Hydrophobic Interaction Chromatography: Effects of Mixed Electrolytes on Protein Separations

TOYOPEARL
APPLICATION NOTE

Introduction

While the productivity of ion exchange chromatography (IEX) and other chromatographic modes have been improved due to advanced surface modifications of newly developed resins (such as the TOYOPEARL GigaCap® series of resins), hydrophobic interaction chromatography (HIC) has undergone comparably fewer performance improvements to the resin itself.

For HIC separations, parameters other than resin surface modifications can be employed to enhance performance. While the capacities and recoveries of HIC applications cannot compete with those of modern IEX resins, its performance can be improved by adjusting the operating parameters. The general method complexity of HIC allows for various strategies to be used when approaching this goal. For many applications, the selectivity of HIC can be striking.

This application note addresses the electrolyte composition of the mobile phase as one parameter responsible for protein adsorption and desorption. The results presented illustrate the benefits regarding capacity and selectivity in HIC of often neglected salts and their mixtures.

The salt most recognized for use in HIC separations is ammonium sulfate ($(NH_4)_2SO_4$). Its salting-out (kosmotropic) potential is well-known and it is also used in non-chromatography based purification methods such as protein precipitation.

In addition to sodium citrate and sodium sulfate, which also show great salting-out potential for many proteins, salts like sodium acetate and sodium chloride are common mobile phase salts used in manufacturing scale purifications. The latter two compounds are not typically used with HIC as their kosmotropic properties are relatively weak.

Dynamic Binding Capacity

The impact of the electrolyte composition of the mobile phase in HIC on the dynamic binding capacity (DBC) for lysozyme and a monoclonal antibody (mAb) was investigated. The solubility-limiting concentration of ammonium sulfate was optically determined to be 2.2 mol/ L for lysozyme and 1.1 mol/ L for the mAb. Hence, concentrations of 2.0 mol/L and 1.0 mol/L ammonium sulfate were chosen as a baseline value for the capacity comparison. *Tables 1a & 1b* list model dynamic binding capacities that could be achieved using ammonium sulfate and mixtures of ammonium sulfate, sodium citrate, sodium sulfate, sodium acetate and sodium chloride for protein adsorption onto TOYOPEARL® PPG-600M, TOYOPEARL Phenyl-600M and TOYOPEARL Butyl-600M.

Capacities were measured at 10% breakthrough, pH 7.0, and a protein concentration of 1.0 g/L. The feed stream was set to 150 cm/h. The corresponding resins were packed to a total column volume of 1 mL with an inner diameter of 0.66 cm.

The dynamic binding capacities for the two presented proteins can be increased for certain salt mixtures and resins. Improvements of up to roughly 50% are possible for some combinations.

Table 1a. Lysozyme dynamic binding capacity

Salt	TOYOPEARL PPG-600M Capacity (g/L)	TOYOPEARL Phenyl-600M Capacity (g/L)	TOYOPEARL Butyl-600M Capacity (g/L)
2.0 mol/L $(NH_4)_2SO_4$	30	46	15
1.0 mol/L sodium sulfate + 1.0 mol/L sodium acetate	39	63	18
1.0 mol/L $(NH_4)_2SO_4$ + 1.0 mol/L NaCl	31	54	10
0.9 mol/L trisodium citrate + 0.9 mol/L NaCl	38	43	20

Lysozyme dynamic binding capacities measured at 10% breakthrough. Capacities were evaluated in various mobile phases with TOYOPEARL PPG-600M, TOYOPEARL Phenyl-600M, and TOYOPEARL Butyl-600M resins. The capacity measured in 2.0 mol/L ammonium sulfate serves as a point of reference for the other samples.

Table 1b. mAb dynamic binding capacity

Salt	TOYOPEARL PPG-600M Capacity (g/L)	TOYOPEARL Phenyl-600M Capacity (g/L)	TOYOPEARL Butyl-600M Capacity (g/L)
1.0 mol/L $(NH_4)_2SO_4$	18	20	18
1.7 mol/L NaCl + 0.7 mol/L $(NH_4)_2SO_4$	30	25	15
1.3 mol/L NaCl + 0.6 mol/L $(NH_4)_2SO_4$	25	20	12

Monoclonal antibody dynamic binding capacities measured at 10% breakthrough. Capacities were evaluated in various mobile phases with TOYOPEARL PPG-600M, TOYOPEARL Phenyl-600M, and TOYOPEARL Butyl-600M resins. The capacity measured in 2.0 mol/L ammonium sulfate serves as a point of reference for the other samples.

Selectivity

The point of elution of a certain protein is not solely dependent on the mobile phase salt molarity. Factors like pH and temperature are known to influence protein desorption. Furthermore, the standard protein separations presented in *figures 1 - 3* illustrate that the protein elution does not correlate linearly with the decreasing salt concentration in the mobile phase for ammonium sulfate and the model salt mixtures.

The different figures each refer to one commonly used TOYOPEARL HIC ligand: PPG (polypropylene glycol), phenyl, and butyl. For selectivity testing, a mixture of cytochrome C, ribonuclease A, and lysozyme were separated. 1.0 mL columns with an inner diameter of 0.66 cm were used. 10 g/L of each protein were loaded. A constant flow of 150 cm/h was applied.

Figure 1 illustrates the use of TOYOPEARL PPG-600M with three different mobile phases. Lysozyme, which is represented by peak 3, is retained the longest and ribonuclease A (peak 2) elutes right after the gradient starts for the mixture of sodium sulfate and sodium chloride. When using ammonium sulfate and sodium acetate, the cytochrome C and ribonuclease A are not resolved and the lysozyme peak elutes midway through the gradient. Using only 1.8 mol/L ammonium sulfate, the order of elution remains the same, but the resolution and selectivity are noticeably different.

A mobile phase using a mixture of sodium sulfate and sodium chloride provides increased resolution for the proteins compared to ammonium sulfate. The same behavior is observed for TOYOPEARL Phenyl-600M *(Figure 2)* and TOYOPEARL Butyl-600M *(Figure 3)*.

Figure 1. Separation of cytochrome C, ribonuclease A, and lysozyme on TOYOPEARL PPG-600M

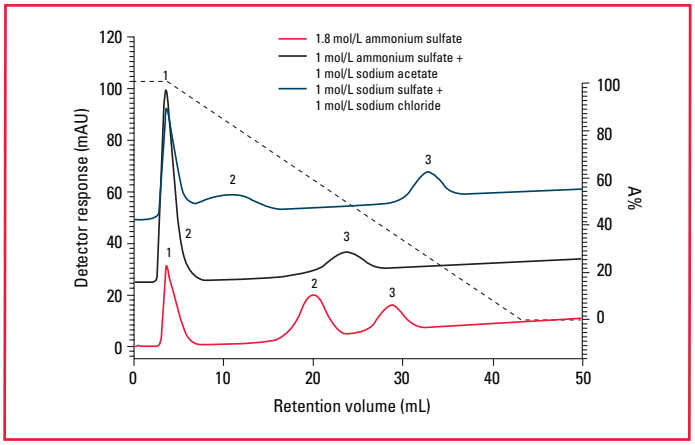


Figure 2. Separation of cytochrome C, ribonuclease A, and lysozyme on TOYOPEARL Phenyl-600M

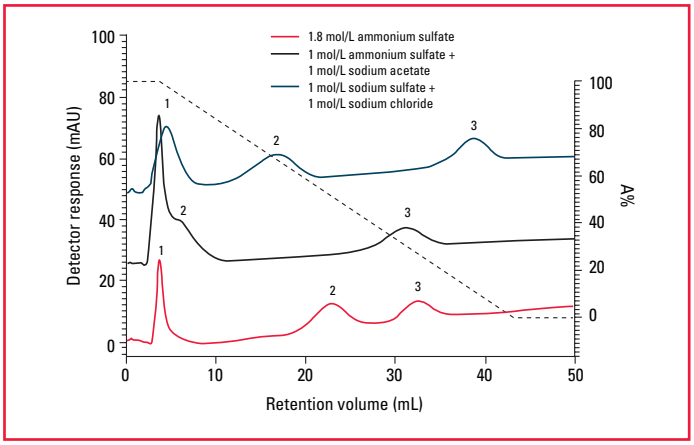
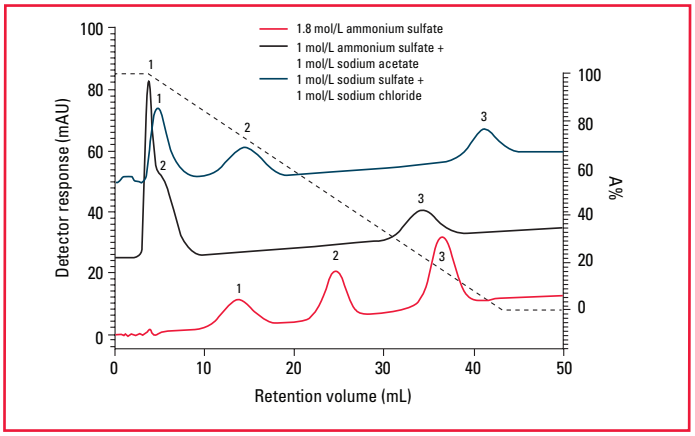


Figure 3. Separation of cytochrome C, ribonuclease A, and lysozyme on TOYOPEARL Butyl-600M



TOYOPEARL Butyl-600M, the most hydrophobic resin among the resins tested, allows binding of cytochrome C for some salts or salt mixtures. Only the mixture of ammonium sulfate and sodium acetate does not promote cytochrome C binding, while lysozyme is almost as strongly retained when compared to ammonium sulfate as a single salt.

Ribonuclease A is only weakly bound to the resin using the mixture of ammonium sulfate and sodium acetate. If it is assumed that lysozyme represents the target molecule, the almost exclusive binding of lysozyme would allow greater adsorption for the target molecule when compared to the other two liquid phase compositions where resin capacity is shared by the other sample components.

Conclusions

Every target protein and its related impurities require distinct salts or salt mixtures to achieve the highest resolution, purity and capacity when performing a chromatographic separation using HIC. On the one hand, this increases the set of variables during process development, but it also allows selectivity modulation to better isolate the target of interest. Capacities can also benefit from salt mixtures. In addition to a more refined binding of the target of interest, general capacities of up to 150% of the ammonium sulfate capacity can be reached. Using mixed electrolytes in HIC introduces an additional opportunity to improve HIC separations as an alternative to traditional HIC applications using ammonium sulfate.

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