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Nota applicativa

Method Development for Hydrophobic Interaction Chromatography (HIC) Based Protein Separations on Waters Protein-Pak Hi Res HIC Columns

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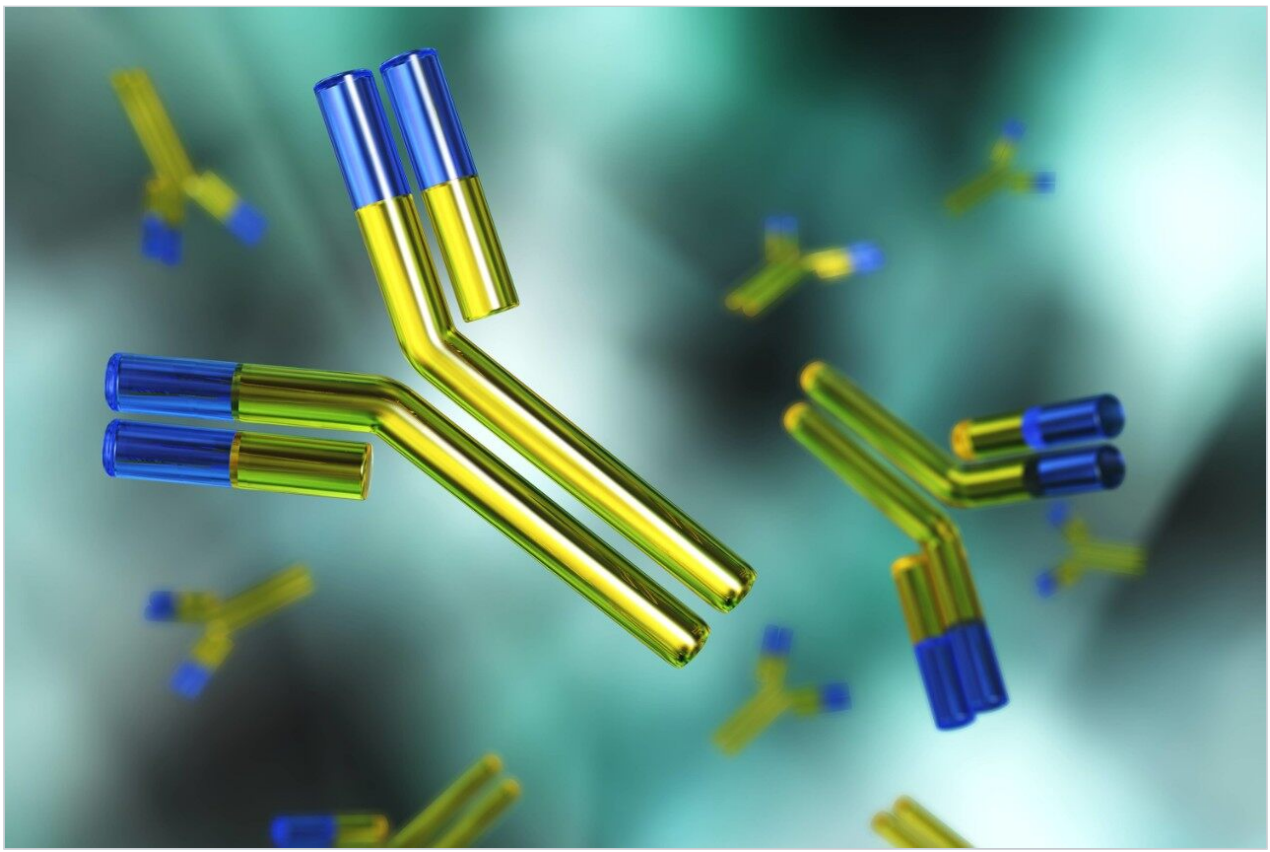


Abstract

This application note describes Hydrophobic Interaction Chromatography (HIC) method development, ideal for protein and conjugated protein characterization using non-denaturing, hydrophobic-based separations.

Benefits

- General guidance of HIC method development
- Ideally suited for protein characterization using non-denaturing, hydrophobic-based separations
- Use of non-porous, 2.5 µm particles deliver fast, highly efficient separations to address high-throughput needs
- HIC Protein Standard used in study is included with Protein-Pak Hi Res HIC Column to help ensure user success

Introduction

Hydrophobic interaction chromatography (HIC) is a technique for separation of proteins, peptides, and other biomolecules based on their relative degree of hydrophobicity. However, unlike reversed-phase chromatography, HIC is a non-denaturing technique. Therefore, the native form of the proteins is expected to be maintained, which is beneficial if one wants to further study the biological characteristics of the separated proteins.

In a HIC separation, the hydrophobic ligands on the stationary phase interact with the hydrophobic regions on the surface of the protein and the retention mechanism is due to adsorption – desorption equilibrium in the presence of salts. In practice, proteins bind to the HIC stationary phase in the presence of high concentration of salt, and are eluted in the order of increasing hydrophobicity by decreasing the salt concentration.

HIC has been used increasingly in protein and conjugated protein (e.g., antibody drug conjugates [ADCs]) separations requiring systematic method development to obtain the optimal separation conditions for these non-denaturing separations. This application note will guide users through the “Tips and Tricks” of HIC method development.

Experimental

Sample description

HIC Protein Standard Test Mix (p/n 186007953): Bovine Ribonuclease A (0.05 mg/vial), Horse Cytochrome c (0.025 mg/vial), Horse Myoglobin (0.05 mg/vial), Chicken Lysozyme (0.03 mg/vial), Yeast Enolase (0.10 mg/vial), Alpha chymotrypsinogen A (0.05 mg/vial)

LC conditions (unless otherwise noted)

LC system:	ACQUITY UPLC H-Class Bio with TUV Detector
Sample temp.:	4 °C
Analytical column temp.:	30 °C
Flow rate:	0.6 mL/min
Injection volume:	2 µl
Column:	Protein-Pak Hi Res HIC, 2.5 µm, 4.6 mm x 100 mm and HIC Protein Standard (p/n 176003576)
Detection:	UV absorbance at 220 nm
Sample collection:	TruView Vial (p/n 186005668cv)
Mobile phase A:	2 M $(NH_4)_2SO_4$ in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.9
Mobile phase B:	50 mM NaH_2PO_4/Na_2HPO_4, pH 6.9
Data management:	Empower Pro (v2)

Gradient table

Time (mL/min)	Flow rate	%A	%B	Curve
0.0	0.6	100	0	
15.0	0.6	0	100	6
18.0	0.6	0	100	11
19.0	0.6	100	0	11
30.0	0.0	100	0	11

Results and Discussion

HIC separation is based on the interaction between the hydrophobic ligands on HIC media and the hydrophobic surfaces on proteins. In pure water or a low-ionic strength buffer, the hydrophobic interactions between ligands and proteins should ideally be weak enough to allow the protein to elute from the column without the use of an organic modifier in the elution buffer. However, certain salts enhance these desired hydrophobic interactions, and adding such salts brings about binding (adsorption) to HIC media. For selective elution (desorption), the salt concentration is lowered gradually via gradient elution and the sample components elute in the order of hydrophobicity with less eluting prior to more hydrophobic components.

Figure 1 shows the chromatogram of the six proteins in the HIC Protein Standard Test Mix (p/n 186007953) using the conditions specified in the Experimental section.

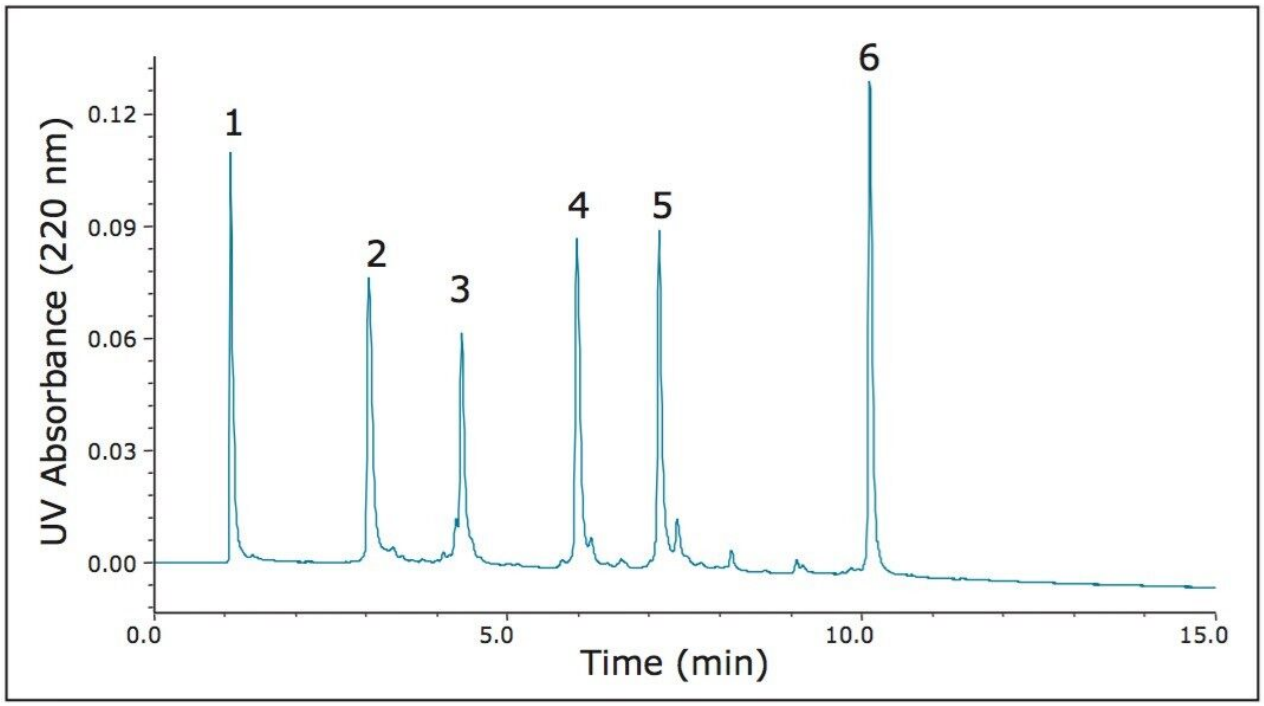
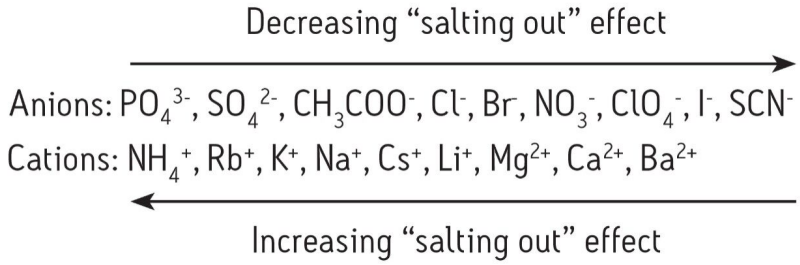


Figure 1. Using the conditions specified in the Experimental section, six proteins in the HIC Protein Standard Test Mix are well separated by the Protein-Pak Hi Res HIC Column. The proteins are: 1) cytochrome c, 2) myoglobin, 3) ribonuclease A, 4) lysozyme, 5) enolase, 6) alpha-chymotrypsinogen A.

Effect of salt type and concentration

The type and concentration of salt used to dissolve the sample, as well as that used in the HIC mobile phases, will influence the separation. Changing the type of salt and the starting salt concentrationcan change the selectivity of the obtained separation.

Some ions are more kosmotropic and therefore are more effective in “salting out” protein and driving hydrophobic interactions compared to use of other salts. The strength of HIC binding follows the order of Hofmeister “salting-out” series for protein precipitation.



While increasing salt concentration increases protein binding capacity via the “salting out” mechanism, too high a salt concentration may result in protein precipitation.

Figure 2 shows a separation of the HIC Protein Standard Test Mix (p/n 186007953) using various NaCl gradient separations. Note that NaCl is not very effective for use in HIC since not all proteins bind to the HIC column even at 3.5 M concentration. By comparison, and as seen in Figure 3, Na_2SO_4 is more effective than use of NaCl for the HIC separation of proteins in this mix. Furthermore, use of $(NH_4)_2SO_4$ (Figure 4) is even more effective in separating the protein mix.

For some strongly hydrophobic proteins, non-polar solvents such as acetonitrile may be required for elution (reference 4).

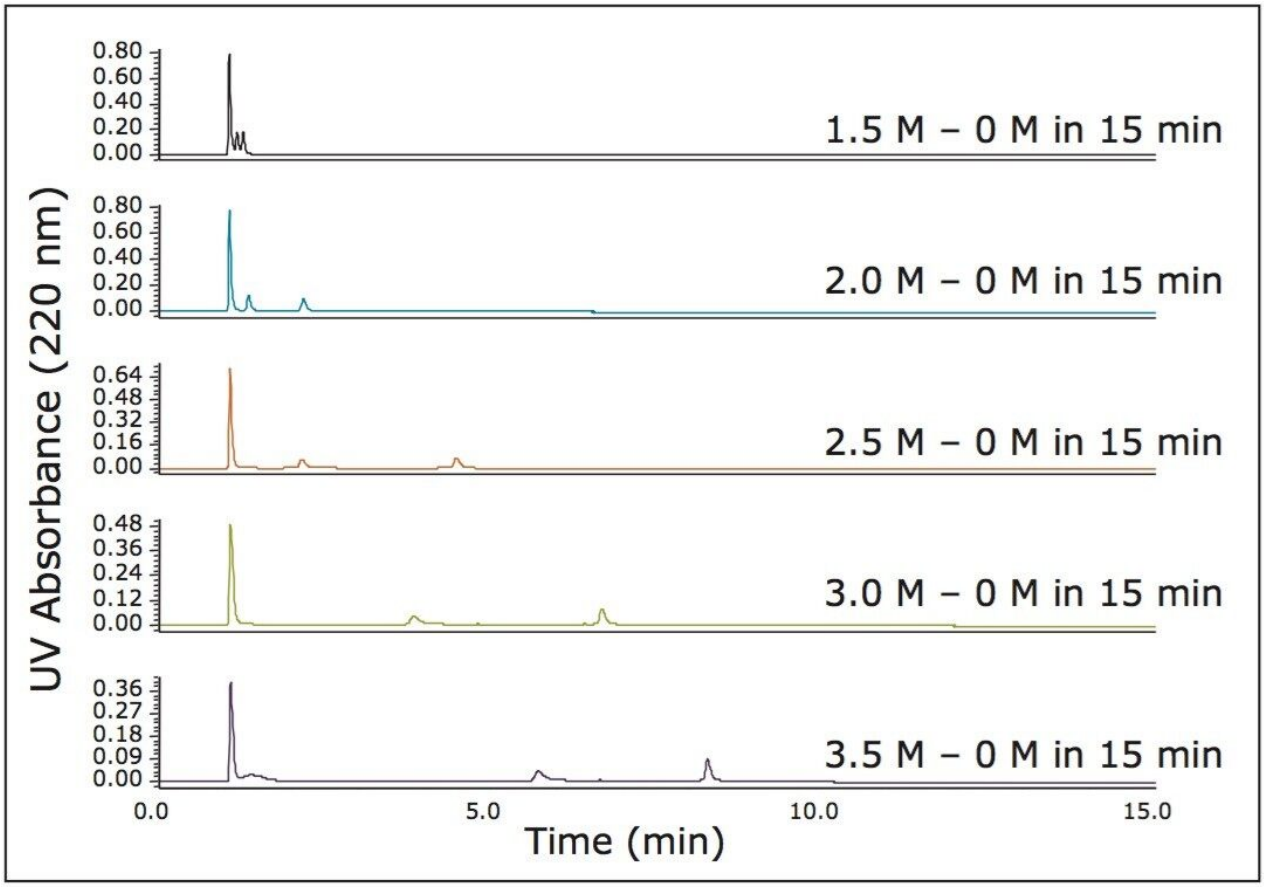


Figure 2. A separation of the HIC Protein Standard Test Mix using NaCl as the salt in the mobile phase. Note that NaCl is not very effective for use in HIC since even at 3.5 M concentration, not all proteins bind to the HIC column and many come through the column unretained.

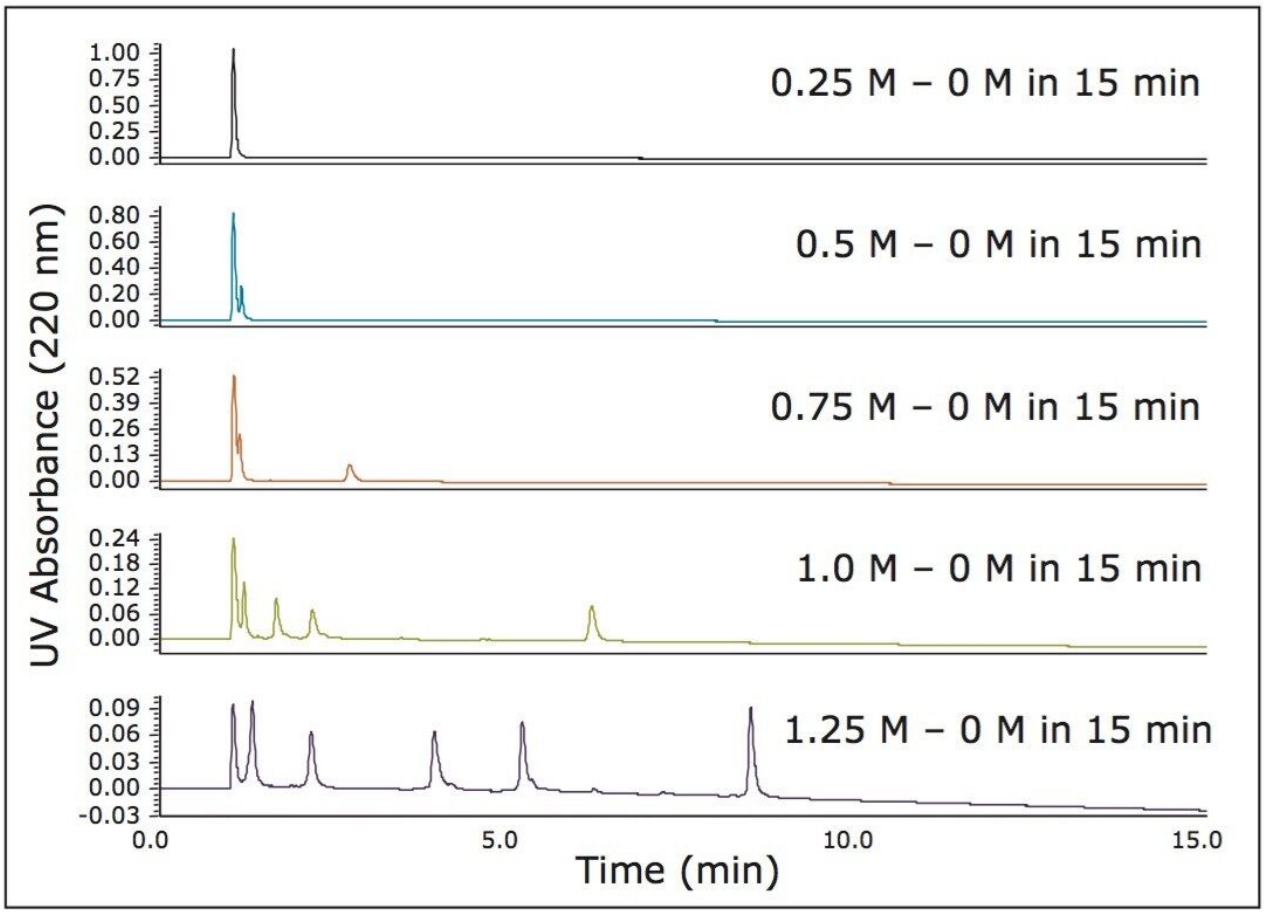


Figure 3. Na_2SO_4 is more effective in separating the proteins than NaCl (see Figure 2).

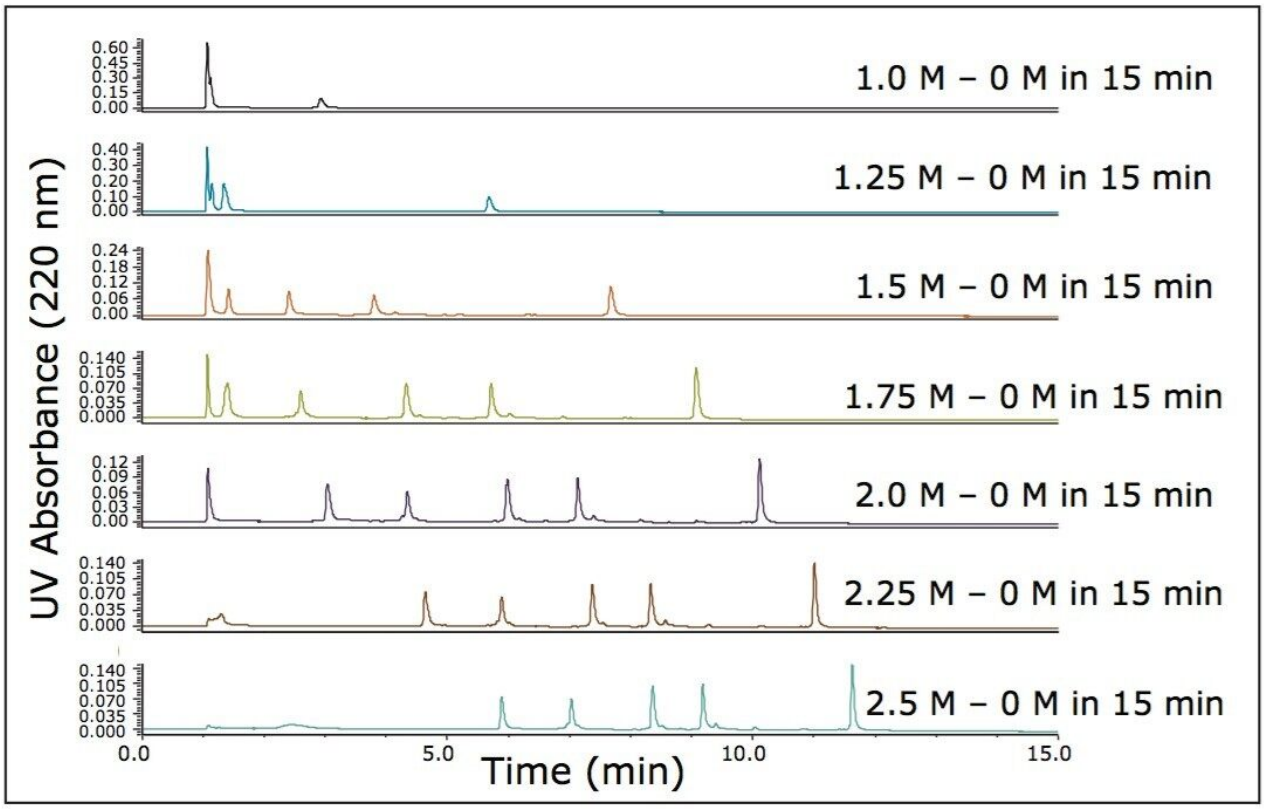


Figure 4. Using $(NH_4)_2SO_4$ as the salt in the mobile phase, proteins are effectively separated

Effect of pH

The effect that pH has on HIC-based protein separations is not straightforward nor is it predictable, yet this variable certainly can affect component selectivity.[1]

Figure 5 shows the results of a single variable experiment where only pH was varied. It is clear from these data that a greater degree of separation between peaks 1 and 2 and between peaks 4 and 5 is at pH 8. However, a greater degree of separation between peaks 2 and 3 and peaks 5 and 6 is seen at pH 6.0.

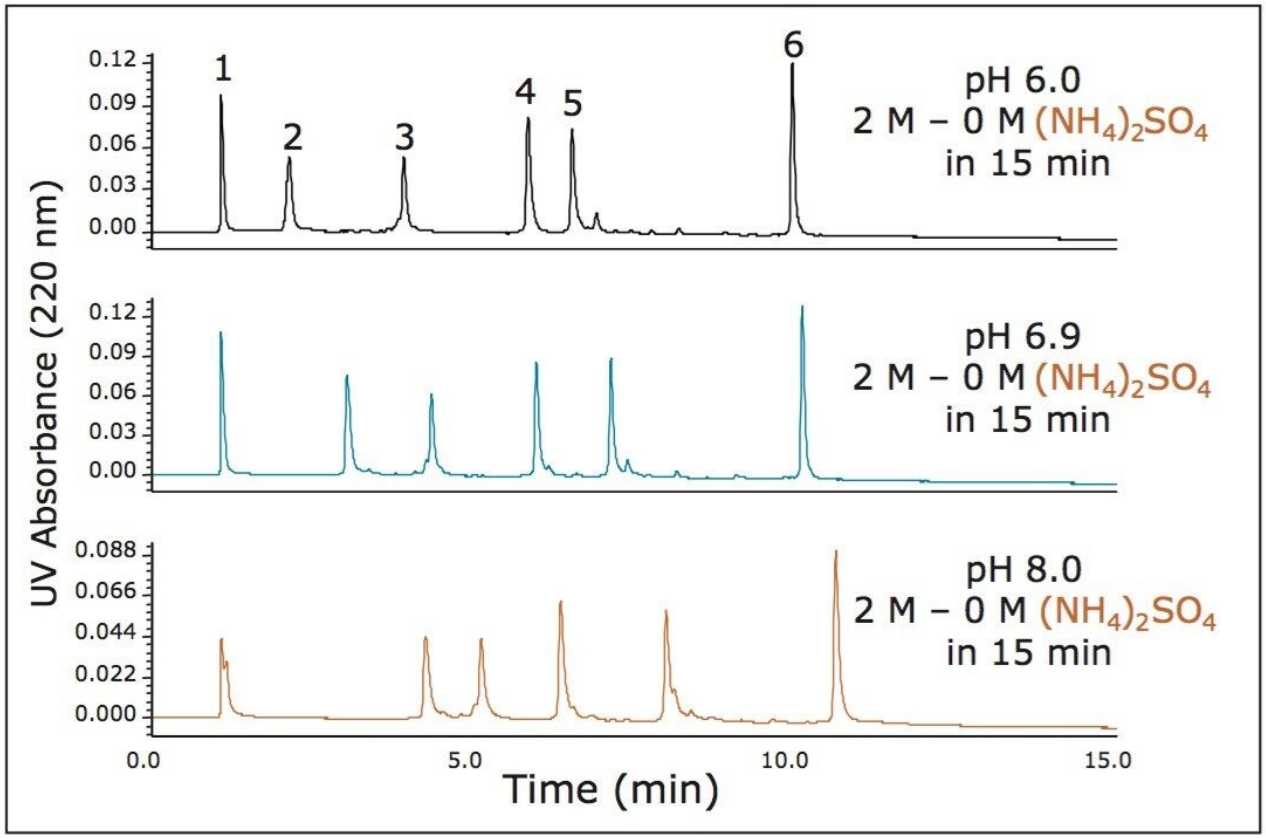


Figure 5. Effect of pH on a protein mix separation using a Protein-Pak Hi Res HIC Column.

Effect of temperature

In general, an increase in temperature increases hydrophobic interaction, as seen in Figure 6 between proteins and HIC Butyl ligand. This can have a significant impact as noted in the overall retention and subtle separation differences observed between 30 °C and 40 °C. It is therefore important to use a column temperature control for HIC separations in order to obtain reproducible results. As suggested by the 50 °C data, higher temperatures may result in a poor chromatographic recovery and peak shape for some proteins.

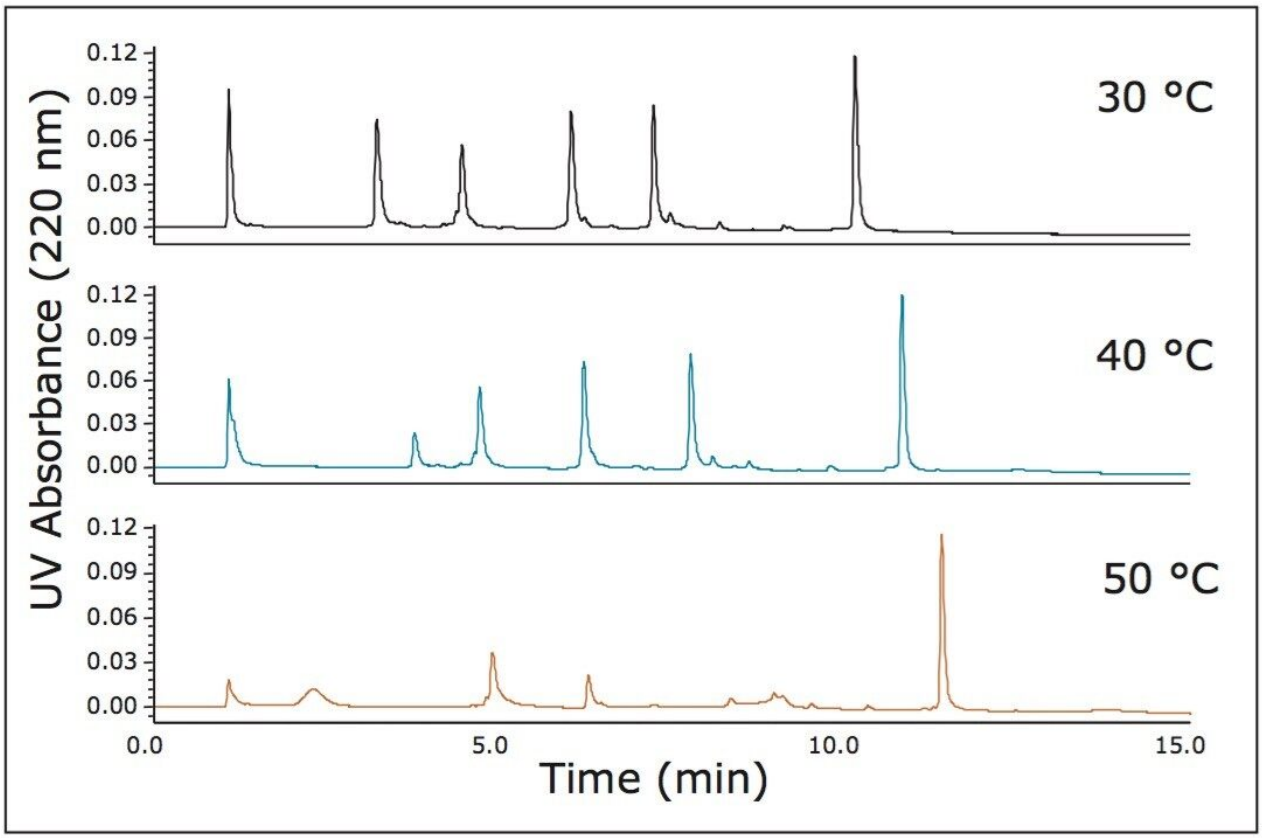


Figure 6. Effect of temperature on a protein mix separation using a Protein-Pak Hi Res HIC Column. All separations performed at pH 6.9 using a 2 M–0 M $(NH_4)_2SO_4$ gradient in 15 minutes.

Conclusion

In summary, in developing a HIC-based separation of a protein mixture, the following suggestions should be considered:

- The HIC medium should bind the protein of interest at a reasonably low concentration of salt. This is often dependent on the type of salt chosen. For example, a higher concentration of NaCl might be necessary to obtain a binding effect comparable to that obtained with ammonium or sodium sulfate.
- Ideally, the sample should be dissolved in the same buffer formulation used to equilibrate and load the HIC column. The loading buffer ideally should not cause precipitation of any of the proteins contained in the sample.

- 1.0 M ammonium sulfate, pH 7 is a good starting point for screening experiments.
- The bound protein should be eluted from the column with salt-free or low-salt buffer and with high recovery. For some very hydrophobic proteins, non-polar solvents may be needed for protein elution from HIC columns.
- pH and the type of salt used in the mobile phases can be utilized to maximize selectivity differences and ultimately the resolution of the proteins to be separated.
- Since hydrophobic interaction is dependent on temperature, it is important that method development work is performed at the intended final working column temperature and that the column temperature be controlled during analysis.

References

1. Ghose, S.; Tao, Y.; Conley, L.; Cecchini, D., Purification of monoclonal antibodies by hydrophobic interaction chromatography under no-salt conditions. *mAbs* 2013, 5 (5), 795–800.
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3. http://www.technologyinscience.blogspot.com/2014/04/ hydrophobic-interaction-chromatography.html#.VEUQyvnF98E
4. Yoshio, K. et al., Operational variables in high performance hydrophobic interaction chromatography of proteins on TSK gel phenyl-5PW. *J Chromatogr A* 1984, 298, 407–418.

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