![CuriRx logo]

This article includes case studies and a discussion of CuriRx's development of new SEC-HPLC methods to eliminate the interaction of proteins or peptides with residual silanols and improve the separation of proteins, peptides, and impurities of those biologics.

For the separation of biologics, the pharmaceutical industry uses Size Exclusion High Performance (SEC-HPLC) Columns to assess the major peaks, aggregates and degradants present in a product.

For the studies referenced in this article, many conditions and additives were evaluated including high ionic strength, organic solvents and ion pairing agents. Several SEC-HPLC columns and mobile phases were evaluated for the assay of proteins and peptides to further diminish interactions with residual silanols. Mobile phase optimization was evaluated based on plates, resolution, symmetry, recovery, RT closer to the real molecular weight and purity and impurity recovery.

This article will include a discussion for developing a denovo method to eliminate the interactions of proteins or peptides with residual silanols and improve the separation of proteins, peptides and impurities. CuriRx's approach mitigates the problems associated with the recovery of the monomeric and high molecular species for these biomolecules, which are retarded or bound on the column during separation. Examples of peptides and antibodies will be presented that demonstrated reduced column interaction with full recovery of the monomeric and the aggregate species.

The issues experienced with SEC chromatography methods depend mainly on the aggregates and degradation of the biologics. But the question is, how *accurate* we are in the quantification of aggregates, or main peak, or degradations. Many times, there is a tendency for proteins, or their degradation products, or high molecular species to bind to the resin of the column. This may be due to their hydrophobicity under stress conditions, thereby rendering quantitation questionable. It is important to know the reason why proteins interact with the column to mitigate it.

Prior to using porous silica, scientists were using soft gels like agarose or sepharose, with long streaking and long separation times. The problem, however, with the porous silica is strong ionic interaction because of the silanols. To overcome this interaction, CuriRx produced a diol functional group to minimize hydrophobic interactions, yet still, residual silanols can remain. High ionic strength mobile phase, or some basic amino acids such arginine are used to diminish this interaction. In rare cases organic solvent with a concentration which will not precipitate the protein is used. Some ionic pairing agents are also typically used.

SEC HPLC development at CuriRx addresses issues such as:

- The effect of salt, pH, ion pairing agent, and flow rates.
- Several SEC columns and mobile phases are evaluated for the SEC-HPLC assay of proteins and peptides to further diminish interactions with residual silanols.
- Identification of additives to the mobile phase like cyclodextrin to help reduce the interactions.

Case study examples cited include:

Example 1) An example of the effect of salt on concentration, on the interaction to the column.

Example 2) The effect of flow rate, pH, salt on a partially purified immunoglobulin, to show impurities.

Example 3) Proteins with a molecular weight of 14 kDa, severely retarded and binding to SEC columns.

Example 4) The delayed elution of a 55 Kda protein due to the column binding.

Example 1) The effect of arginine salt concentration in mobile phase on impurity interaction of an antibody to the column.

Fig. 1

10-1074-LS Iteration 4: Effect of Time, Temperature and Shear - SEC

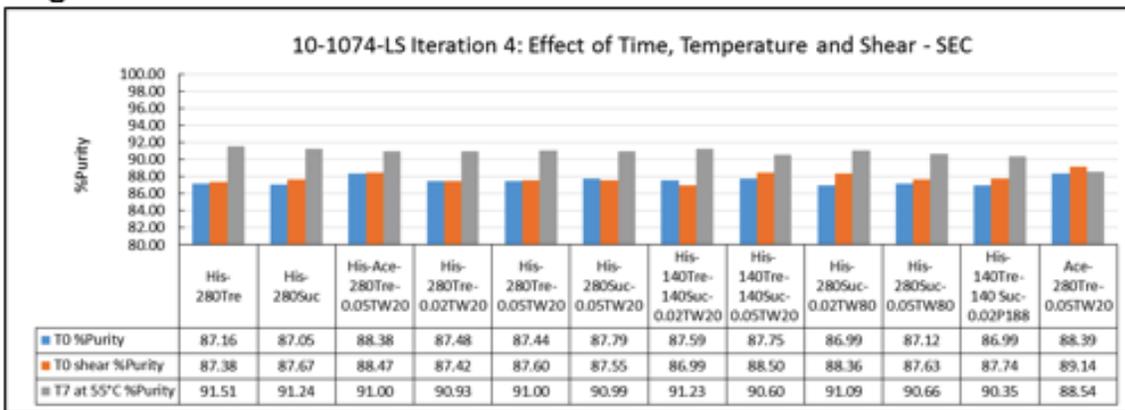|	His-280Tre	His-280Suc	His-Ace-280Tre-0.05TW20	His-280Tre-0.02TW20	His-280Tre-0.05TW20	His-280Suc-0.05TW20	His-140Tre-140Suc-0.02TW20	His-140Tre-140Suc-0.05TW20	His-280Suc-0.02TW80	His-280Suc-0.05TW80	His-140Tre-140 Suc-0.02P188	Ace-280Tre-0.05TW20
T0 %Purity	87.16	87.05	88.38	87.48	87.44	87.79	87.59	87.75	86.99	87.12	86.99	88.39
T0 shear %Purity	87.38	87.67	88.47	87.42	87.60	87.55	86.99	88.50	88.36	87.63	87.74	89.14
T7 at 55°C %Purity	91.51	91.24	91.00	90.93	91.00	90.99	91.23	90.60	91.09	90.66	90.35	88.54

[fig. 1] This is the HPLC screening. We tried to find out which formulation is better. We used Tosoh SWXL 3000 column, 100 millimolar acetate and 100 millimolar sulfate pH 6 as a mobile phase.

You can see after stress, instead of purity going down, the purity goes up in most cases. This is because in this case the impurity preferentially binds to the column. The study shows that the impurity binds to the column, even at 55 degrees. When the samples are incubated at 55 degree centigrade for a week, it should generate more impurities. But you can't see the impurity. The four percent impurity is down because this impurity is preferentially binding to the column. This can be mitigated just by adding 50 millimolar arginine and 10 millimolar histidine. Instead of 100 millimolar acetate, CuriRx included 10 millimolar histidine and 50 millimolar arginine at pH 6. It completely eliminated the interaction of T7 impurity to the column. Similarly, the FDA published a paper on several antibodies. They used sodium perchlorate in the eluent, so that interactions, especially the aggregates to the column is eliminated.

This is the effect of salt concentration of interaction to the column, separation of 2 related antibodies using Tosoh TSK-Gel G3000: [fig 2] The top row is 50 millimolar arginine and 50 millimolar sulfate pH 6 as a mobile phase. As expected, the 150 kD both and antibody eluted at the same time.

When the salt concentration is increased from 50 millimolar to 200 millimolar sodium sulfate, it appears to separate two related antibodies. When the concentration of 400 millimolar sodium sulfate is present, the two antibodies are separated. When we use 550 millimolar, the resolution of the two antibodies are much higher. But as the figure shows, when the salt concentration is increased from 50 millimolar to 500 millimolar, there is more interaction to the column. For example, 50 millimolar salt, the molecular weight is around 150 for both antibodies. But when you increase the concentration to 500 millimolar, both the antibodies bind to the column and are eluted at a later time.

Fig. 2
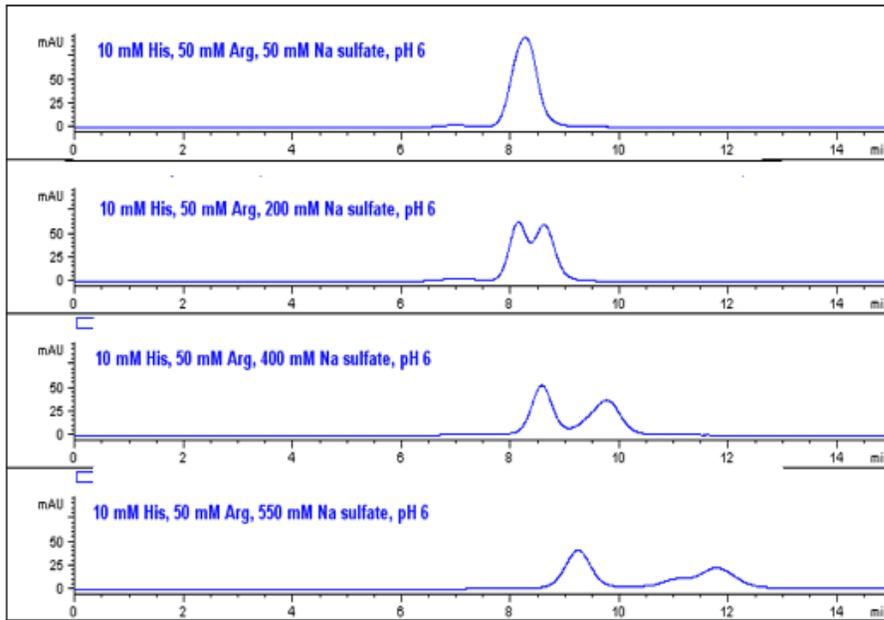
Example 2) The effect of flow rate, pH and salt on a partially purified IgG.

[fig. 2] The top graph is PBS pH 7, with a flow rate of 0.2 mL per minute. It took about 30 minutes to elute the main peak. We can shorten the time using the same pH within 15 minutes by increasing the flow rate without any resolution difference. It eluted in around 12 minutes, but as you can see, it should be at eight minutes to have a molecular weight of 150 k. It eluted later, the reason for this is interaction with the column.

When we changed the mobile phase from PBS to 100 millimolar acetate and 100 millimolar pH 6, the main peak came in at the right molecular weight, 150 k. And, the strict polymer aggregates consist of a sharp peak. You can see from dimer, trimer, and polymer very clearly. Also, the interacting, different product low molecular weight also separated. It showed a clear resolution, so, selecting the right concentration of salt is very important to separate the proteins.

Example 3) 14 kDa protein, which is retarded or binding to the SEC column. This is a very challenging protein.

Fig. 3

# of conditions	Column	Mobile phase	µg peptide injected	Total AUC	Comments
1	Tosoh SWXL 3000	0.1M sodium acetate and 0.1M sodium sulfate pH 6.0	50	373	Very little protein recovered
2	Tosoh SWXL 3000	0.1M sodium acetate and 0.5M sodium sulfate pH 6.0	50	375	Very little protein recovered
3	Waters Acquity BEH SEC	0.1 M Acetate/0.1 M sodium sulfate pH 6.0/10% Ethanol/0.05% TFA	50	1600	14KDa peptide separation but showed carry over suggesting interaction.
4	Waters Acquity BEH SEC	0.1 M Acetate/0.1 M sodium sulfate pH 6.0/10% Ethanol	50	1650	14KDa peptide separation but showed carry over suggesting interaction.

We have used several conditions in this example. [fig. 3 cond. 1 - 4] You can see initially we tried to use Tosoh column with the regular 100 millimolar sodium acetate, 100 millimolar sodium sulfate, pH 6. We injected 50 micrograms, but AUC was only small, meaning it is almost completely binding to the column.

We increased the salt concentration from 0.1 molar to 0.5 molar sodium sulfate. It did not improve at all. Very little protein recovered. [fig. 4 cond. 5-9] Therefore, we changed the column to Water's Acquity BEH SEC column, and also used some organic solvent, 10% ethanol with 0.05% TFA. There was a slight increase in the total AUC, but with lots of carryover on the next injection, even though the metal processes for 20 minutes. Using the ethanol alone, still there is a lot of carryover.

Fig. 4

# of conditions	Column	Mobile phase	µg peptide injected	Total AUC	Comments
5	Waters Acquity BEH SEC	0.1 M Acetate/0.1 M sodium sulfate pH 6.0/10% isopropanol	50	1980	14KDa peptide separation but showed carry over suggesting interaction.
6	Waters Acquity BEH SEC	0.1 M Acetate/0.1 M sodium sulfate pH 6.0/10% Methanol	50	1770	14KDa peptide separation but showed carry over suggesting interaction.
7	Waters Acquity BEH SEC	0.1 M Acetate/0.1 M sodium sulfate pH 6.0/10% Acetonitrile/0.5% TFA	3	1360	14KDa peptide separation but showed additional protein eluting after the included volume
8	Waters Acquity BEH SEC	0.1 M Acetate/0.1 M sodium Sulfate, pH 6.0 10% acetonitrile/0.005 Tetrabutylammonium phosphate	5	2478	14KDa peptide separation but showed additional protein eluting after the included volume
9	Waters Acquity BEH SEC	0.1 M NaAcetae/0.1 M NaSulfate 1% hydroxypropyl cyclodextrin	0.7	1511	14KDa peptide separated with no carry over or protein eluting after the included volume

We changed to isopropanol and, again, a lot of carryover, even though the total AUC slightly improved from 300 to 2,000. So, we changed to methanol and, still, without any improvement. When we changed to acetonitrile, even with a three-microgram injection, the response was good. Still, however, there is a

drawback, with carryover to the next column injections. Then, we added tetrabutylammonium phosphate, total AUC increased, but still, there is a carryover. Finally, we used the same mobile phase without any organic solvent. We included one percent cyclodextrin, hydroxypropyl cyclodextrin in the mobile phase. Even with a 0.7 microgram injection, we had a good total recovery, and with no carryover at all.

The conclusion is that 14 kDa protein highly interacts with the column. pH, salt, ion pairing agent, or the organic solvent did not improve, preventing the column interaction. But adding a complexing agent in the mobile phase, completely eliminated this interaction.

We used a bridged ethyl hybrid particles BEH column. The silanol group is reduced significantly here. According to the literature, it requires less salt additive to minimize ionic interaction with the proteins. Moreover, the high mechanical strength enables a reduction in the particle to 1.7, so the column efficiency will be increased. But, still, it needs a mobile phase to prevent the interaction of the protein.

At CuriRx, we have identified cyclodextrin as a very novel agent in the mobile phase to eliminate the interactions of proteins or peptides with residual silanols, and improved separation of proteins, peptides, impurities like aggregates, or degradants. The good thing about inclusion of cyclodextrin in the mobile phase is not only a reduced or elimination of the column interaction of the protein, avoiding the underestimation of the impurities, but also the apparent molecular weight of the analyte is very, very close to the theoretical molecular weight.

[fig. 5] While we were optimizing the SEC separation, we used several formulations. One of them, of course, is PBS. We used the BEH column, with 0.1 molar sodium acetate and 0.1 molar sodium sulfate and 10% acetonitrile, 0.005 molar tetrabutylammonium phosphate as a mobile phase. As you can see from this figure, the red line is the peptide formulated in PBS.

Fig.5
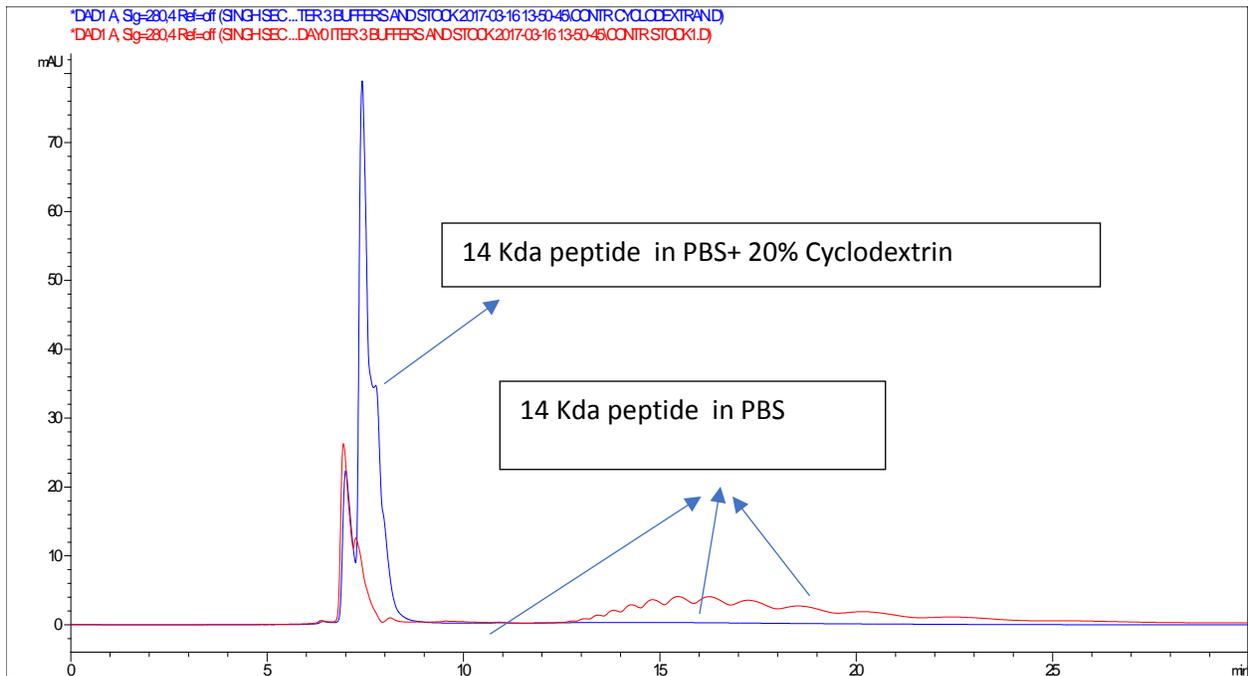
You can see a peak here, with streaking after 10 minutes. There is a huge silanol interacted protein. We collected the fraction of this protein and checked using the reverse rate, and it appeared that whatever streaked is the real 14 kDa protein, and the impurity did not streak. But when it is formulated in 20% cyclodextrin, you don't see that streaking. Everything came out as a single peak, and the impurities.

We postulated that including the cyclodextrin in the mobile phase may mitigate any protein interaction with the column.

[fig. 6] As we proceeded, with cyclodextrin, everything came out at 10 minutes. That means the molecular weight is less than a water. But when you use cyclodextrin, you clearly separated the impurity as a pre-peak and main peak, and the molecular weight is very close to 14 kDa. Here, there is a complexing reagent that resolved the problem of interacting the peptide with the column.

Fig 6. Chromatogrphic profiles of 14 Kda peptide formulation on Acquity BEH column using 0.1M NaAcetate/0.1M Nasulfate pH 6.0/0.1%hydroxypropyl cyclodextrin
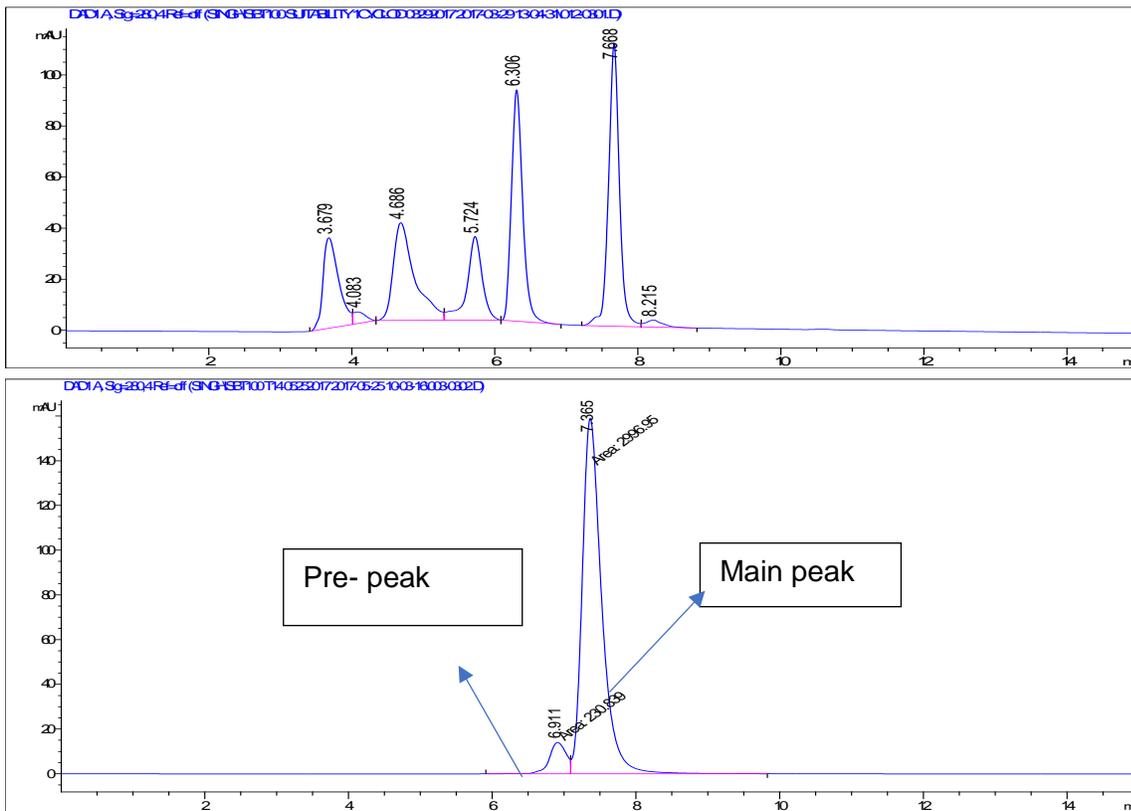
We tried several pH to eliminate the problem, and salt also. Finally, based on our previous experience with complexing reagent, it eliminated the column interaction. You will see this example in the slides to follow.

The top graph is a molecular weight marker. The bottom graph is a protein. Here, this protein elutes even after the buffer peak. The huge peak that you see is a buffer peak. It comes out after the buffer peak. Checking the molecular weight, it is less than the lot vitamin B12, which is 1350.

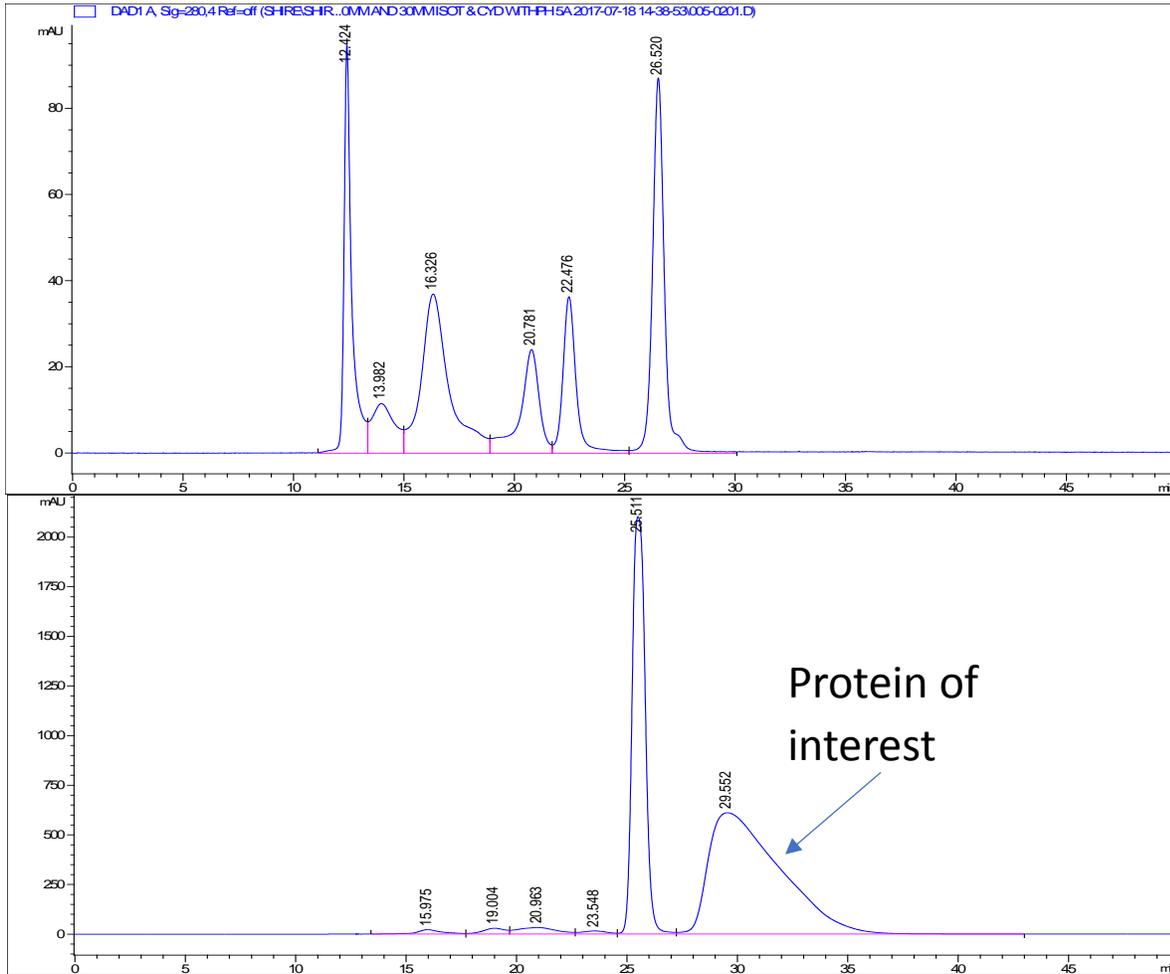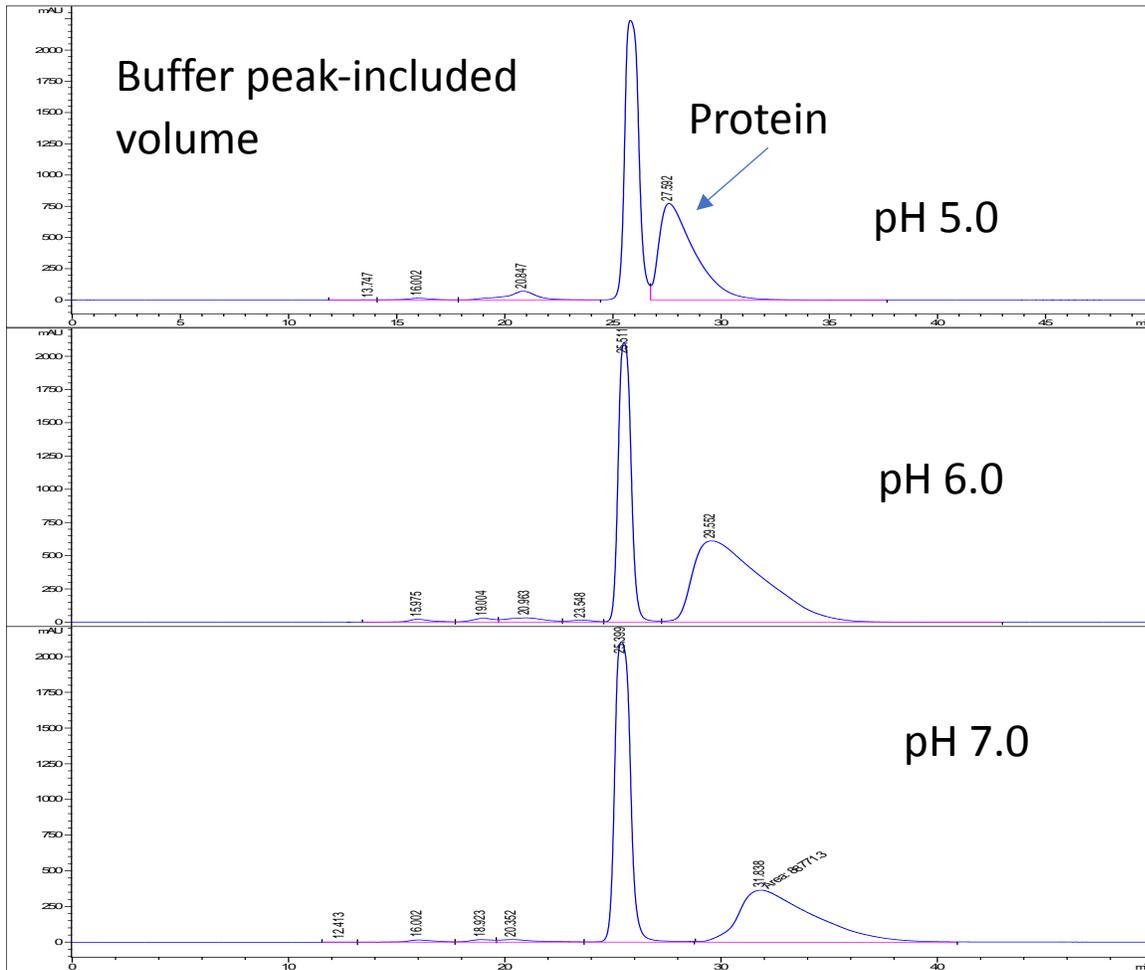**Fig.7 Water Protein Pak 300SW Column (8X300 mm)**

We did several pH: pH 5, 6, and 7. As you can see, still the protein comes after the buffer peak, and the protein eluted later, when the pH is increased and it streaks farther down. It is not total symmetry, and it is interacting with the column more at higher pH.

Fig 8 Waters Protein Pak 300SW column (8X300 mm)

The conclusion of this effect of pH is: at higher pH, it streaks more. We can optimize the pH, but still, that is not enough.

^fig. 9 Then we included 1% cyclodextrin in the mobile phase. The buffer peak comes after the main peak. Looking at the protein peak, it is very symmetrical and has good resolution.

The molecular weight is very close to the theoretical weight. That is very important. For example, it elutes before ovalbumin, which is around 44,000 molecular weight. It is not eluting less than 1,300. The "take-home message" in this, is we have to optimize the SEC method, and cyclodextrin played a major role in mitigating most of the problem whether it is a protein or peptide or antibody.

Fig 9: Mobile phase at pH 5.0 and 1% cyclodextrin Waters Protein Pak 300SW column (8X300 mm)
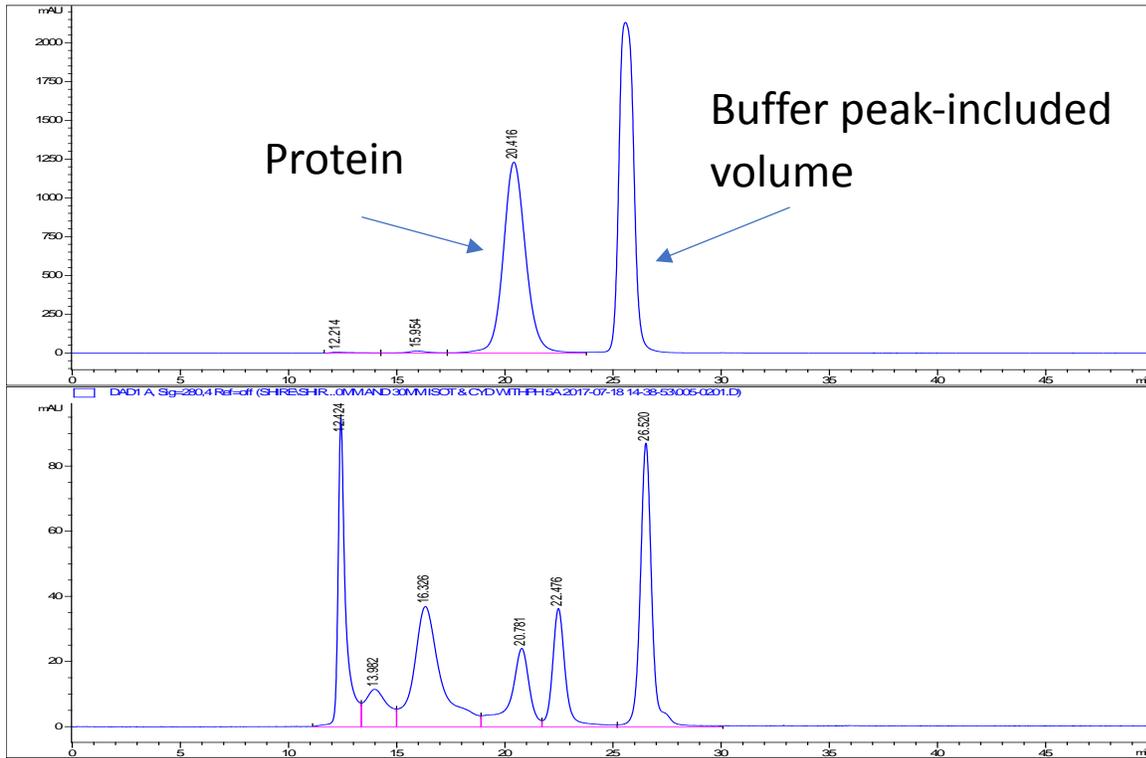

fig. 10 Here, in presence of cyclodextrin (this is the zoomed chromatogram) You can evaluate the purity precisely. Unlike when it is streaking, you do not know how much the aggregates are adsorbed to the column.

Fig10 Mobile phase at pH 5.0 and 1% cyclodextrin Waters Protein Pak 300SW column (8X300 mm): Zoomed chromatogram to show the impurities: Aggregates and Main peaks are assayed very precisely
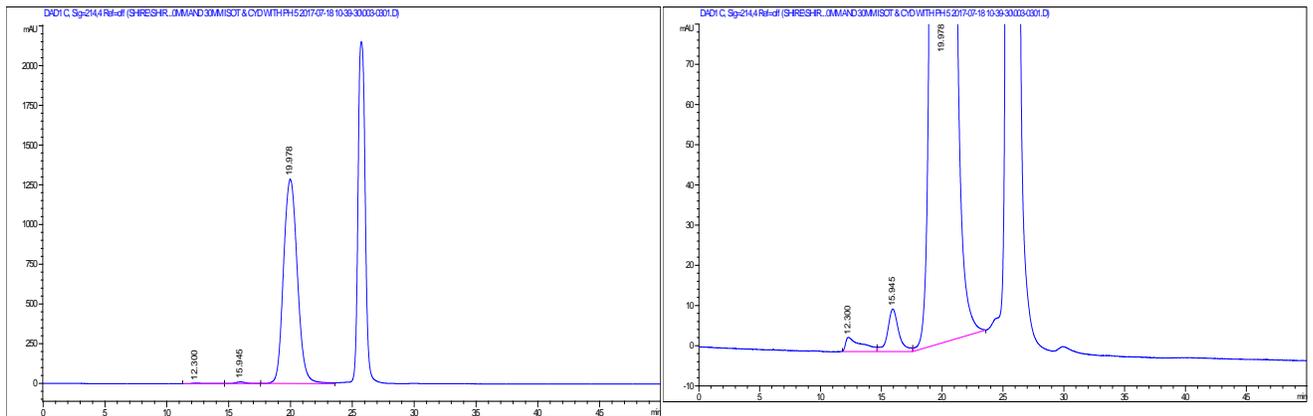
#	Time	Area	Height	Width	Area%	Symmetry
1	12.3	324.9	3.5	1.2341	0.325	0.186
2	15.945	673.4	10.6	0.9525	0.673	0.832
3	19.978	99047.2	1284.7	1.2275	99.002	0.896

^{fig. 11} This is a molecular weight, apparent molecular weight. In percent's of cyclodextrin, the molecular weight is very close to the real molecular weight at pH 5. We hope to optimize pH 5 complexing reagent to bring the protein to the real molecular weight and without any interaction with the column.

Fig. 11

Calculation of the Apparent Molecular weight using Waters protein Pak 300SW column (8x300 mm)

Protein	Apparent Molecular Weight (Daltons)
pH 5 without cyclodextrin	> 1350
1% CD @ pH 5.0	49082.8
1% CD @ pH 6.0	40657.6

The conclusion of this method is mobile phase pH 5 is improved resolution, and inclusion of 1% cyclodextrin with the mobile phase pH 5 to 6 eliminated completely the interaction with the column.

1% cyclodextrin at pH 5 in the mobile phase is a better mobile phase based on the plates in terms of resolution, symmetry, purity, as well as recovery.

In summary:

First, we hope to select a suitable column. That is very important, and commercially there are several columns available. We hope to check the suitable column for this to minimize the column interaction and understand the effect of pH of the solvent on the column.

Understand the effect of salt on column interaction of the protein or peptide. In some cases, people may think higher salt can elute anything. But higher salt can favor column interaction also. We have shown that example in this presentation, a favoring the separation of two related antibodies.

Understand the effect of ion pairing agent is also very important in some cases. Organic solvent also. Most importantly, the complexing reagent cyclodextrin played a major role on preventing the interaction of protein or peptide to the column.