# About the resolving power of non-porous HIC columns for intact protein analysis

Hydrophobic interaction chromatography (HIC) regained attention due to its non-denaturing conditions making it a first choice for protein analysis. HIC usually employs a weak hydrophobic stationary phase, such as YMC's BioPro HIC phases. The short chained modified resin interacts with hydrophobic moieties of the protein. The interaction between resin and protein can be facilitated with a high initial salt concentration.

An inverse salt gradient ensures that the hydrophobic interactions decrease with time, resulting in the elution of the protein. This explains the alternative name of salting-out chromatography.

In the work by Ewonde et al. the separation performance of HIC columns for intact protein analysis was investigated. Since the influencing parameters have not been addressed in depth for HIC mode yet, they were the focus in this study:

1. **Influence of salt concentration**
2. **Temperature impact on retention factor**
3. **Flow rate and gradient time effect on separation performance**
4. **Column length and particle diameter**

YMC's BioPro HIC BF and BioPro HIC HT were used in order to determine the effect on intact protein retention. The proteins used for evaluation were cytochrome c, myoglobin, ribonuclease A, lysozyme, carbonic anhydrase, trypsinogen and α-chymotrypsinogen A in varying combinations.

*Reference:*
*R. E. Ewonde, J. De Vos, K. Broeckhoven, D. Eßer, S. Eeltink, "Assessment of the resolving power of hydrophobic interaction chromatography for intact protein analysis on non-porous butyl polymethacrylate phases", J. Chrom. A., 2021, 1651, 462310.*

## 1. Influence of salt concentration

At high salt concentration, retention follows the hydrophobicity index (carbonic anhydrase > lysozyme > ribonuclease A). For the chosen proteins, the retention factor increased with increasing salt concentration (Figure 1). Higher salt concentrations ensure that the shielding water layers around the protein are disrupted, resulting in more accessible hydrophobic moieties and consequently in a stronger retention. By reducing the salt concentration, these water layers are restored, resulting in decreased hydrophobic interactions.

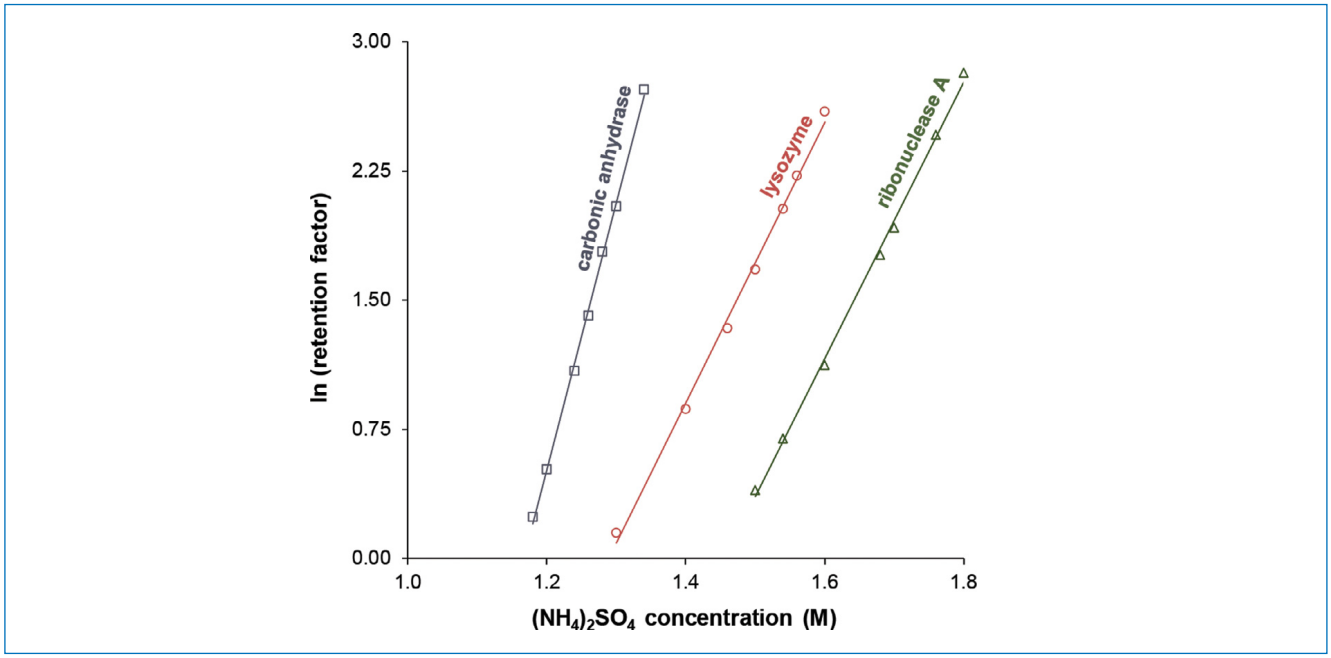


*Figure 1: The increase in retention factor with increasing ammonium sulphate concentration for carbonic anhydrase (□), lysozyme (○) and ribonuclease A (△).*

## 2. Temperature effect

Of course, changes in temperature influence any equilibrium state – that counts also for the retention of intact proteins in HIC mode. In order to quantify the impact, different temperatures were used for the separation of a mixture of 5 proteins using an inverse salt gradient for 30 min (Figure 2). For all proteins, an increase in temperature resulted in a slight increase in retention time due to a decrease in Gibbs' free energy.

In addition, the peak area for myoglobin and carbonic anhydrase was significantly reduced. A broad peak was observed at 40 °C and 50 °C (marked with arrow heads) which belongs to the unfolded myoglobin. This protein is known to be denatured at elevated temperatures, with more hydrophobic moieties accessible to interact with the stationary phase. It results in an increased retention factor and peak broadening.

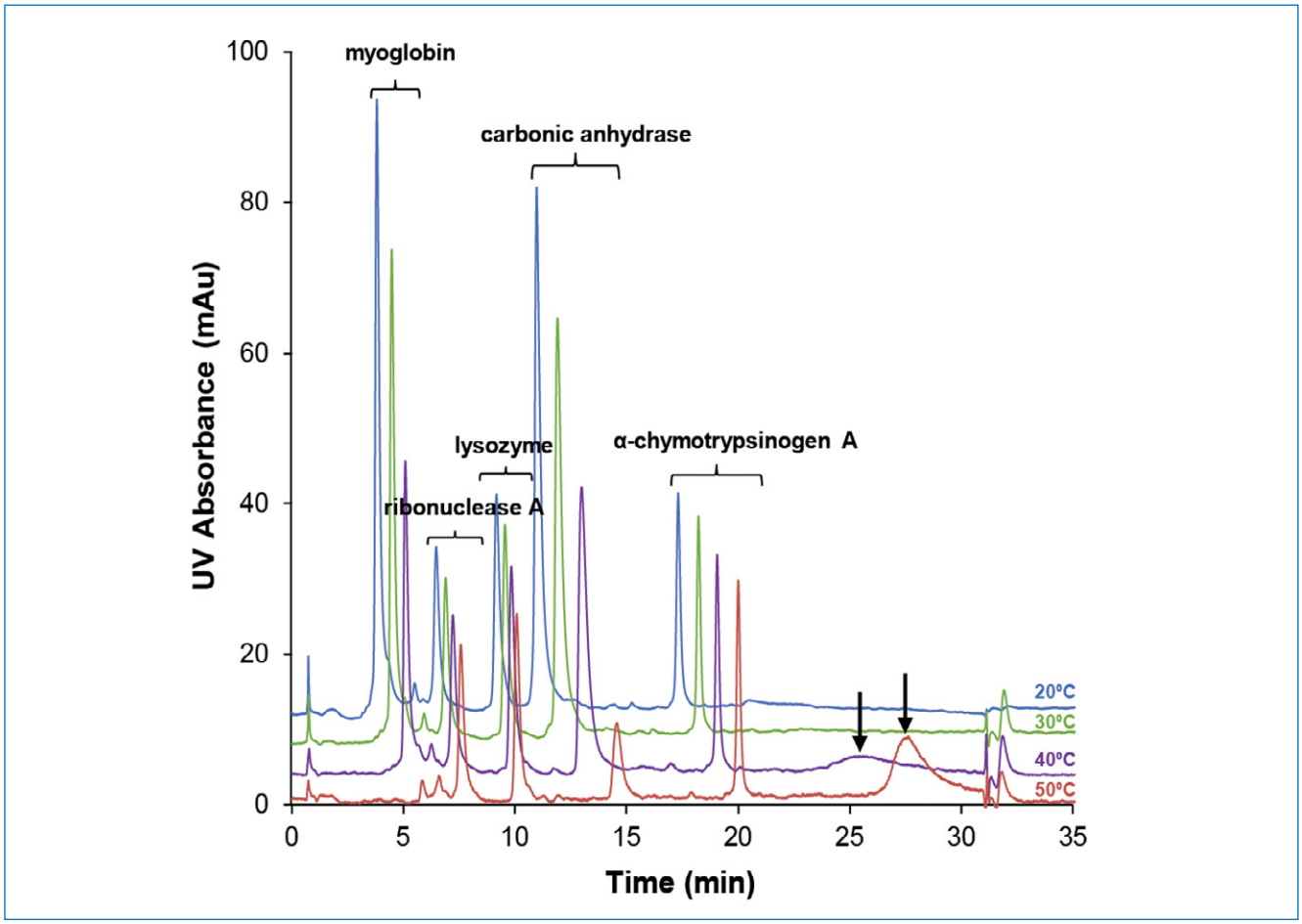


*Figure 2: Overlay of chromatograms of the 5 proteins myoglobin, ribonuclease A, lysozyme, carbonic anhydrase and α-chymotrypsinogen A at 20 °C (blue), 30 °C (green), 40 °C (purple), 50 °C (red).*

The corresponding van't Hoff plots show linearity. Therefore, no abrupt change in protein confirmation can be assumed within the selected temperature range. Otherwise, there would be a non-linear increase in retention since more hydrophobic areas of the protein were accessible.

**Important finding:**
**Except for myoglobin, the van't Hoff plots show that the slope is rather shallow with increasing temperature. Conversely, a small increase in salt concentration provides a steeper slope for the retention factor indicating a much stronger retention. Comparing the impact of salt concentration and temperature, it is noticeable that increasing the salt concentration is a more powerful tool to increase retention compared to temperature.**

## 3. Effect of flow rate and gradient time

As a next step, flow rate and gradient time were examined in order to determine their influence on protein analysis.
Firstly, the gradient time ($t_G$) and flow rate (F) was varied with observing the peak width at half height ($w_{1/2}$). Increase in $t_G$ cause a linear increase in $w_{1/2}$.
Narrow peaks are gained with short gradient times and high flow rates. This finding is in accordance with reversed phase chromatography (RP).

Taking peak capacity in consideration to qualify separation performance, the following observations have been made. At a fixed flow rate, the peak capacity increases with increasing gradient time, whereas the increase levels off with higher gradient duration. The optimum flow rate for each gradient time varies. For short gradients it is the maximum applied flow rate, for longer gradients the optimum flow rate is lower (Figure 3).

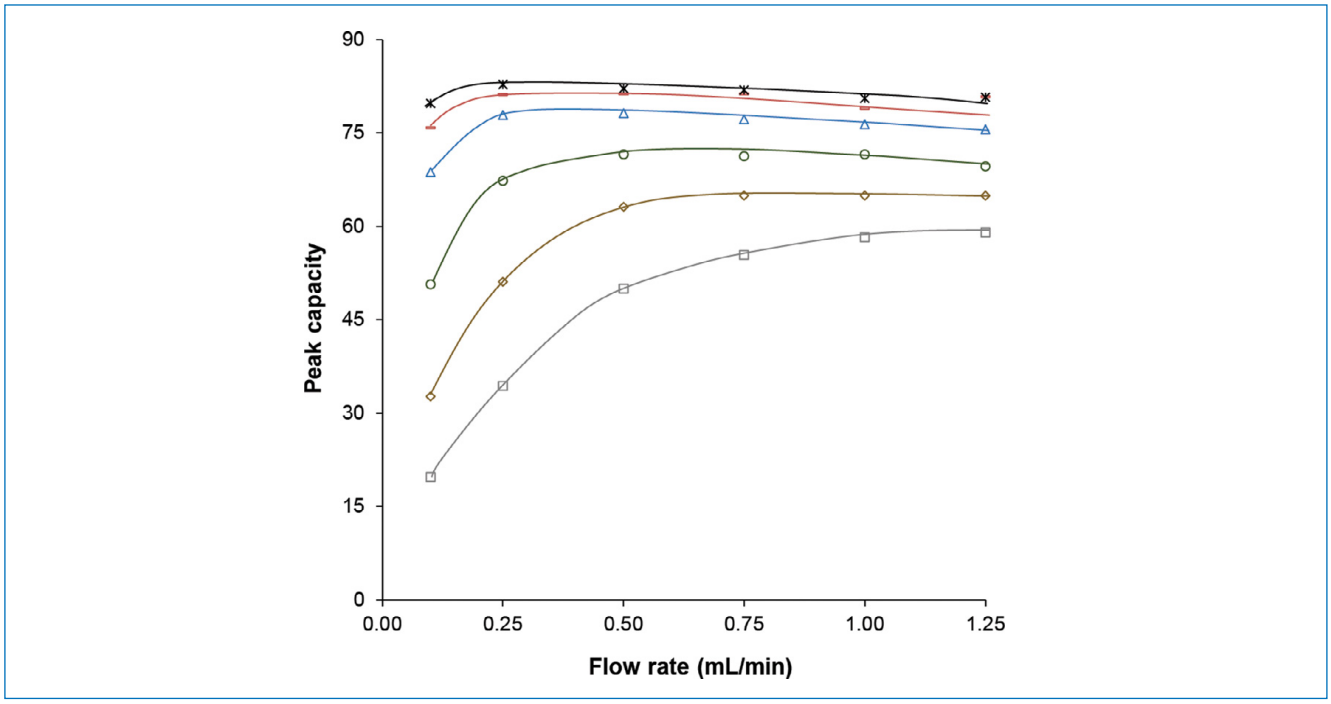


*Figure 3: Peak capacity for the analysis of ribonuclease A as a function of flow rate for different gradient times of 7.5 min (□), 15 min (◇), 30 min (○), 60 min (△), 90 min (-), 120 min (✳).*

Again, in accordance with RP where peaks elute relatively earlier ($t_R/t_G$), proteins elute relatively earlier in HIC with increasing gradient time, too. In contrast to RP, the salt content is higher as an inverse gradient is applied.
In this example the optimum flow rate for $t_G = 120$ min is F = 0.25 mL/min. Whereas the optimum flow rate F = 1.25 mL/min is determined for $t_G = 7.5$ min. As the peak capacity is proportional to the square root of the plate number as well as the ratio of $t_G/t_0$ ($t_G$ = gradient time, $t_0$ = retention time of an unretained compound), the effect of the flow rate on both was also demonstrated.

The van Deemter plots show that even at a flow rate of 0.06 mL/min the minimum plate height was not reached (Figure 4). The mobile phase composition was approximated from earlier gradient separations, respectively. The steep increase for carbonic anhydrase (MW = 30 kDa) compared to lysozyme (MW = 14.3 kDa) and ribonuclease A (MW = 13.7 kDa) is due to the molecular weight of the proteins, and consequently to the molecular diffusion. Carbonic anhydrase has a much higher molecular weight which obviously influences the mass transfer. Therefore, increasing the flow rate results in much higher increase in plate height compared to the much smaller proteins lysozyme and ribonuclease A.

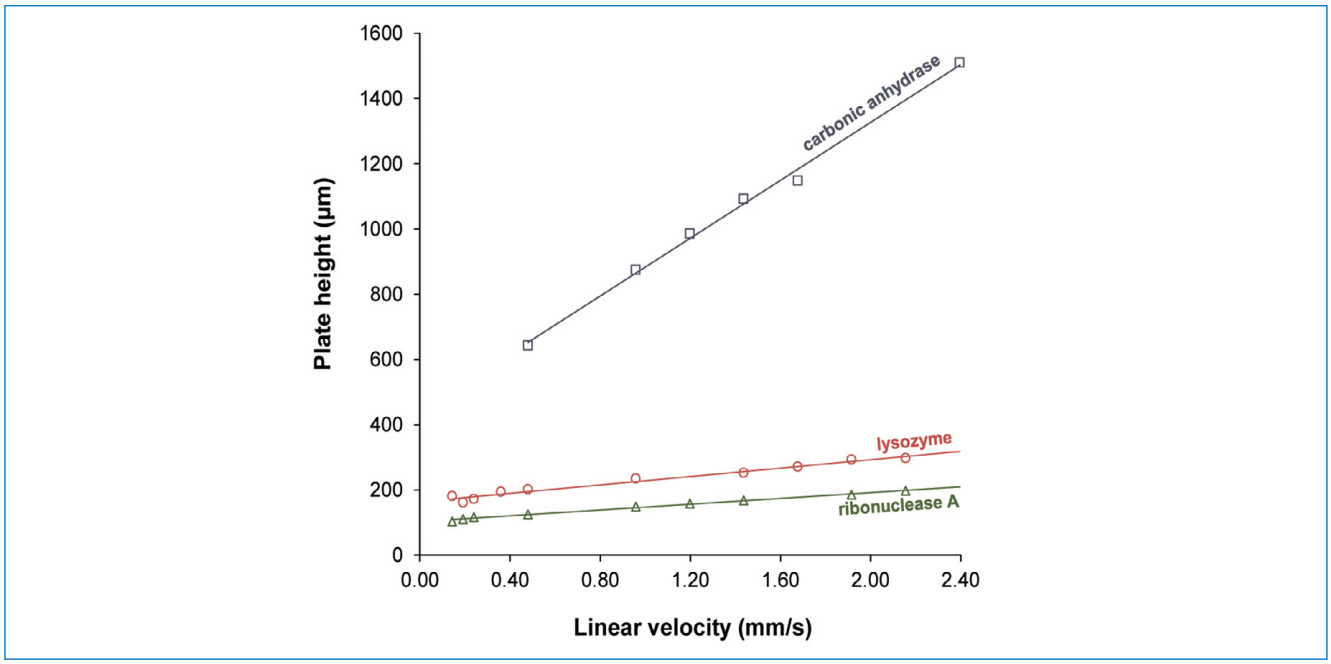


*Figure 4: Van Deemter plots of ribonuclease A (△), lysozyme (○) and carbonic anhydrase (□).*

To compare the results from the experiments in isocratic mode to gradient mode, additional experiments were made to determine the theoretical plate height in gradient mode. Therefore, the influence of two gradient slopes (15 and 30) and the flow rate on theoretical plate height were calculated. These results show a similar behaviour as for isocratic mode. The increase of $H_{gradient}$ is comparable, for the steep increase for carbonic anhydrase as well as for lysozyme and ribonuclease A, indicating a larger resistance to mobile phase mass transfer. However, the steeper the gradient, the shallower is the increase in $H_{gradient}$ due to its increased performance.

## 4. Effect of column length and particle size

The data from section 3 indicates that an increase in gradient time results in higher peak capacities. However, it could also be seen that this trend levels off, if the gradient times are not significantly longer. For example, at a flow rate of 0.5 mL/min the peak capacities for a gradient time of 60 minutes and 120 minutes only differ slightly (79 vs. 82, marked in Table 1 with red circle) for a column with 100 mm length and a particle size of 4 µm.

Increasing the flow rate also showed no significant change, whereas increasing the column length did. When increasing the column length by coupling two columns of 100 mm length the peak capacity could be increased up to 105, which means a 33 % increase compared to 79.

In Figure 5 A the separation of seven globular proteins is shown using a short gradient of 15 min on a single column, whereas in Figure 5 B a long gradient of 120 min on two coupled columns was used.

*Table 1: Selection of peak capacities obtained for separation of ribonuclease A using various flow rates, particle diameters, gradient times and column lengths.*

| Flow rate | Particle diameter | Gradient time | Peak capacity for L=100 mm | Peak capacity for L=200 mm |
|---|---|---|---|---|
| 0.75 mL/min | **BioPro HIC HT: 2.3 µm** | 15 min | 104 | |
| | | 30 min | 133 | |
| | | 60 min | 176 | |
| | | 120 min | 238 | |
| 0.5 mL/min | **BioPro HIC BF: 4 µm** | 15 min | 63 | |
| | | 30 min | 71 | |
| | | 60 min | 79 | 88 |
| | | 120 min | 82 | 105 |

The peak marked with ** corresponds to myoglobin, which is denatured due to the long residence time at 37 °C, since it was not observed for the 15 min gradient.

The peak marked with * corresponds to an aggregate, which was determined in further experiments.

Another approach was made by decreasing the particle diameter. Using a smaller particle diameter implies the reduction of the flow rate due to an increase in backpressure. For a flow rate of 0.75 mL/min the separation using a column with 2.3 µm particles is shown in Figure 5C. The corresponding peak capacities show that the smaller particle diameter leads to a significantly higher peak capacity using a column with 100 mm length (marked in Table 1 with orange circle).

The peak capacity is comparable to those for the two coupled columns, but it is reached within a significantly shorter retention time (15 minutes vs. 120 min).

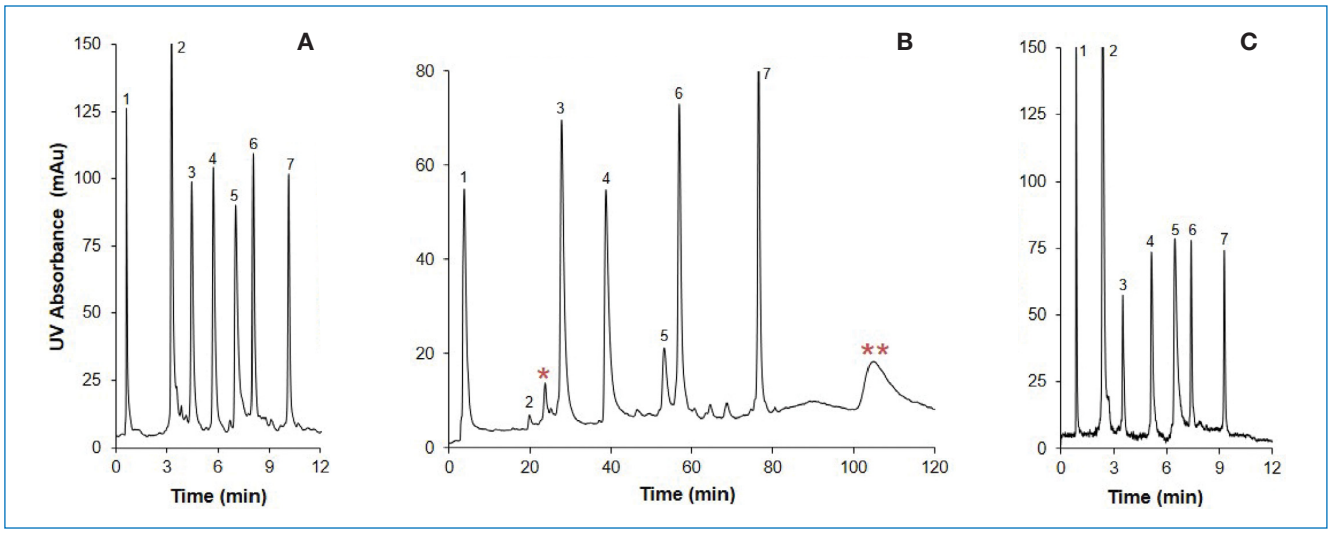


*Figure 5: Chromatograms obtained for different particle diameters and column lengths.*
*Peak identification: 1: cytochrome c, 2: myoglobin, 3: ribonuclease A, 4: lysozyme, 5: carbonic anhydrase, 6: trypsinogen, 7: α-chymotrypsinogen A. 5A: L = 100 mm, $d_p$ = 4 µm, F = 1.25 mL/min, $t_G$ = 15 min. 5B: L = 2 x 100 mm, dp = 4 µm, F = 0.5 mL/min, t = 120 min. 5C: L = 100 mm, $d_p$ = 2.3 µm, F = 0.75 mL/min, $t_G$ = 15 min.*

## 5. Conclusions

**The study by Ewonde et al. provides an overview of how many parameters can influence the resolving power for intact protein analysis by HIC.**

- Increasing the salt concentration and temperature leads to an increase in retention
- Salt concentration has a higher impact than temperature
- Flow rate and gradient time affect each other
- Short gradients benefit from high flow rates and steep gradients
- Increase in column length is beneficial for peak capacity, but requires longer analysis times
- Use of smaller particles allows for increasing peak capacities with shorter analysis times.