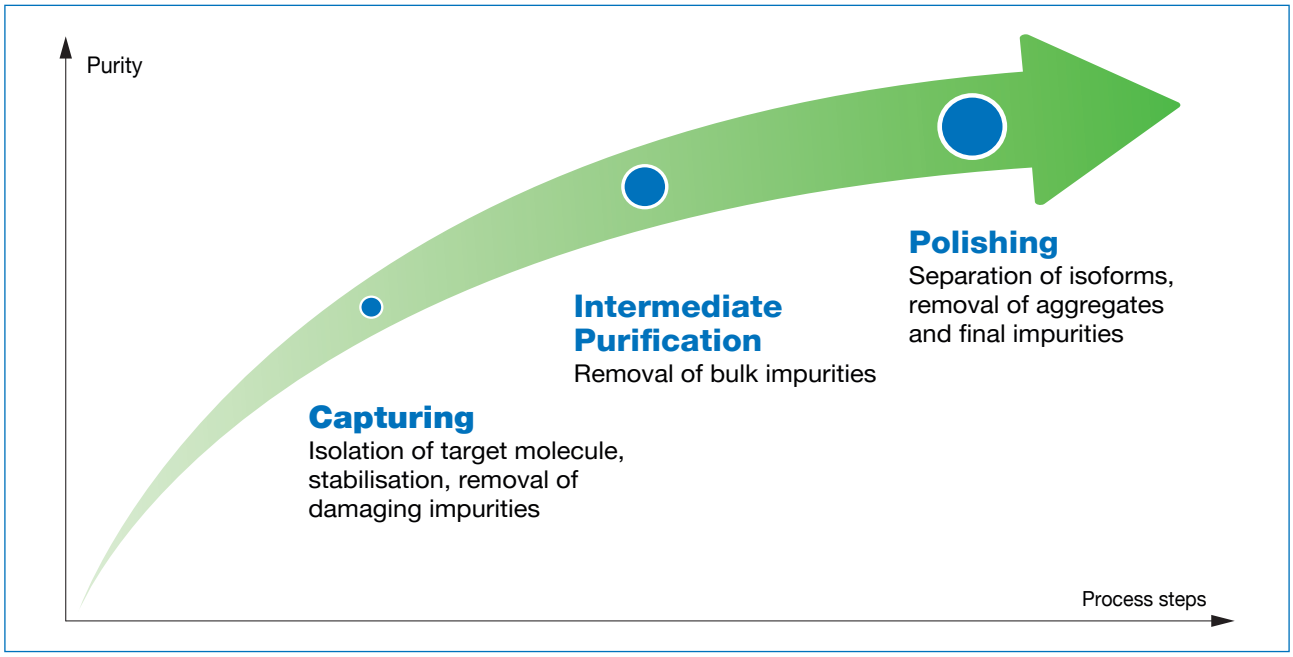


Downstream Processing: Purification of Biomolecules

The purification of biomolecules is a challenging process. The target molecules are generally produced via expression in dedicated cells. The feed solution after the upstream process is a very heterogeneous matrix consisting of various host-cell proteins (HCP), DNA contaminations and metabolites. The composition of the feed solution depends on the expression system and type. The purification process of the target molecule from this solution is called downstream process (DSP) and includes various separation and preparation steps until the final purity level is achieved. In a typical DSP, a cascade of up to three chromatographic steps is implemented.

Sample preparation

Before the solution is applied onto a chromatography column, the feed solution has to be prepared appropriately. Generally, an initial filtration as well as a buffer and pH-adjustment are recommended to remove insoluble components. Furthermore, the target molecule is stabilised to ensure an appropriate binding to the chromatographic column.



Capturing

During this initial purification step, the target molecule is captured and isolated from the feed solution. As a result, the target is concentrated and small molecule impurities including metabolites and damaging impurities such as proteases are removed. The target is stabilised and partially purified.

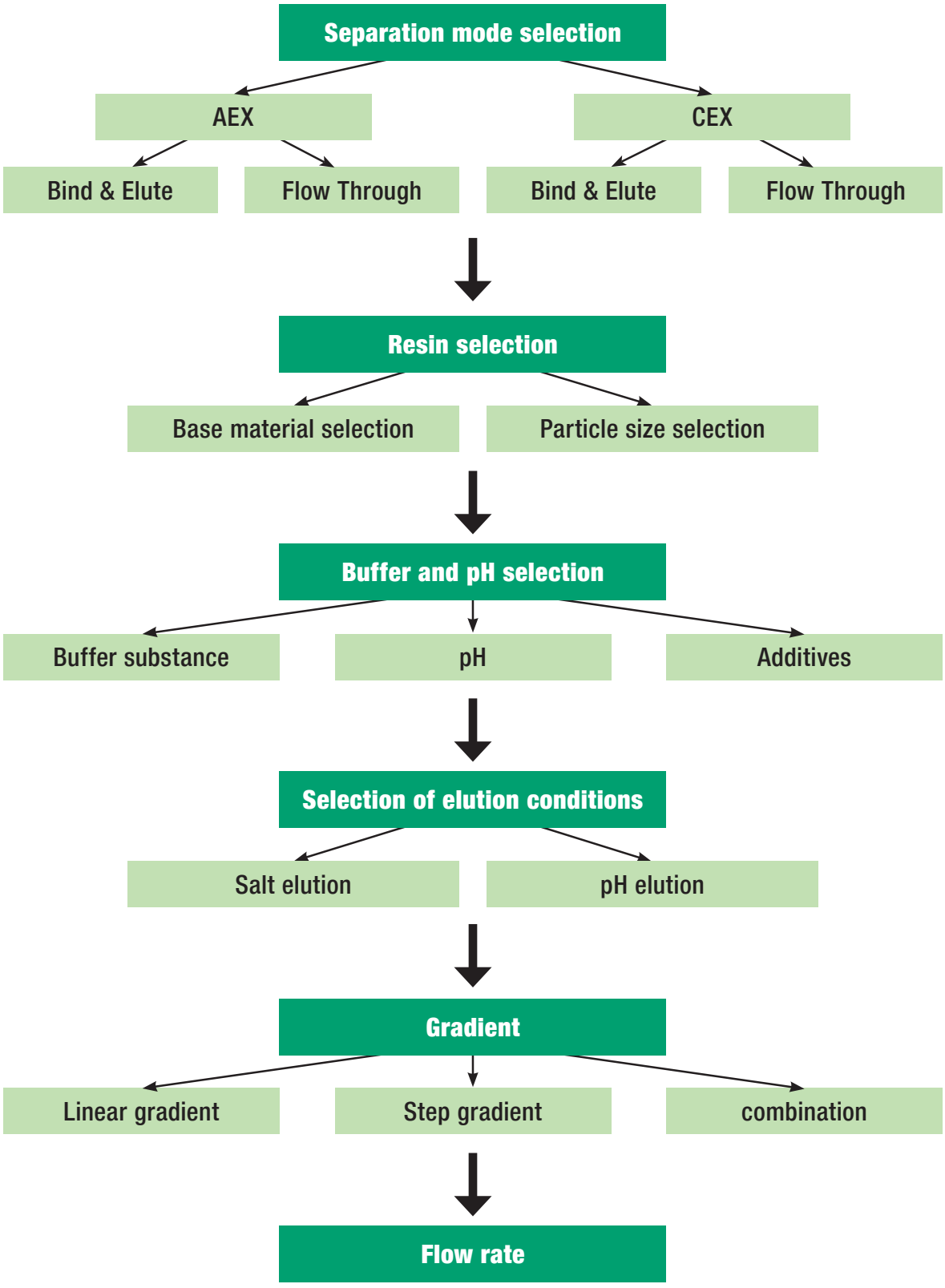
Intermediate Purification

This purification step removes the bulk impurities. The content and variety of HCP is very complex and therefore the chromatographic purification has to separate various proteins with different characteristics with high resolution. After this step, the purity level of the sample should be greatly increased.

Polishing

The polishing step is required to separate highly similar impurities such as isoforms, multimers and also degradation products. Therefore, a separation technique is required that can distinguish between these similar molecules and also provides high resolution. With this purification step, the final purity level of the target protein is achieved.

2. Method development steps using IEX



In this chapter, all important aspects of method development for a robust and efficient purification via ion exchange chromatography are discussed. The following steps are explained with theoretical and practical examples:

1. **Downstream strategies for successful purification**
2. **Applicable separation modes**
3. **Selection of the resin type and particle size**
4. **Buffer composition and pH**
5. **Definition of the elution conditions**
6. **Gradient adaption and elution optimisation**
7. **Flow rate optimisation**
8. **Cleaning strategies**

After defining the general method setup, initial loadability studies at analytical scale and scale-up considerations for larger scale are the next steps towards the finalised purification method. These aspects are discussed in the chapters 3 and 4.

The most important factor of a successful and cost-efficient purification is the selection of an appropriate resin. Usually, the most suitable resin is determined within a comprehensive screening with different resins. The following check list might be helpful for choosing resins for the screening process. The resin that is finally selected should fulfil the listed criteria.

Important Check List

Before choosing a phase for a method screening in preparative LC, the following check list might be helpful:

- ✓ **1. Availability of the resin**
- ✓ **2. High productivity**
- ✓ **3. Reproducibility**
- ✓ **4. Good packing characteristics**
- ✓ **5. Supply guarantee**

2.1. Implementation of IEX principles in a purification process

An efficient downstream process generally requires multiple purification steps until the desired purity of the target molecule is achieved. Frequently different separation techniques are coupled to separate the target molecule from the impurities. The most versatile separation technique within a DSP is ion exchange chromatography. This separation is relatively fast method, so it can be implemented for any step within the overall purification process. Ion exchange chromatography is also well suited in combination with other separation methods.
The following scheme shows that IEX is the only purification method which is applicable to the capturing step, intermediate purification and polishing method:

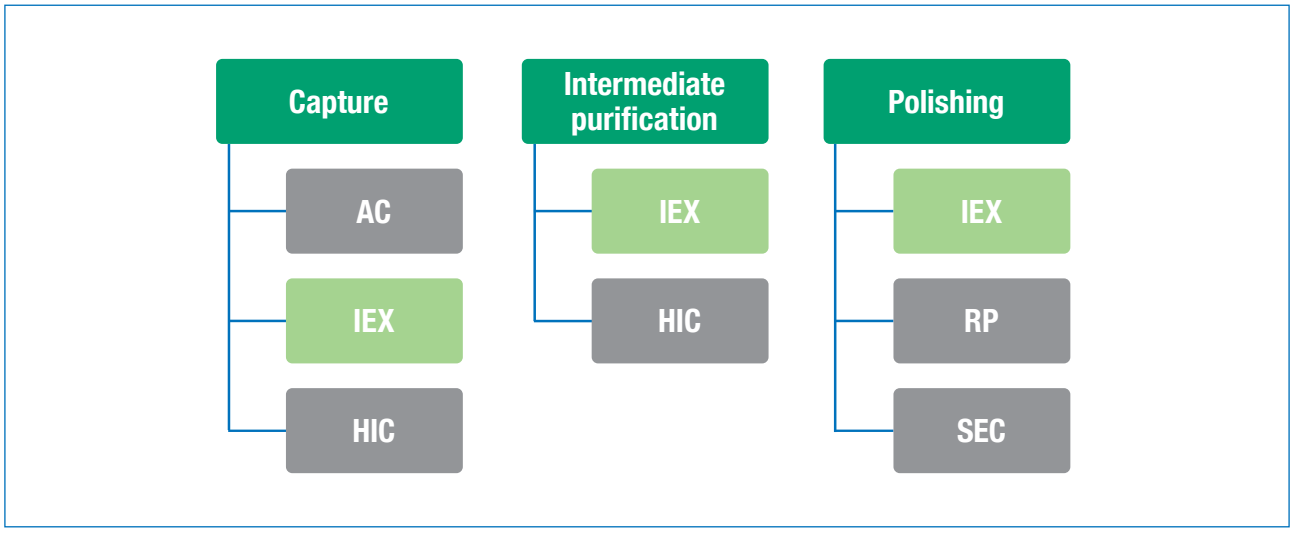


Figure 1: Typical chromatographic separation modes for the single steps during a downstream process:
AC = affinity chromatography;
HIC: = hydrophobic interaction chromatography;
IEX = ion exchange chromatography;
RP = reversed phase chromatography;
SEC = size exclusion chromatography

Combination of IEX with HIC

Hydrophobic interaction chromatography (HIC) requires high salt concentrations for the loading of the feed onto the column. As elution during IEX is usually performed by increasing the salt concentration, the elution fraction is ideally suited for a direct application onto a HIC column. This of course also applies in the other direction: the eluted target fraction from a HIC purification run usually contains low salt concentrations and can be combined with a subsequent IEX step.

Combination of IEX with SEC

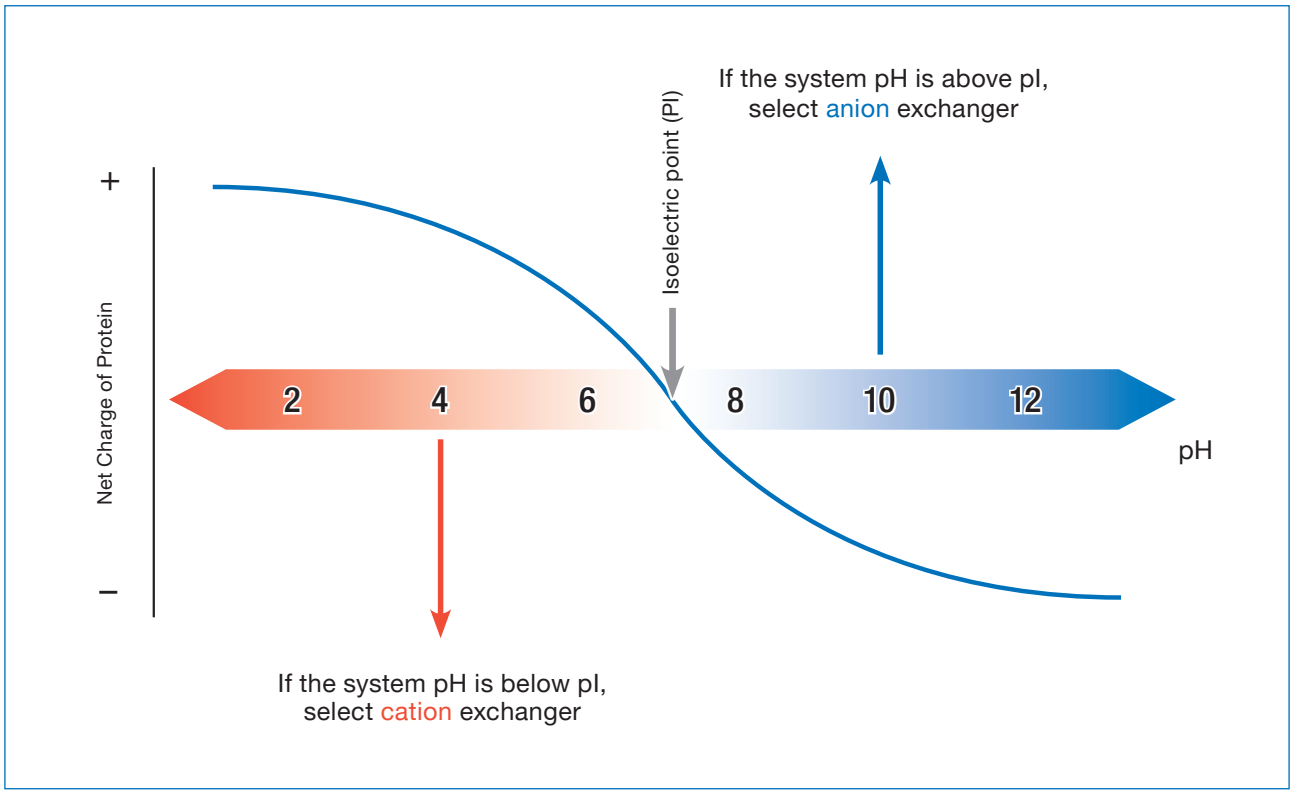
IEX is also suited as a prior step before a size exclusion chromatography (SEC) run. The generally low concentrated buffer and higher concentrated salt conditions are good starting conditions for a subsequent a SEC purification. Additionally, the desalting effect during the SEC separations reduces the high salt concentration of the sample solution after the IEX purification.

2.2. Separation mode: exchanger type and binding conditions

The next step during process development is the determination of the separation mode to identify if an anion or cation exchanger is required. This depends on the protein itself but also on the impurities that have to be removed. In this context, the choice between a Bind-and-Elute and a Flow-Through method setup is included.

2.2.1. CEX vs. AEX

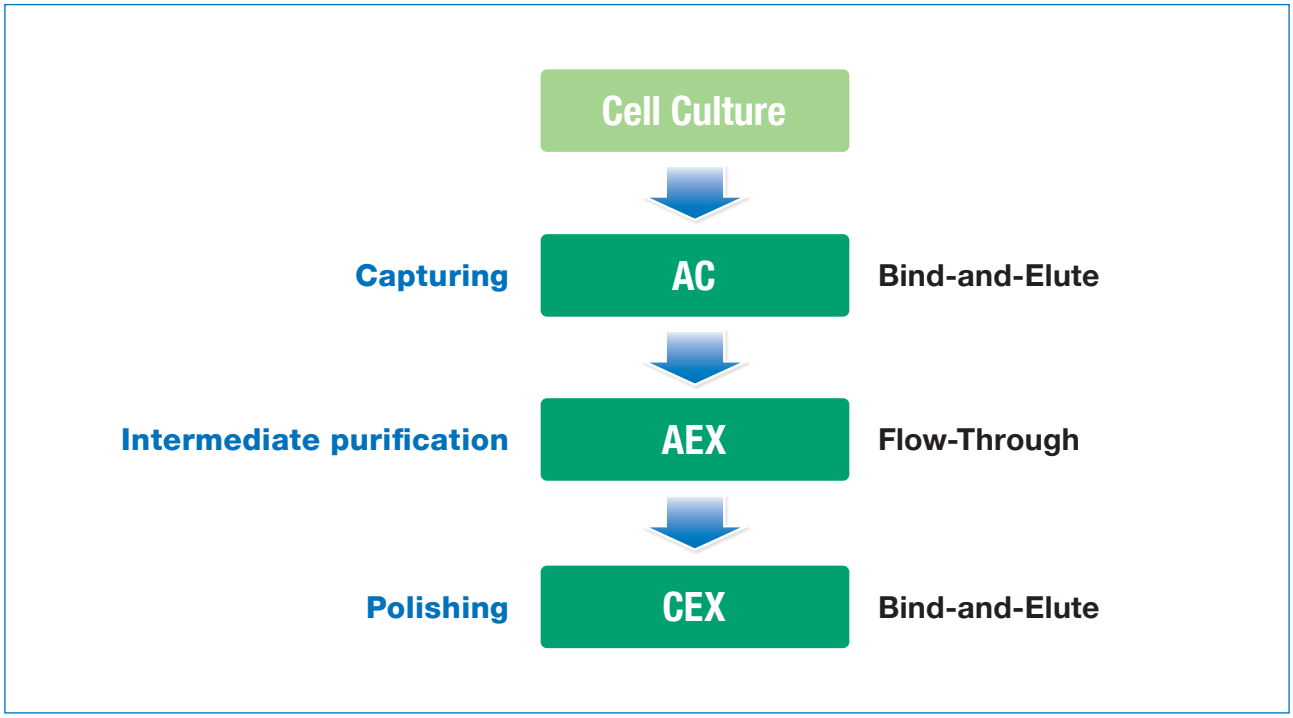
The decision between cation exchange chromatography (CEX) and anion exchange chromatography (AEX) depends on the overall charge of the target molecules – and on the impurity profile. The overall charge of the target protein or antibody is defined by its isoelectric point (pI). This is the pH value at which the net charge of the target molecule is zero, with all negative and positive charges cancelling each other. If the pH of the buffer in which the proteins are dissolved, is above the pI of the target, the negative charge of the target predominates. In this case, an anion exchange resin with its positively charged surface modification is the exchanger of choice to bind the target. However, if the system pH is below the pI, the positive charges predominate and therefore the negatively charged cation exchange resin is the preferable stationary phase to bind the target. The pI of a protein or antibody directly depends on its amino acid sequence and can be calculated by online tools. Modifications to the proteins and antibodies can influence the pI and therefore the overall charge of the target molecule.



By selecting the exchange type and the system's pH, the binding of the target molecules and the impurities can be modified. Therefore, the most appropriate binding conditions are selected.

Practical example:
DSP strategy with two IEX steps for efficient mAb purification

For the efficient purification of monoclonal antibodies (mAb), ion exchange chromatography is very well suited. It is also possible to combine two different IEX steps – an anion exchange step and a cation exchange step – to maximise the overall efficiency of the process. In this case, the initial capturing is performed by affinity chromatography using a Protein-A resin. Afterwards, the vast majority of impurities are removed by an AEX step. The final polishing purification including the removal of final impurities is done by CEX.
During this step, the antibody is bound to the resin and by optimising the elution conditions, the remaining proteins are separated.



2.2.2. Bind-and-Elute and Flow-Through applications

The classical ion exchange application is based on the binding of the target molecule to the ion exchange resin and the subsequent elution and separation from other bound molecules. During this application, the target substance and molecules with a similar charge profile interact with the column whereas other molecules with no significant or different charges do not bind and therefore flow through the IEX column. This approach is called the **Bind-and-Elute** (BE) application.
On the other hand, it is also possible to switch the application mode. In a **Flow-Through (FT)** application, the target substance does not interact with the stationary phase and flows through the column by selecting an ion exchange resin with the same charge modification. At the same time, the impurities of interest bind to the column and therefore are separated from the target. The advantage of this application is its speed of operation. Additionally, the loading and fractionation conditions remain the same for the target during the FT mode so that the elution conditions such as high salt concentration or buffer change have no influence on the target molecule. After the overall feed has been pumped through the column or the binding capacity is reached, the bound impurities elute in a single step by a washing with an eluent with high elution strength. Afterwards the column is regenerated for the next purification run.

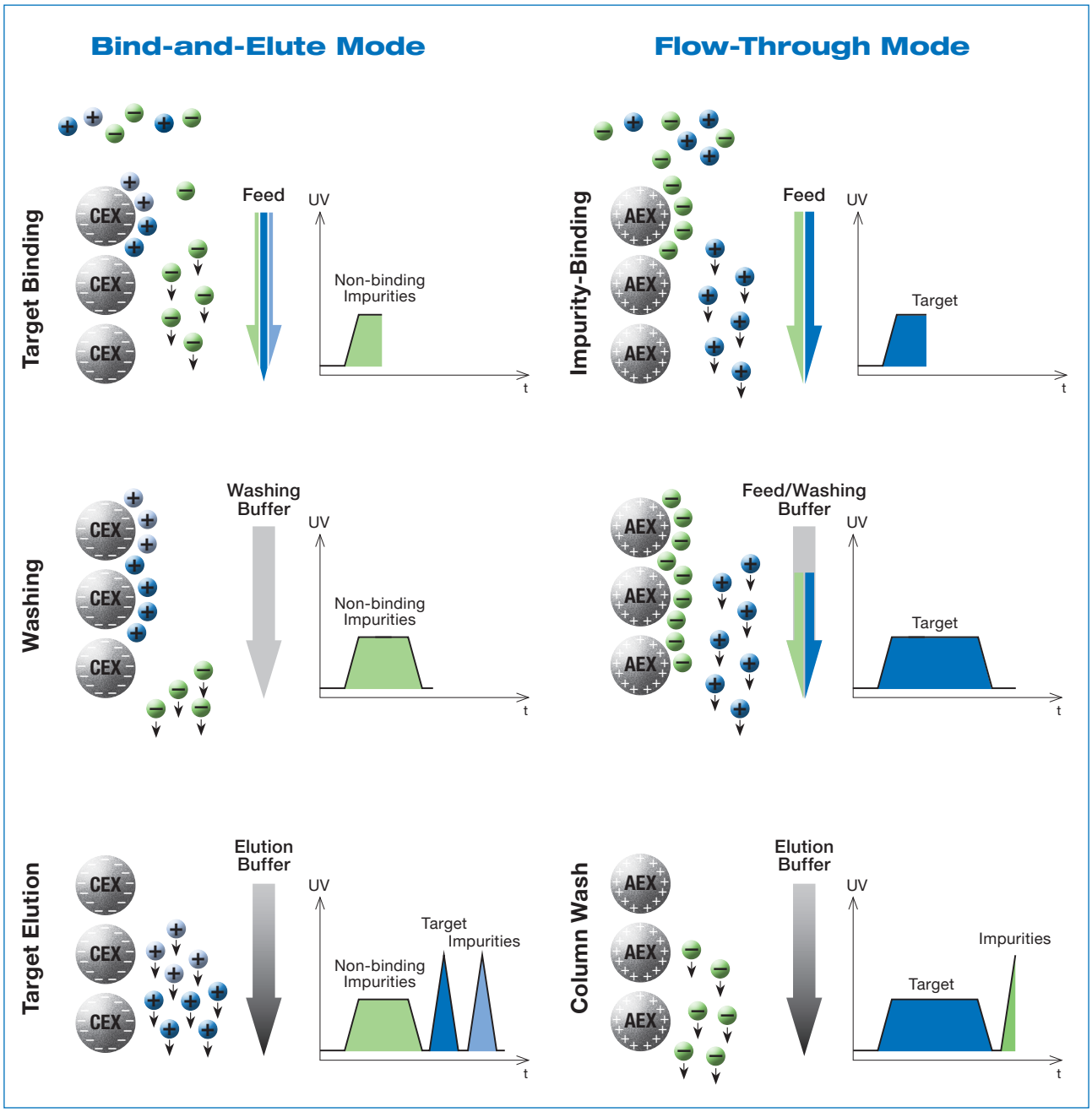


Figure 2: Schematic principle of Bind-and -Elute and Flow-Through applications.

The principle is shown for a positively charged target (dark blue). During the Bind-and-Elute mode (left), the sample feed is loaded onto the CEX-column. The positively charged target and similarly charged impurities bind to the stationary phase whereas negatively charged impurities flow through. The bound molecules are then eluted and separated by their specific charge characteristics. During a Flow-Through application, the positively charged target does not bind to the column and the negatively charged impurities are captured. The target is collected within the Flow-Through fraction and the impurities are eluted later within the column wash step.

The selection of the most suitable application depends on the target and the impurity profile. If DNA with its high negative charge is a major impurity, a Flow-Through application where the DNA is bound to the column in AEX mode and the target flows through can be a beneficial purification strategy.

2.3 Selection of an appropriate resin

The most important factor for a successful and efficient IEX method is the choice of the most appropriate resin. Various IEX resins are available on the market which are based on different bead base materials and with different particle sizes and modifications.

Important considerations for resin selection and scale-up

Even at the early stage of initial screenings, the final scale of the process should be considered. This includes the check for important resin features and criteria for the supplier of the material (see also the important check list on page 7 with general criteria):

- ✔ **Dynamic binding capacity**
- ✔ **Pressure-flow characteristics**
- ✔ **Availability of different particle sizes**
- ✔ **Security of supply and availability of large-scale quantities**
- ✔ **Regulatory affairs support and technical documentation**

Selection of resin type and an appropriate base material

IEX resins are based on different bead base materials. Some beads have an agarose basis, other are based on synthetic polymers such as polystyrene/divinylbenzene (PS/DVB) or polymethacrylate. The base particle material has a massive impact on the resin itself and its characteristics. Synthetic polymers generally provide a high chemical and mechanical stability which is especially important for alkaline cleaning-in-place procedures (CIP) and higher pressure-flow characteristics. Hydrophilic polymers allow for easy column packing with 100% aqueous conditions. Therefore, polymethacrylate – a hydrophilic polymer with excellent characteristics – is a common base material of modern process resins.

Selection of the particle size

Smaller particles in general provide better separation properties and therefore higher resolution ability. However, smaller bead sizes generate elevated backpressures. Therefore, the particle size selection is a compromise between sufficient resolution and the resulting backpressure being appropriate for the chromatographic system whilst allowing efficient flow rates.

Important considerations for scale-up

The impact of particle size on the backpressure should be considered even at early stage of process development, as the overall setup and the system at process scale has to cope with the pressure. In consequence, the ideal choice for the particle size typically depends on the existing ratings.

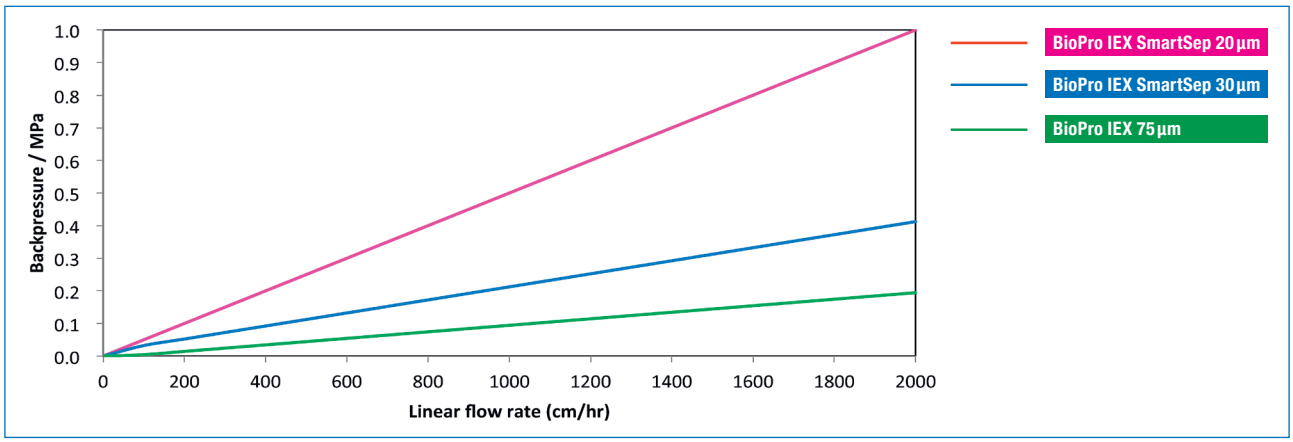


Figure 3: Pressure-Flow curves for the different particle sizes (20µm, 30µm and 75µm) of BioPro IEX packed into a 5x0.5cm ID column. The linear flow rate was increased from 0 to 2000cm/h and the resulting backpressure (in MPa) was monitored. The pressure-flow curve for the 20µm material shows the steepest slope.

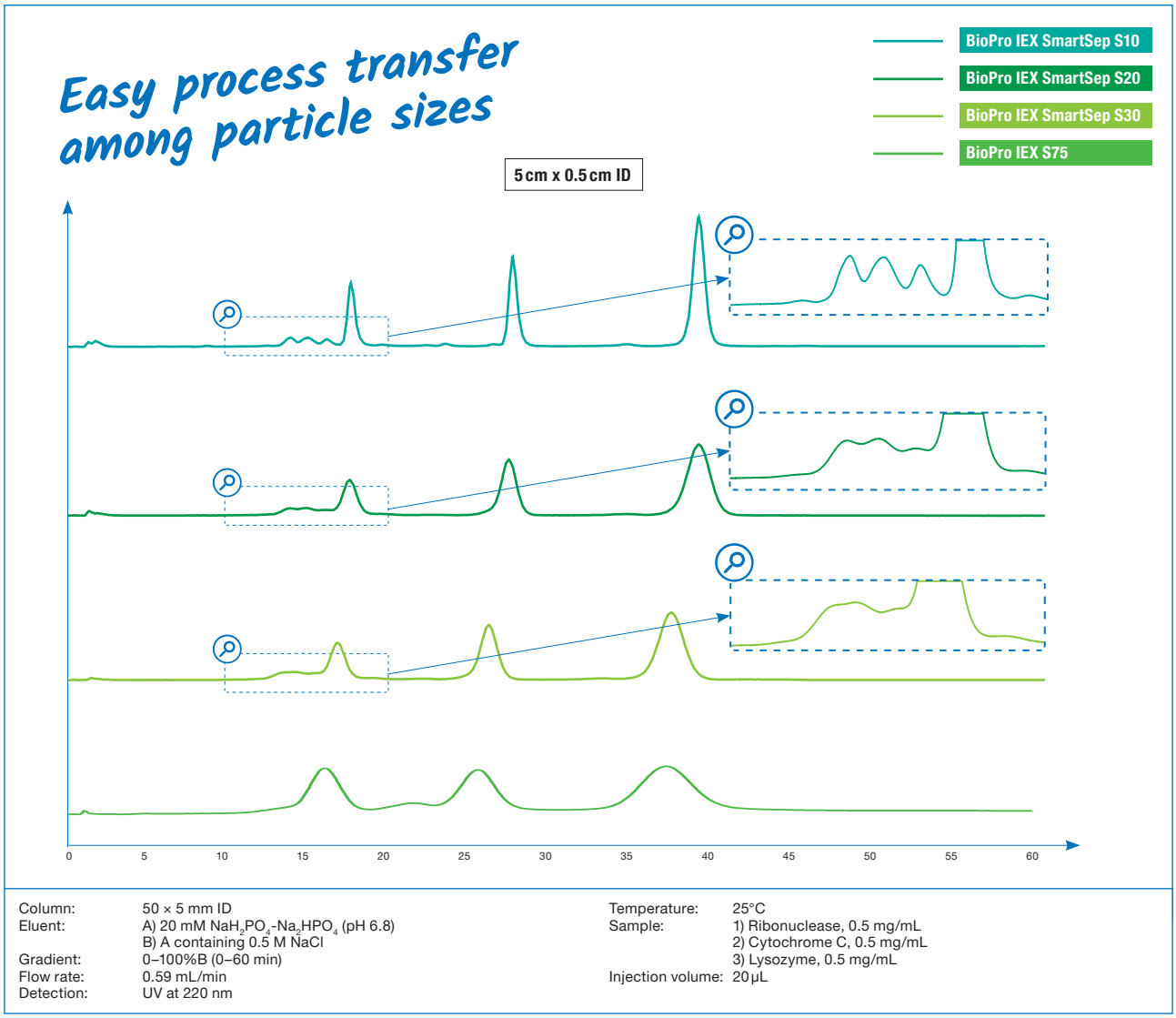
For initial capturing processes, large particles are generally the first choice. These separations require a high binding capacity whereas a maximum resolution is not of major importance. This is the same for Flow-Through applications.

For intermediate and polishing purification steps, high separation efficiency and therefore high resolution is required. In these cases, the use of smaller particle sizes is beneficial to improve the purity and productivity.

Expert Tip: Use an IEX resin for screenings that is available in different particle sizes for increased flexibility

Some resins are available at different particle sizes. If these resins with the different particle sizes are fully scaleable, it can be highly advantageous for process development. This property provides an identical retention behaviour across all particle sizes. Depending on the required resolution the optimal bead size can be selected. This enables highly flexible implementation of the resin, resulting in a perfectly tailored solution.

An illustrated example is shown below for the three enzymes ribonuclease, cytochrome c and lysozyme. This shows that the separation of the main peaks remains stable across the four different particle sizes. If the highest resolution is required, 10 µm particles are the optimum choice. 10 µm particles allow the isolation of trace impurities. During a capturing process the 75 µm material demonstrates adequate separation. The three main peaks are well separated.



Column:	50 × 5 mm ID	Temperature:	25°C
Eluent:	A) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8) B) A containing 0.5 M NaCl	Sample:	1) Ribonuclease, 0.5 mg/mL 2) Cytochrome C, 0.5 mg/mL 3) Lysozyme, 0.5 mg/mL
Gradient:	0–100%B (0–60 min)	Injection volume:	20 µL
Flow rate:	0.59 mL/min		
Detection:	UV at 220 nm		

2.4. Selection of the mobile phase conditions

The mobile phase conditions define if the target molecule will bind to the ion exchange column or if it will flow through. Additionally, the strength of the binding can be adjusted by modifying the mobile phase conditions. To ensure optimal target binding by charge-charge interactions, the most suitable buffer and pH conditions as well as additive and salt concentrations have to be evaluated.

2.4.1. Buffer and pH

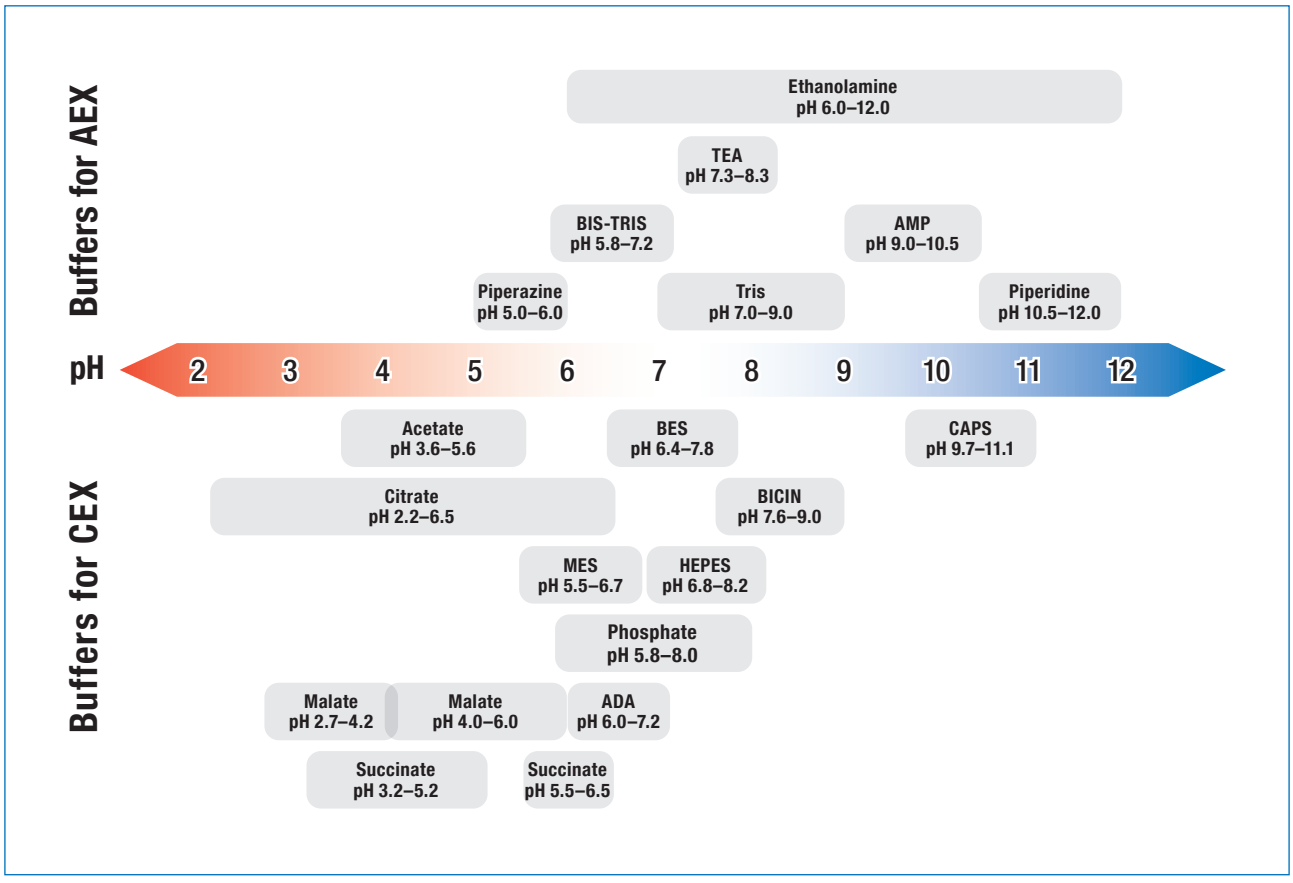
As ion exchange chromatography is based on the separation of the different charges of the target molecules, the selection of an appropriate buffer and pH are crucial steps for an efficient purification process.

The pH of the solution has a direct influence on the ionisation and the binding of the target as well as the impurities to the ion exchange media. Therefore, a suitable buffer and pH has to be chosen that allows an optimal binding.

General recommendations:

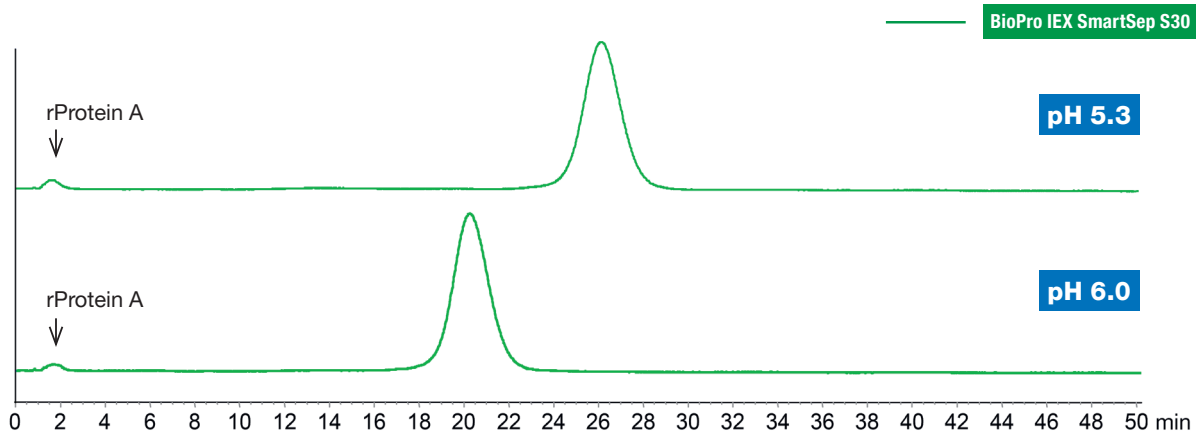
- ✔ **Choose a pH that is more than 0.5–1 pH unit below or above the pI of the protein or antibody (if known) to ensure that the molecule is soluble and ionised.**
- ✔ **The concentration of the buffer should be as low as possible but also high enough to ensure sufficient buffering capacity. Generally, the initial buffer concentration should be approx. 15–20 mM, depending on the buffer type and additives.**
- ✔ **The ionic strength of the buffer should be low so that the binding of the target to the ion exchange material is not inhibited/reduced (for Bind-and-Elute applications).**

The following graphic shows typical buffer systems for AEX and CEX applications as well as the typical working pH-range.

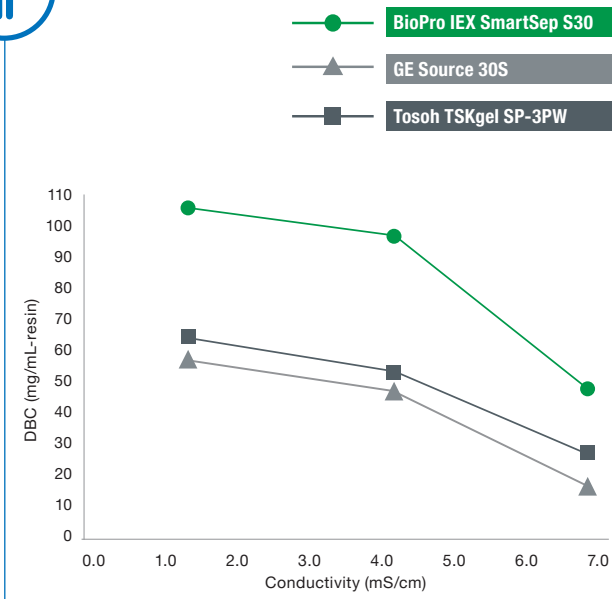


Practical example: Modifying the retention behaviour of antibodies by varying the pH-value

The influence of the pH of the buffer used as mobile phase can be immense. In this example, the retention time of an IgG-antibody was monitored at two different pHs. Even though the difference in pH value is only 0.7 units, the influence on the retention time is obvious. Therefore, the retention behaviour of a target substance can directly be influenced by changing the pH value of the mobile phase. A screening of different pHs starting from the initial pH can be beneficial.



Column:	50 x 5.0 mm ID	Detection:	UV at 280 nm
Eluent:	A) 20 mM citric acid-NaOH (pH 5.3) B) Eluent A containing 0.5 M NaCl	Sample:	Anti-human TNF-α monoclonal antibody (after affinity chromatography)
Gradient:	0–100%B (0–30 CV)	IgG Load:	0.1 mg
Flow rate:	180 cm/hr (0.59 mL/min)	Injection:	0.25 mL
Temperature:	ambient		



Flow rate:	200 cm/hr (0.66 mL/min)
Sample:	1.5 mg/mL human polyclonal IgG in equilibration buffer containing 0.25, 50 mM NaCl

2.4.2. Salt concentration

As the addition of salt increases the ionic strength of the solution, the interaction of the target protein or antibody with the stationary phase can be reduced. Therefore, to ensure an optimal binding of the target, the salt concentration of the feed solution should be as low as possible.
In many cases, this cannot be avoided as the salt concentration depends on the previous treatment steps and some proteins need a defined salt concentration for stabilisation.

The figure on the left shows the influence of different salt concentrations on the dynamic binding capacity of different resins. The overall capacity decreases with higher conductivity. Resins that generally have a higher dynamic binding capacity can also allow for higher salt concentrations within the feed solution.

2.5. Selection of elution conditions

Generally, ion exchange chromatography is based on Bind-and-Elute methods. Therefore, the target molecules bind to the ion exchange media whereas other molecules do not interact and therefore flow through the column. Of course, this can also be operated vice versa (see chapter 2.1.). The elution of bound molecules is performed by reducing the interaction between the charged molecules with the charged stationary phase so that the binding dissociates. This can generally be done in two different ways: increasing the ionic strength of the mobile phase by **increasing the salt concentration** or **by changing the pH value of the mobile phase**. A combination of both elution methods is possible, too.

2.5.1. Elution by increasing ionic strength / salt concentration

This elution method is the most common type of elution in IEX. The increasing salt concentration leads to an increase in the ionic strength of the mobile phase and therefore to a dissociation of the bound proteins from stationary phase.

Low salt concentration for initial loading
➜ Low ionic strength ensures efficient target binding

Increasing salt concentration during elution
➜ Salt ions compete with the target for binding to the stationary phase
➜ Desorption of the target and elution with the mobile phase

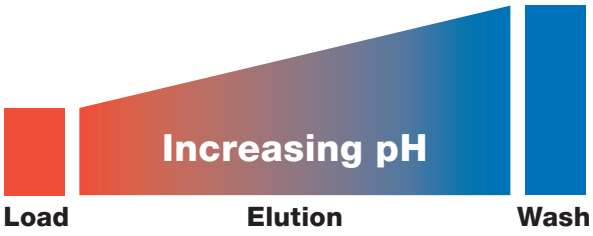
2.5.2. Elution by changing pH values

Another elution method is based on changing the pH of the mobile phase. In this case, the pH of the buffer is adjusted so that the target molecule is less ionised and therefore less charged. Consequently, the bound biomolecules dissociate from the charged resin surface and elute.

Cation Exchange

Low pH for initial loading
➜ increased positive charge of the protein

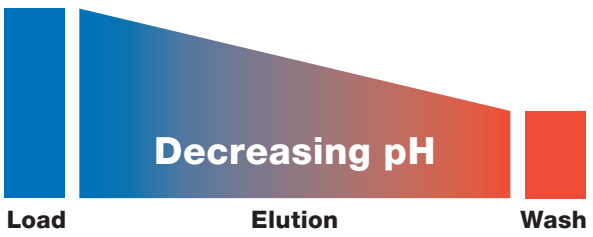
Increasing pH during elution
➜ decreased positive charge of the protein
➜ desorption from negatively charged resin



Anion Exchange

High pH for initial loading
➜ increased negative charge of the protein

Decreasing pH during elution
➜ decreased negative charge of the protein
➜ desorption from positively charged resin



The implementation of a stable linear pH gradient is a challenging task. The mixing of buffers with different pHs does not allow a linear pH gradient (except for specialised multi-component buffers). Additionally, the monitoring of the pH gradient needs special equipment and is not as accurate as the monitoring of changing conductivity.

This makes the pH elution by gradient highly sophisticated. For large scale purifications, a method that is developed at small scale has to be transferred in larger scales with the same chromatographic performance – in the case of pH gradients, this is hard to apply as the fluctuation range is large in this case.

Important considerations

- ✔ Salt elution is the most common elution as it is easy and cost-efficient
- ✔ Elution by pH gradient is highly sophisticated as an accurate gradient is hard to apply
- ✔ The reproducibility and scale-up of pH gradients is more complicated
- ✔ The monitoring of changing conductivity during salt elution is easier
- ✔ pH elution can be highly beneficial for challenging separations (e.g. the separation of aggregates)

Expert Tip: Combination of salt gradient elution and pH-elution

As the changing of the pH of the elution buffer can improve the separation in many challenging cases but on the other hand is complicated to apply, a combination of both elution techniques can be an efficient solution.

In this approach, the elution is performed by a salt gradient but the pH of the binding buffer and elution buffer differs, so that the overall elution can be refined. This approach allows for a more robust method compared to an elution by a pH gradient.

2.6. Gradient and Step Elution: how to perform and optimise target elution

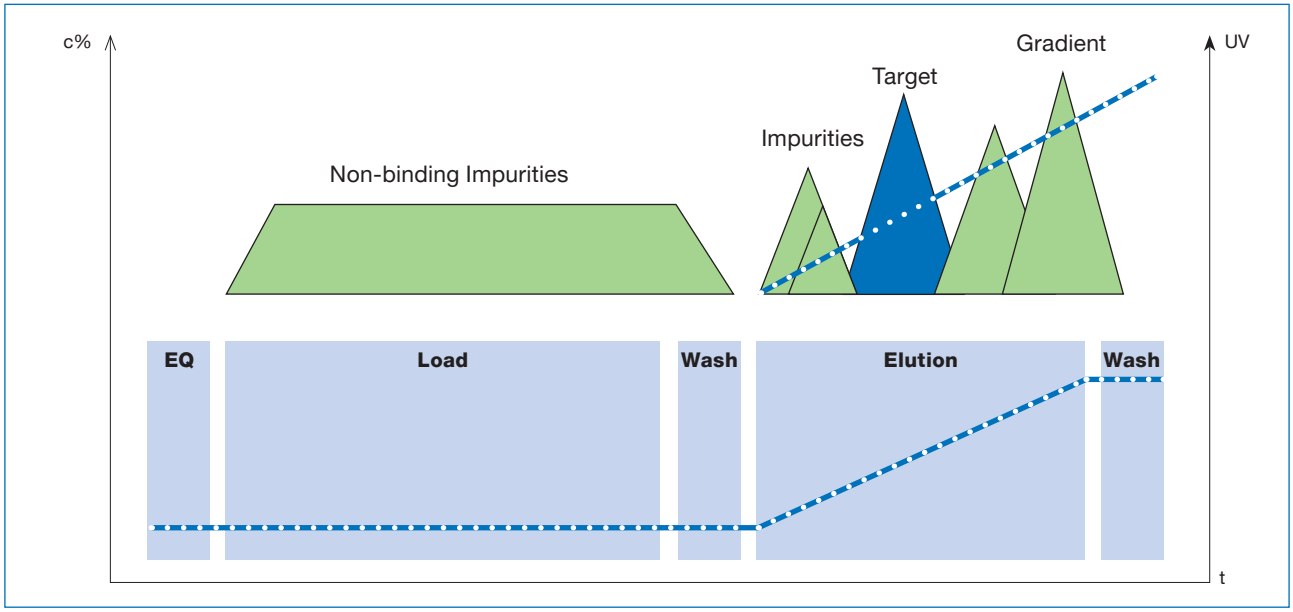
For Bind-and-Elute methods, the optimal elution conditions determine the success of the purification. Generally, the elution can be performed by a linear gradient of increasing elution conditions, or by a step elution. A combination of both methods is also possible to optimise resolution and recovery but also time and eluent consumption. In this chapter, the principles and optimisation of elution conditions starting from the initial gradient are explained.

2.6.1. Initial gradient and gradient optimisation

The recommendation for initial elution experiments is a shallow linear gradient with increasing elution strength for approx. 20–30 column volumes (CV). After loading the feed solution, a washing step is recommended to wash out remaining unbound and loosely bound impurities before starting with the elution gradient. The loading buffer (Buffer A) should have low ionic strength (low salt concentration), whereas the buffer B should have high ionic strength. During the initial elution experiment, the elution profile and the elution time of the target is determined.

Initial elution experiment

Buffer A: **as low ionic strength as possible (0–50 mM NaCl)**
Buffer B: **high ionic strength considering separation efficiency (e.g. 0.5–1 M NaCl)**
Gradient: **0–100%B in 20–30 CV**



2.6.2. Linear gradient: optimisation

The initial linear gradient elution is performed to identify the elution behaviour of the target molecule. However, these initial gradients are not practicable for a final process as they are very time consuming. Therefore, an optimisation of the initial gradient depending on the elution profile is recommended. A good rule is to condense the gradient before and after the target peak. The ionic strength of the starting point of the gradient should be slightly below the target starts eluting (e.g. a few mS/cm below the conductivity at the peak starting point). The endpoint of the gradient should be slightly above the ionic strength after a complete elution of the target. Run the gradient for 5 CV. Shallow gradients result in larger fraction volumes.

Optimised elution experiment

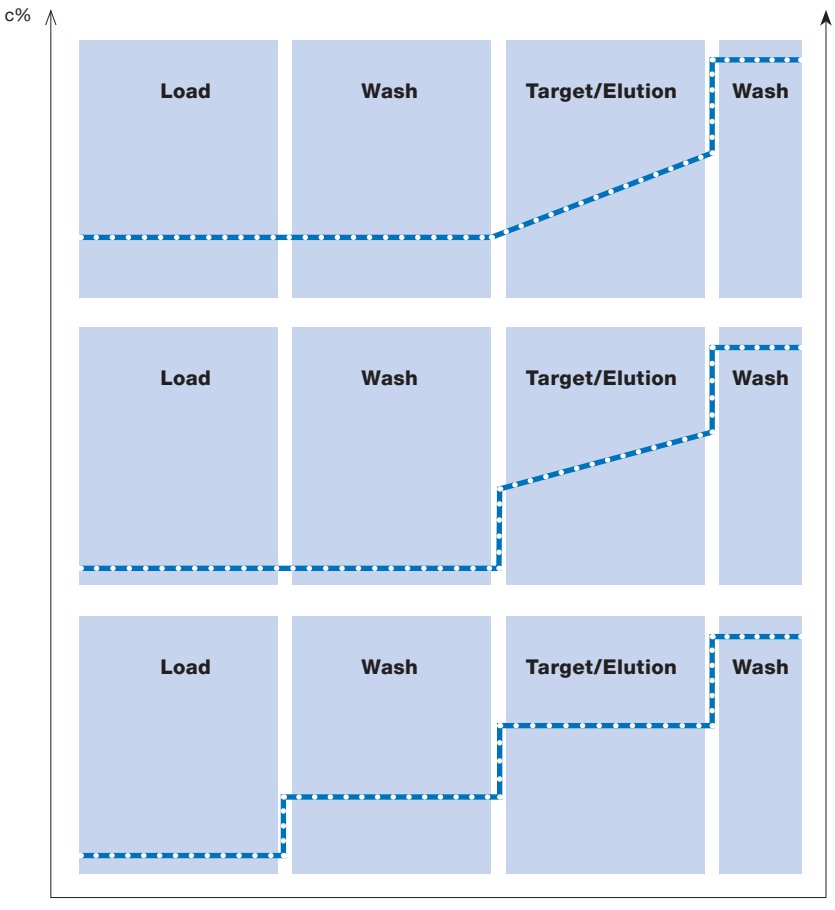
Buffer A: **ionic strength that is slightly below the elution concentration of the target molecule**
Buffer B: **ionic strength that is slightly above the elution concentration of the target molecule**
Gradient: **0–100%B in 5 CV**

To make sure that the impurities that are eluting before and after the gradient are washed out of the column, washing steps with sufficient amount of washing buffer are recommended.

2.6.3. Step elution and combination with gradient elution

The alternative to gradient elution is a step elution. In this case, a step with a defined conductivity or elution strength is performed that leads to an elution of all molecules with this elution point and lower binding at once. The elution point is the concentration of the elution buffer (e.g. the conductivity) at which the target starts to elute. This point is defined within the initial gradient where the elution of the target and the impurity peaks are monitored over a broad gradient of increasing amount of elution buffer.
The elution step is performed with the conductivity that corresponds to the elution point.

This leads to the elution of the target molecule and all impurities that were eluted before the target in the initial gradient. Additional steps with lower and higher conductivity can be implemented to remove further impurities that are eluting before and after the target peak in the initial gradient. During step elution, the eluted peaks are sharper and the elution is faster and less resource consuming but the separation efficiency is lower.
In practice, a combination of step elution and gradient elution is often used as optimised method. Examples for elution principles can be found in the following scheme.



Gradient Elution
- ➜ Linear gradient in the elution region of the target
- ➜ Loading buffer slightly below elution strength

Gradient Elution with previous wash step
- ➜ Linear gradient in the elution region of the target
- ➜ Additional washing step with slightly lower ionic strength than elution strength

Step Elution
- ➜ Step elution at elution region of the target
- ➜ Additional washing step with slightly lower ionic strength than elution strength

2.7. Flow rate optimisation

The flow rate generally has a massive impact on the resolution and also the productivity of a purification run. The faster the purification run, the higher is the resulting productivity as more target material can be purified within a time frame. On the other hand, the flow rate also has an immense impact on the dynamic binding capacity. The higher the flow rate during the binding, the lower is the binding of the molecules to the stationary phase because the contact time is reduced. The following figure shows the elution of BSA after the loading with varying linear velocities. The binding is decreased with increasing flow rates.

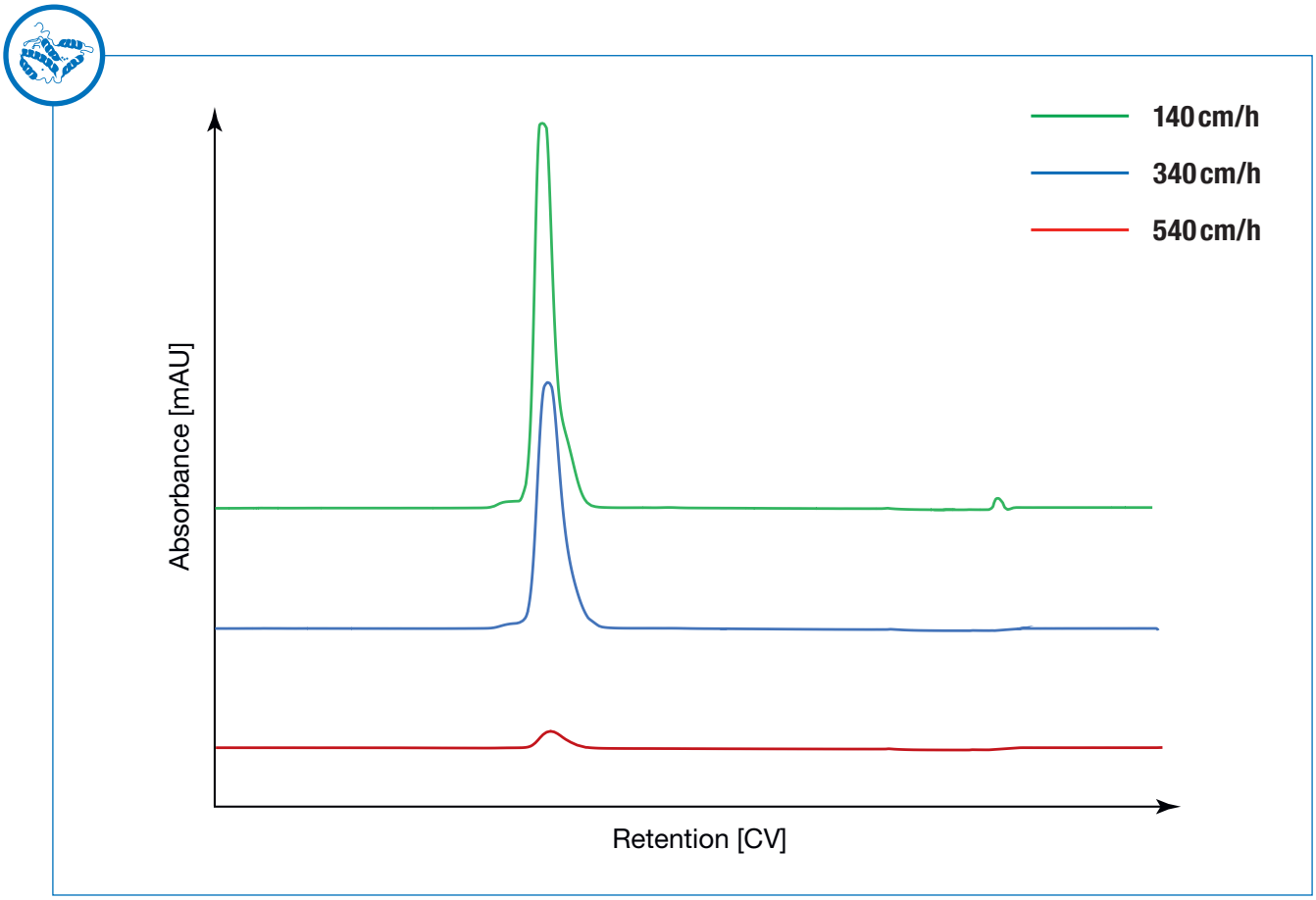
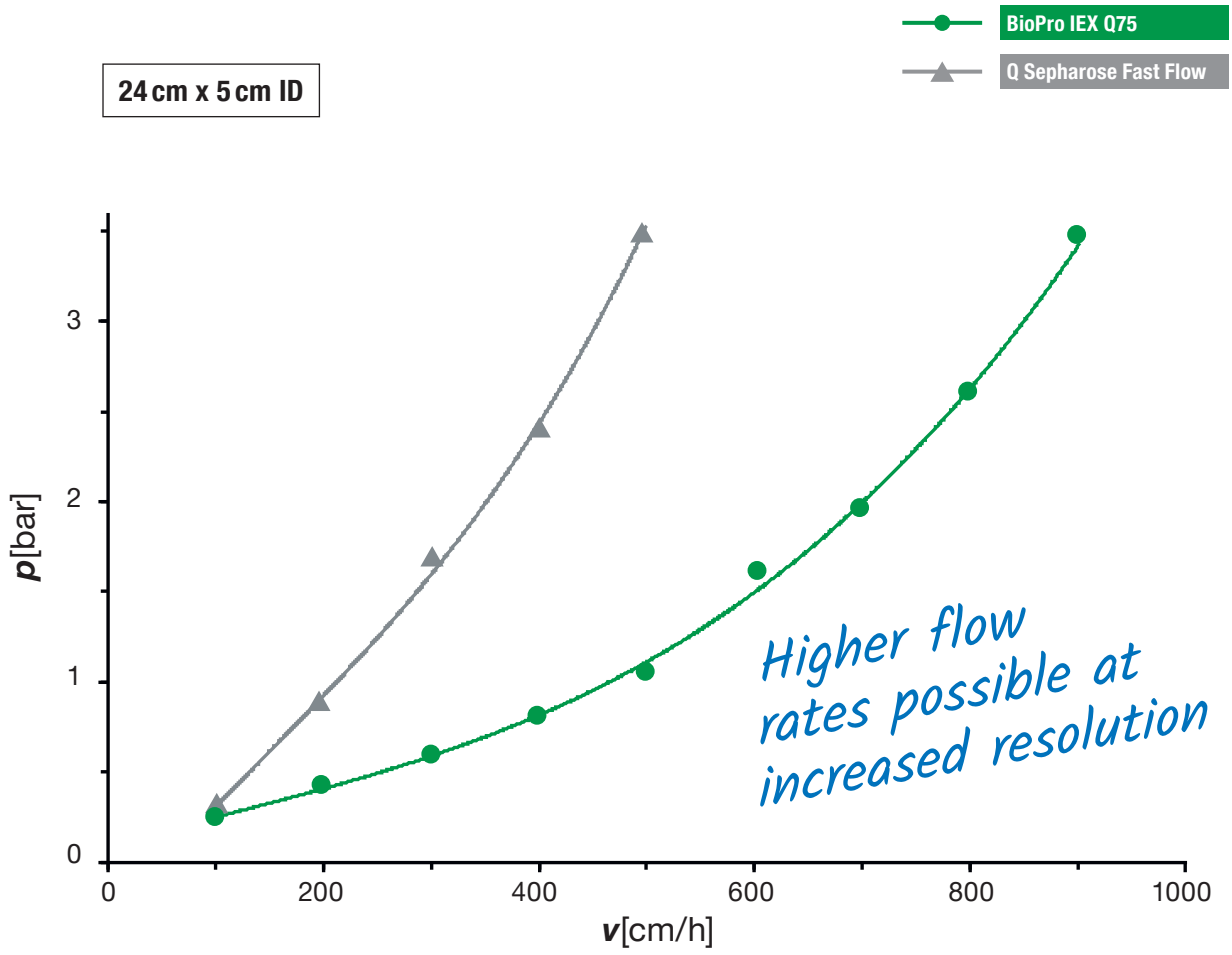


Figure 4: Loading and subsequent elution of a BSA sample feed using different flow rates from 140–540 cm/h with increasing flow rates, the amount of BSA that is bound on the column decreases.

The flow rate is always a trade-off between process speed, efficient binding and sufficient resolution during elution. In some cases, a higher flow rate can lead to increased resolution whereas for other target separations, the same method setup including the same linear velocity can result in decreased resolution and therefore lower yields and recovery. Therefore, the testing of varying flow rates is an important step towards robust and efficient method development. It is also possible to vary the flow rate of the different steps (equilibration, sample loading, washing, elution and regeneration). For example, the loading maybe more efficient at a lower linear velocity whereas the elution is performed at higher linear flow. This has to be adjusted for each method to ensure the development of the most efficient process.

Important considerations for scale-up

Resins with good pressure-flow characteristics allow for more flexibility and higher productivity. Resins producing lower backpressures generally enable higher flow rates. Therefore, the pressure-flow characteristics as well as the pressure ratings of the resin are important factors that should be considered at early stages of process development. The practical example in the following figure shows the pressure-flow curves of two process resins: in this case, the BioPro IEX Q75 resin shows a much lower backpressure compared to the Q Sepharose FF. As a consequence, the maximum flow rate can be increased. The smaller particle size of BioPro IEX Q75 (75 µm) compared to the Q Sepharose FF (90 µm) additionally allows for higher resolution.



Column:	ECO^{PLUS}, 50 mm ID, max. bed length 250 mm, AB version
Resin amount (gravity settled in 1 M NaCl):	470 mL
Slurry concentration:	40 %
Consolidation flow velocity:	60 cm/h
Consolidated bed height:	24 cm
Mobile phase:	1 M NaCl solution

2.8. Cleaning-in-Place Procedures

Cleaning-in-place (CIP) is essential for the economic use of packed chromatography columns. Efficient cleaning procedures increase the lifetime of the separation process and thereby contribute to the overall cost effectiveness. Furthermore, powerful CIP procedures strongly increase the safety and productivity of every downstream process.

The cleaning procedure depends on the impurity profile and therefore on the type of impurities that are bound to the resin.

Strongly bound impurities and highly charged impurities (such as nucleic acids)
For strongly bound impurities that do not elute with 100% of the elution buffer, higher ionic strength like 1–2 M NaCl or 1 M $NaClO_4$ can help to remove the impurities from the column.

Hydrophobic impurities, lipids and lipoproteins
Organic solvents are most suitable to elute hydrophobically bound impurities such as hydrophobic proteins, lipoproteins or lipids. For this purpose, 20–70% ethanol or 30% isopropanol can be used.

Precipitated proteins
Precipitated materials can be solubilised and removed by NaOH solutions. Chaotropic salts and agents (compounds which in aqueous solutions can disrupt the hydrogen bonding network between water molecules such as 8 M urea or 6 M guanidium-HCl) are the next step. If these procedures are not sufficient to remove the precipitated proteins, an enzymatic digest with, for example, pepsin can be beneficial.

Removal of metal ions
Citric acid is the favoured solution to remove typical metal ions (such as iron) from anion exchange resins. Additionally, chelating agents such as EDTA in low concentrations can help to completely remove metal ions from the resin, if necessary.

Viruses and endotoxins
Potential viruses and endotoxins have to be removed efficiently from the column. The application of NaOH solutions is an efficient way to remove them.

Sanitisation of the column

Another important factor why CIP procedures are highly important in the field of DSP is the risk of microbial growth. Therefore, the column has to be sanitised. The sanitisation can be done by multiple ways. Most commonly, a treatment with alkaline solutions (including 0.1–1 M NaOH) is used for sanitisation. For long-term storage, 20% ethanol is recommended to inhibit microbial growth.

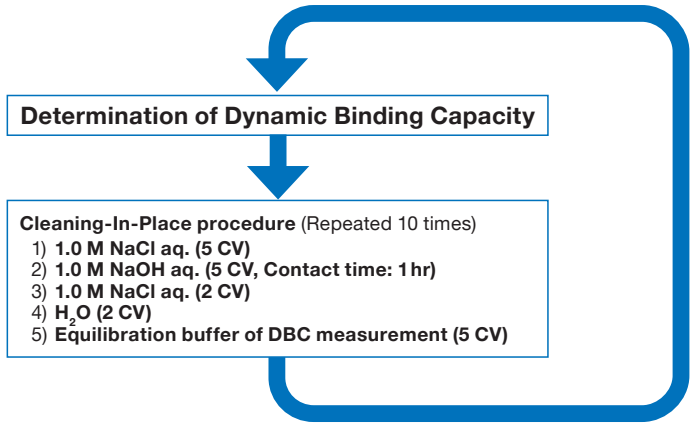
Recommended standard protocol

The most efficient CIP method for standard purifications is the treatment of the resin with strong ionic strength (1–2 M NaCl) and with sodium hydroxide (0.1–1 M NaOH):

1) **1.0 M NaCl aq. (5 CV)**
2) **1.0 M NaOH aq. (5 CV, Contact time: 1 hr)**
3) **1.0 M NaCl aq. (2 CV)**
4) **H_2O (2 CV)**
5) **Equilibration buffer of DBC measurement (5 CV)**

Practical example: Stability towards alkaline CIP procedures

In this study, the stability of the BioPro IEX resins towards cleaning with 1 M NaOH as the standard method was investigated. The effect of multiple cleaning cycles on the binding of a monoclonal antibody on the cation exchange resin was tested with the following protocol:



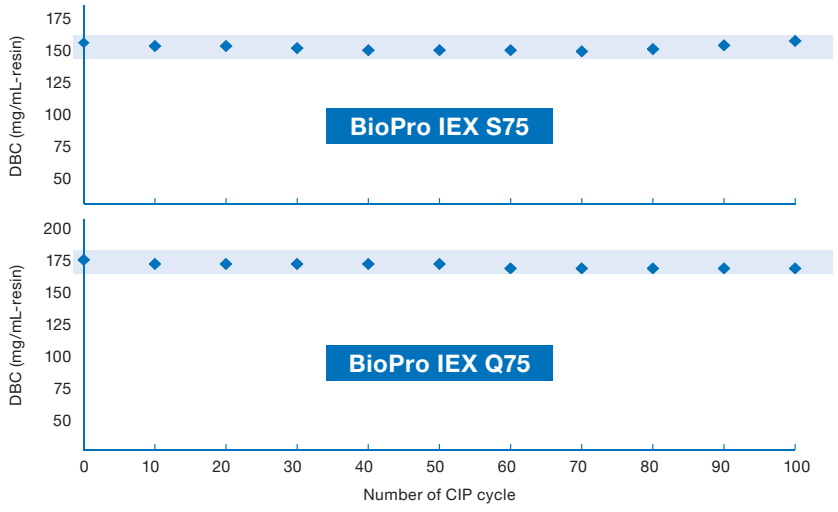
DBC (IgG) (BioPro IEX SmartSep S30, BioPro IEX S75)

Column:	50 × 5.0 mm ID	Temperature:	25°C
Equilibration buffer:	20 mM citric acid-NaOH (pH 5.3)	Detection:	UV at 280 nm
Elution buffer:	Equilibration buffer containing 0.5 M NaCl	Sample:	1.5 mg/mL human polyclonal IgG in equilibration buffer
Flow rate:	200 cm/hr (0.66 mL/min)		

CIP cycle

Column:	50 × 5.0 mm ID
Flow rates:	200 cm/hr (1.0 M NaCl, H_2O, Buffer)
	30 cm/hr (1.0 M NaOH)
Temperature:	25°C

The DBC of the BioPro IEX resins for IgG was constantly very high. No decrease of the binding capacity was found for both resins even after 100 CIP-cycles.



The resins are highly stable towards CIP procedures with 1 M NaOH. Therefore, the column lifetime is not affected by this very harsh but also very effective cleaning procedure which allows high productivity and robust antibody purification.

3. Loadability Studies

After the general method setup is defined, loadability studies on the analytical scale are the next step during process development with ion exchange chromatography. This step defines the maximum amount of feed solution that can be loaded and eluted with sufficient resolution.
The maximum amount of target that can be loaded onto an IEX column is limited by the overall binding capacity of the resin and by the resolution of the eluted peaks. The higher the dynamic binding capacity of the resin, the more substance can be bound to the stationary phase and the higher is the maximum loadability.
On the other hand, the resolution of the target and the impurity peaks during the elution is the other factor determining the maximum loading. Both are investigated at analytical scale to save time, money and target sample.

Determination of the dynamic binding capacity

The DBC of a resin as most important factor for the productivity is determined by pumping a defined amount of substance through the column and measuring the breakthrough point of the target.
The DBC per mL resin can then be calculated. The scheme and the corresponding formula for the DBC determination is shown in figure 5.

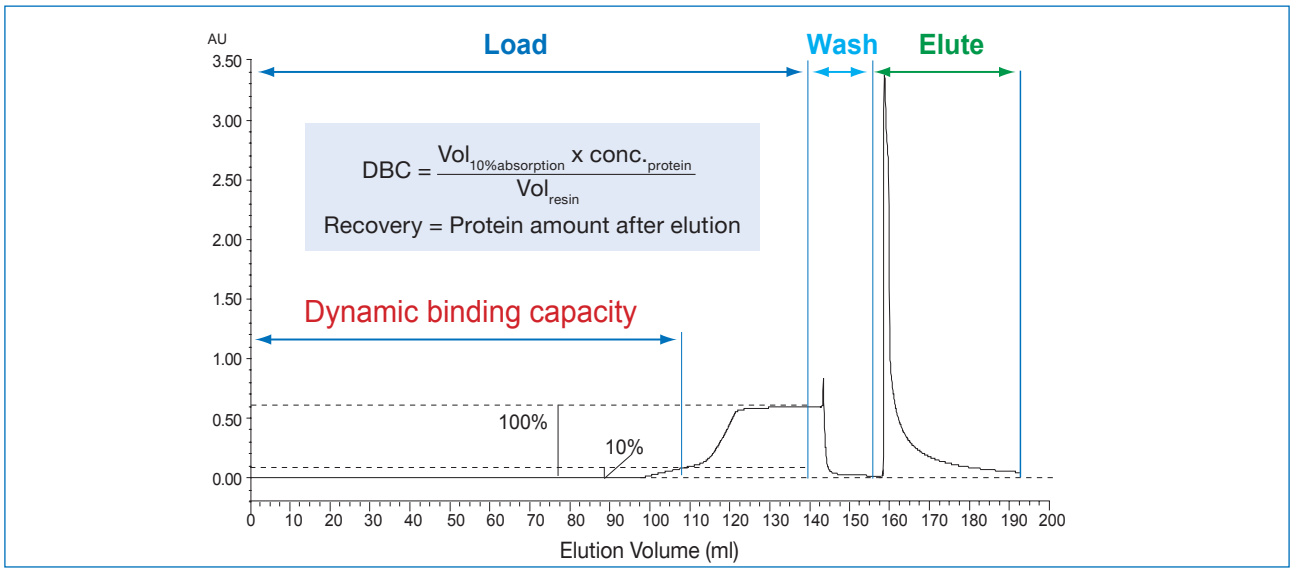
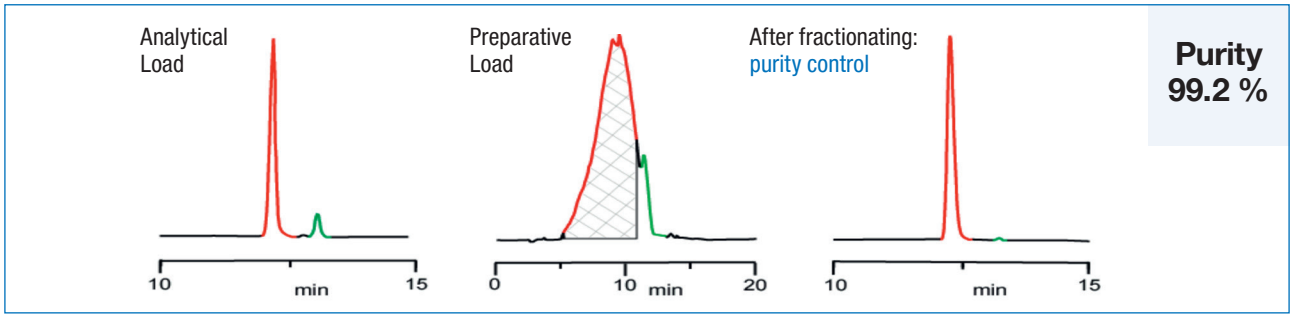


Figure 5: Procedure to determine the dynamic binding capacity with a breakthrough at 10%

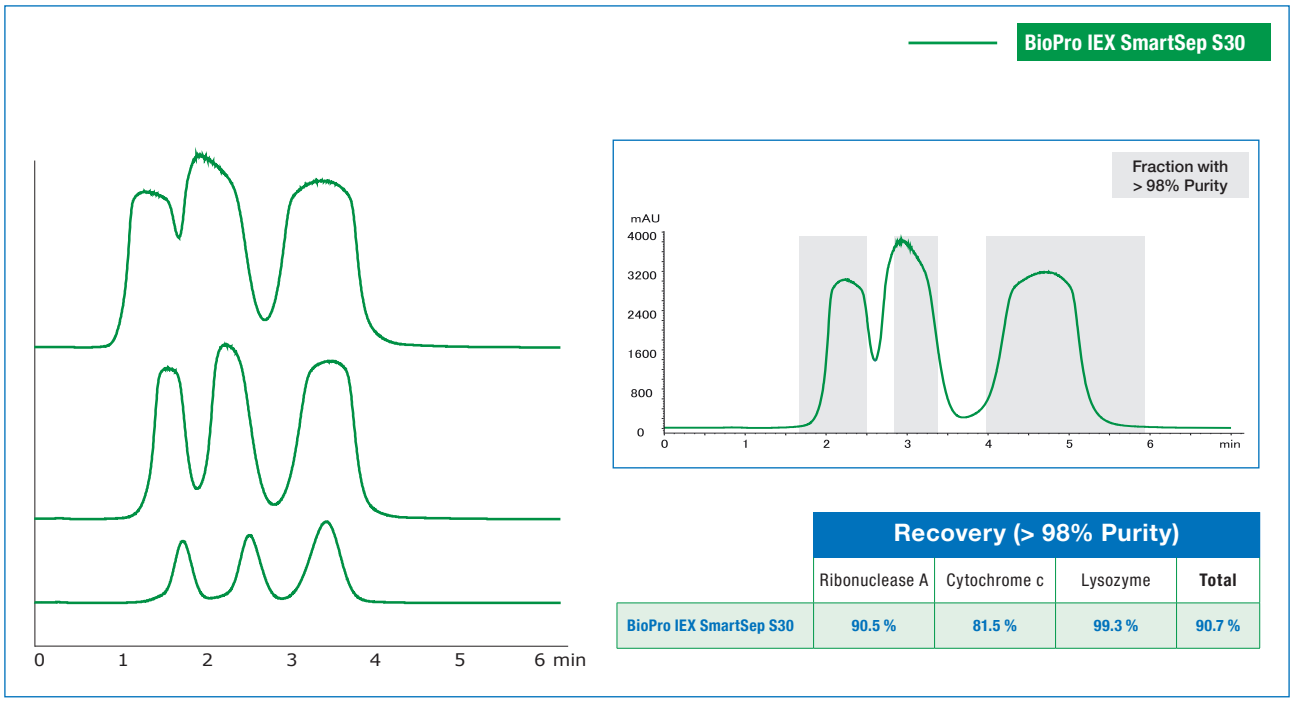
Optimised Loading and pooling criteria

The following example shows the chromatogram of an analytical loading with a good resolution. During the subsequent overloading studies the maximum overload had been determined. In order to control the purity of the collected fraction(s), purity checks have to be carried out by an analytical method. Based on the determined purity level, the fractionation can be optimised to increase the overall yield.



Practical example: increased protein loadings and the effect on the resolution, purity and recovery

This example shows the separation of three different target proteins. The elution profile at initial loading amounts (3 mg protein) shows three well-separated peaks. At higher loading (15 mg protein), the resolution decreases as a result of larger peak areas. With increased protein loading of up to 30 mg protein, the resolution especially between the peaks 1 and 2 further decreases. Nevertheless, the protein recovery of all three proteins is still at a high level (90.7% recovery at >98% purity).



	Recovery (> 98% Purity)			
	Ribonuclease A	Cytochrome c	Lysozyme	Total
BioPro IEX SmartSep S30	90.5 %	81.5 %	99.3 %	90.7 %

Figure 6: Separation of three target proteins at different loadings. With the highest loading of 30 mg loading, high recovery levels are achieved

The resolution of the eluted peaks also depends on the elution itself and the gradient that is applied. Therefore, it is important to improve the elution conditions and the resulting resolution during the gradient optimisation step. A higher resolution subsequently allows for higher loadings and therefore for hgher recovery.

Flow-Through Applications

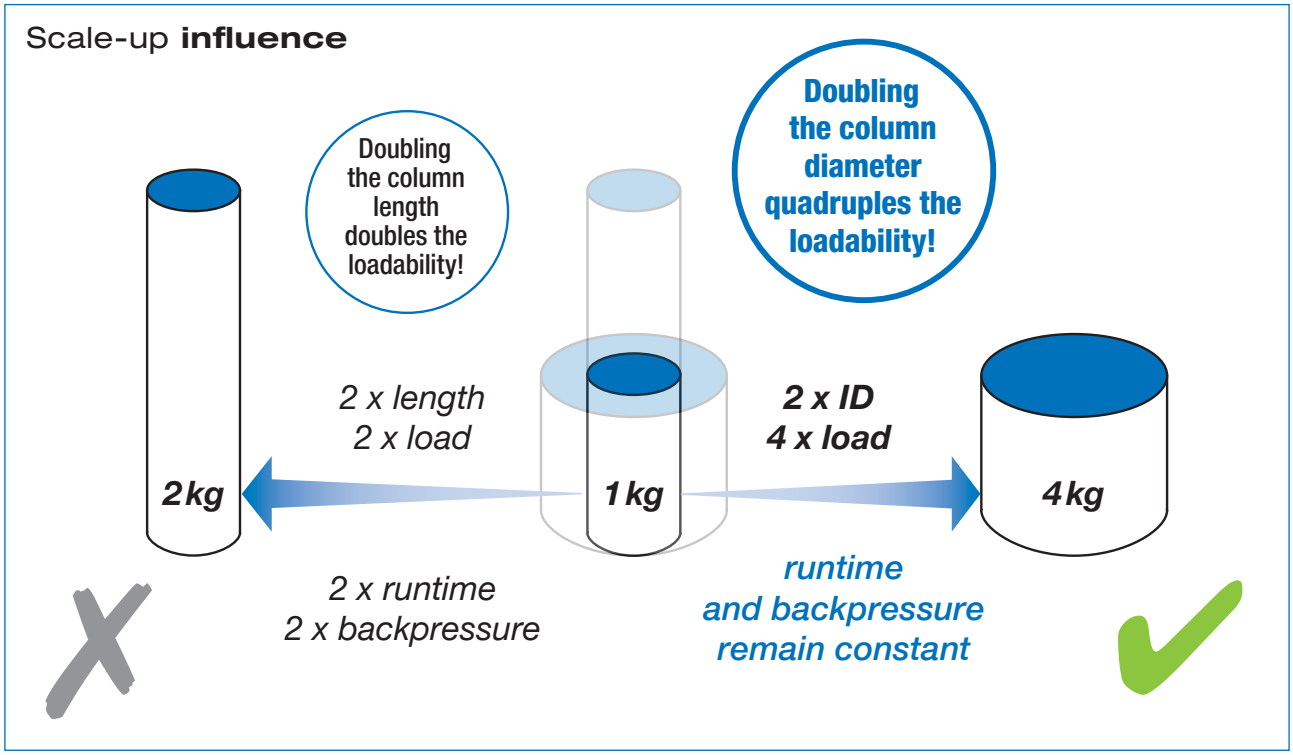
In the case of Flow-Through applications the resolution is not relevant as the target flows through. The impurities are bound the column and subsequently eluted by a single washing step. Therefore, the maximum loadability is determined by the maximum binding capacity of the resin for the relevant impurities. The higher the dynamic binding capacity of a resin, the higher is the productivity of the process because more feed solution can be processed per purification run.

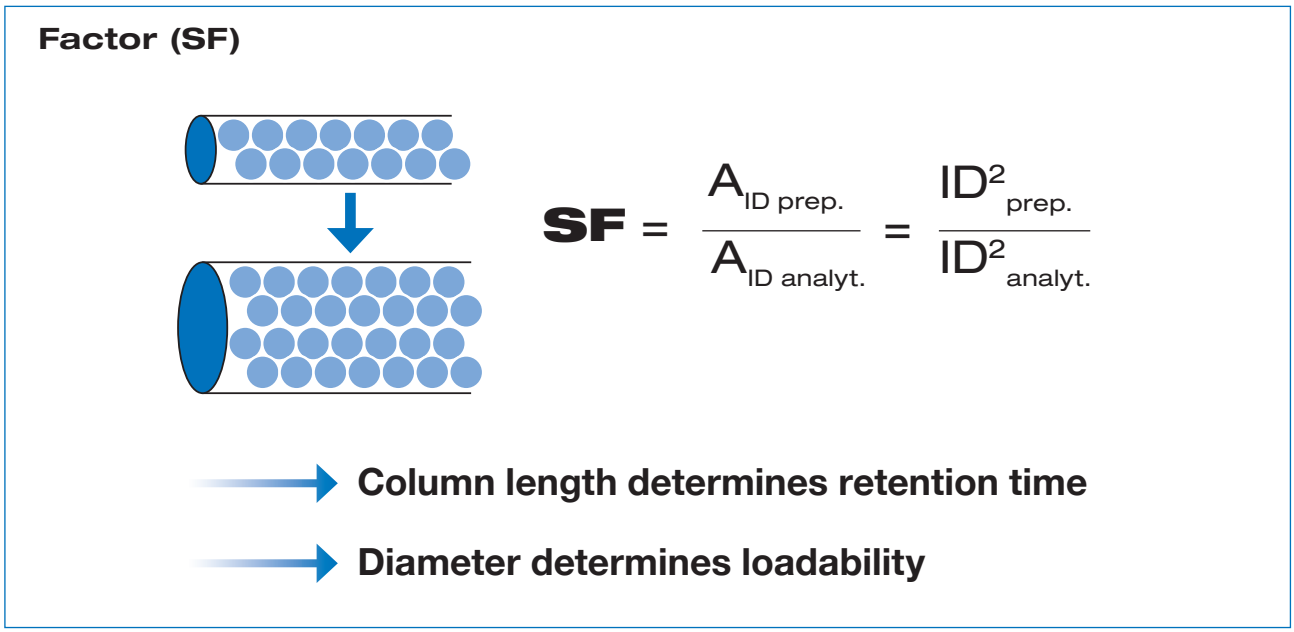
4. Scale up

After the method parameters have been defined, the process can be scaled-up. The linear scale-up method is a simple approach that allows the transfer of an existing method to a larger scale.

4.1. Linear Scale-Up

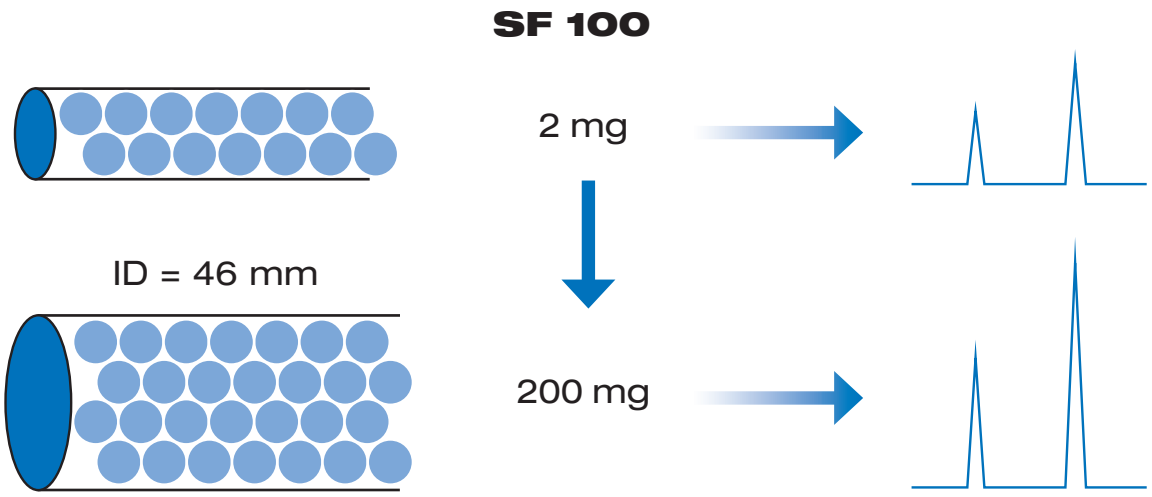
Within the linear scale-up process, column length is maintained, as is the particle size which was used during the method development and loading studies. The most efficient approach is to increase the column inner diameter to increase the capacity. By increasing the column diameter, the loadability increases as shown in the following diagrams.





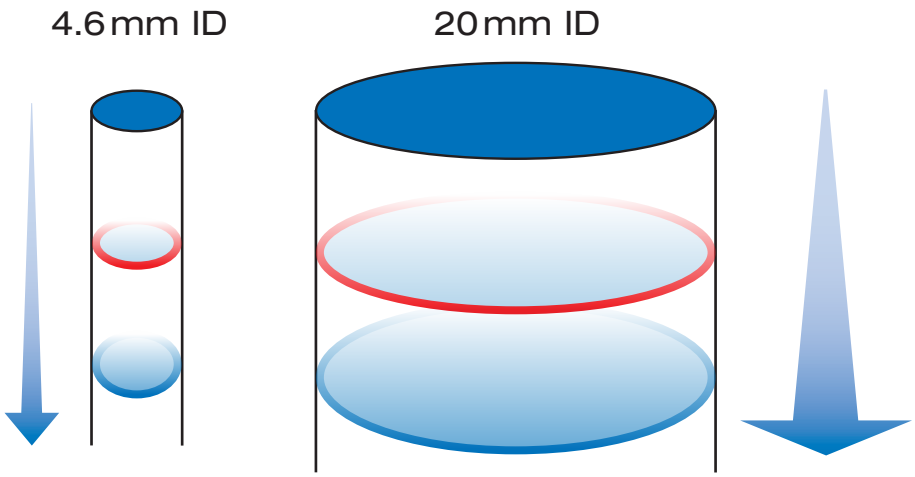
Linear Scale-up: **Loading**

$$\mathbf{SF} = \frac{ID^2_{prep.}}{ID^2_{analyt.}} = \frac{m_{prep.}}{m_{analyt.}} \rightarrow \mathbf{SF} = \frac{(46\ mm)^2}{(4.6\ mm)^2} = \frac{200\ mg}{2\ mg} = \mathbf{100}$$



Flow rate and retention time

$$\mathbf{SF} = \frac{ID^2_{prep.}}{ID^2_{analyt.}} \rightarrow SF = \frac{(20\,mm)^2}{(4.6\,mm)^2} = \mathbf{18.9}$$



Flow rate: 1 ml/min **18.9** ml/min

5. Conclusion

The key to successful and efficient purifications with ion exchange chromatography is the definition of the purification parameters and the selection of the most appropriate IEX resin.

The method development and optimisation of the different steps are performed at small scale and include the following steps:

1. **Downstream strategies for successful purification**
2. **Applicable separation modes**
3. **Selection of the resin type and particle size**
4. **Buffer composition and pH**
5. **Definition of the elution conditions**
6. **Gradient adaption and elution optimisation**
7. **Flow rate optimisation**
8. **Cleaning strategies**

The first step during process development is the selection of the separation mode followed by the **evaluation of appropriate resins**. This can be done within in a comprehensive resin screening. The particle size determines the resolution but also the backpressure.

The next step is the determination of the most appropriate **mobile phase conditions** including buffer, pH and initial salt concentration to ensure an optimal binding of the target to the resin. The elution in IEX can generally be performed by gradient or step elution of increasing salt concentration or changing pH. A combination of these methods is also possible.

An initial gradient as starting point is helpful to optimise the elution parameters. The flow rate should be adjusted for an optimal binding as well as elution and is in general a powerful tool to improve the efficiency of a purification process. Regularly applied Cleaning-in-Place (CIP) procedures restore the column efficiency. Resins with high stability towards alkaline CIP procedures improve the cost efficiency of IEX processes.

After defining the method parameters of the IEX purification, **loadability studies** are performed to improve the overall productivity. The **linear scale up procedure** is an easy and effective tool for the transfer of the process to larger scales.

More about YMC – Ion Exchange Resins from YMC

BioPro IEX SmartSep for intermediate purification and polishing

BioPro IEX Series	BioPro IEX Q75	BioPro IEX SmartSep Q30	BioPro IEX SmartSep Q20	BioPro IEX SmartSep Q10	BioPro IEX S75	BioPro IEX SmartSep S30	BioPro IEX SmartSep S20	BioPro IEX SmartSep S10
Ion exchange type	Strong anion exchanger				Strong cation exchanger			
Charged group	-R-N$^+$(CH$_3$)$_3$				-R-SO$_3^-$			
Matrix	Hydrophilic polymer beads							
Pore size	Porous							
pH Range	2–12							
Particle size	75 µm	30 µm	20 µm	10 µm	75 µm	30 µm	20 µm	10 µm
Pressure resistance	0.3 MPa	2 MPa Max. 3 MPa		3 MPa Max. 4 MPa	0.3 MPa	2 MPa Max. 3 MPa		3 MPa Max. 4 MPa
Typical flow rate	200–1000 cm/hr Max. 2000 cm/hr							
Ion-exchange capacity	0.10 meq/mL Resin	0.08 meq/mL Resin			0.10 meq/mL Resin	0.08 meq/mL Resin		
Dynamic binding capacity	Min. 160 mg/mL Resin (BSA)	Min. 100 mg/mL Resin (BSA)			Min. 160 mg/mL Resin (BSA)	Min. 100 mg/mL Resin (BSA)		

Ordering Information

Strong anion exchanger: BioPro IEX Q

Product	Particle Size	Code	Pack Sizes*					
			50 ml	250 ml	1 L	5 L	10 L	20 L
BioPro IEX SmartSep Q10	10 µm	QSA0S10	✔	✔	✔	✔	✔	✔
BioPro IEX SmartSep Q20	20 µm	QSA0S20	✔	✔	✔	✔	✔	✔
BioPro IEX SmartSep Q30	30 µm	QSA0S30	✔	✔	✔	✔	✔	✔
BioPro IEX Q75	75 µm	QAA0S75	✔	✔	✔	✔	✔	✔

* Larger or customised pack sizes are available on request.

Strong cation exchanger: BioPro IEX S

Product	Particle Size	Code	Pack Sizes*					
			50 ml	250 ml	1 L	5 L	10 L	20 L
BioPro IEX SmartSep S10	10 µm	SSA0S10	✔	✔	✔	✔	✔	✔
BioPro IEX SmartSep S20	20 µm	SSA0S20	✔	✔	✔	✔	✔	✔
BioPro IEX SmartSep S30	30 µm	SSA0S30	✔	✔	✔	✔	✔	✔
BioPro IEX S75	75 µm	SPA0S75	✔	✔	✔	✔	✔	✔

* Larger or customised pack sizes are available on request.

Regulatory support file available under non-disclosure agreement. Used in validated cGMP-manufacturing processes. Customised material available on request. DMF registered with FDA.

Your local contact:

YMC CO., LTD.
www.ymc.co.jp

YMC Europe GmbH
www.ymc.eu

YMC America, Inc.
www.ymcamerica.com

YMC Schweiz GmbH
www.ymc-schweiz.ch

YMC India Pvt. Ltd.
www.ymcindia.com

YMC Shanghai Rep. Office
www.ymcchina.com

YMC Korea Co., Ltd.
www.ymckorea.com

YMC Taiwan Co., Ltd.
www.ymctaiwan.com

YMC Singapore Tradelinks Pte. Ltd.
www.ymc.sg

Whitepaper PPA|IEX_03/2025_Int