Isolating mAb Variants for Identification or Producing Standards with Twin-column Chromatography

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Introduction

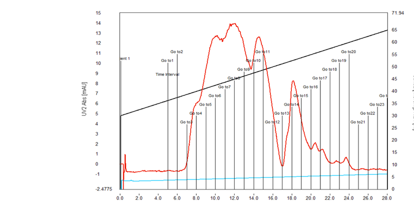
MAb variants can be difficult to characterize since it is not possible to directly synthesize them. These compounds need to be isolated from crude parent material. Compounds being developed for pharmaceutical use are required by regulatory agencies to identify related impurities that are present greater than the Identification Threshold. This can be a difficult task with unknown impurities that are present at low levels within early to mid-development drug substances or drug products. Prep chromatography can certainly be used for isolation of these components, but it can generate a large number of isolated fractions. The entire process can become very challenging and labor intensive, when dealing with the large number and substantial volume of very dilute fractions. N-Rich® technology is a twin column technique that concentrates low level components within the chromatographic system before any fractions are isolated.

3-Step N-Rich Technology Development

Step 1: Single column batch run - Select region of chromatogram for enrichment

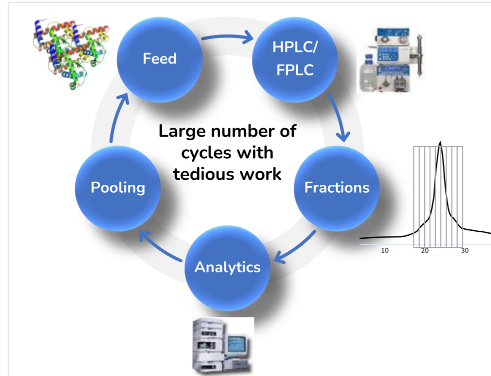
Step 2: N-Rich technology accumulation – Run multiple cycles to enrich the target compounds

Step 3: Fractionation methodology – Elute the target components and collect fractions



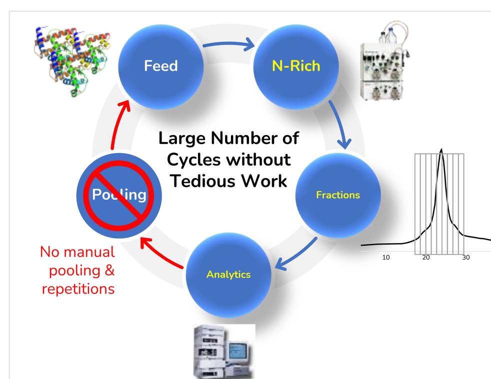
N-Rich Technique Overview

Typical Batch Process



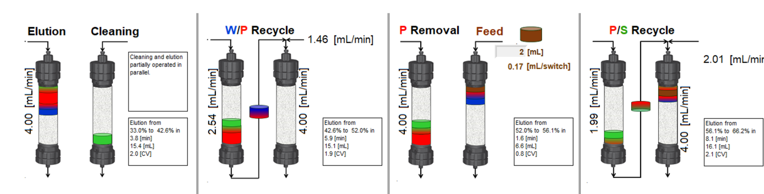
A typical related substance batch process collects all of the targeted components. Many different batch runs need to be performed to obtain enough material. This produces many isolated fractions that are very dilute for every run.

N-Rich Process

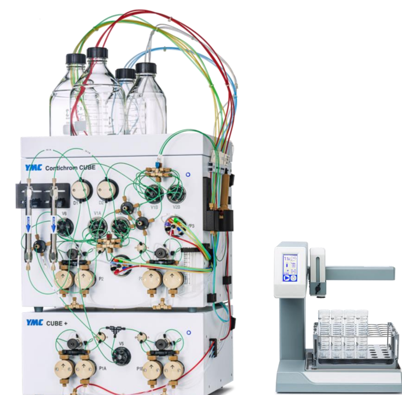


An N-Rich process accumulates the targeted components within the 2 chromatography columns. The portions of the chromatogram with targeted early and late eluting components are sent to a second column while adding more feed material, thus the purification proceeds in a continuous fashion.

2-Column N-Rich Process



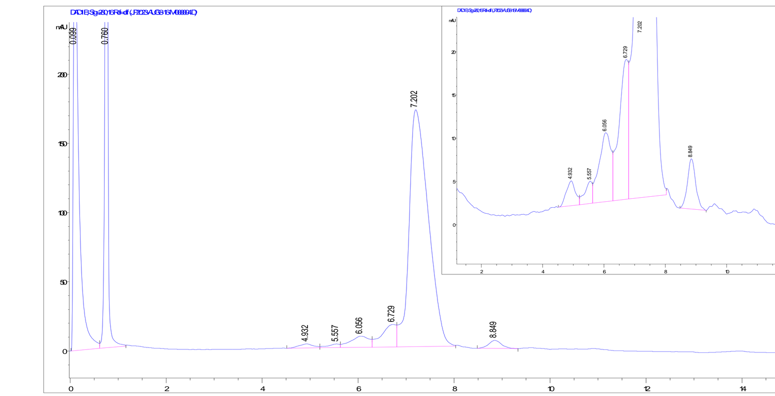
The right column is cleaned when the left column starts to run a sample. The desired early-eluting portion is recycled. The undesired portion is removed while adding more feed to the second column. Finally, the late-eluting portion is recycled. The next step is running the right column while the left column is being cleaned.



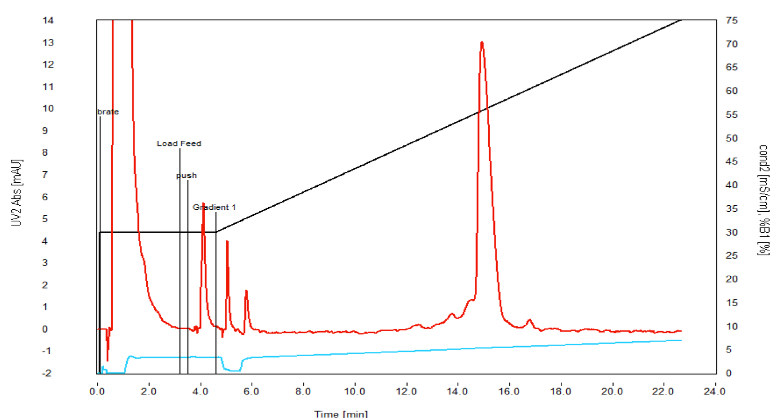
CUBE: Bench-Top System
Available as 37 or 100 mL/min maximum flow rate. Capable of running MCSGP, single column batch, N-Rich technology and CaptureSMB® process.

Step 1: Batch Methodology

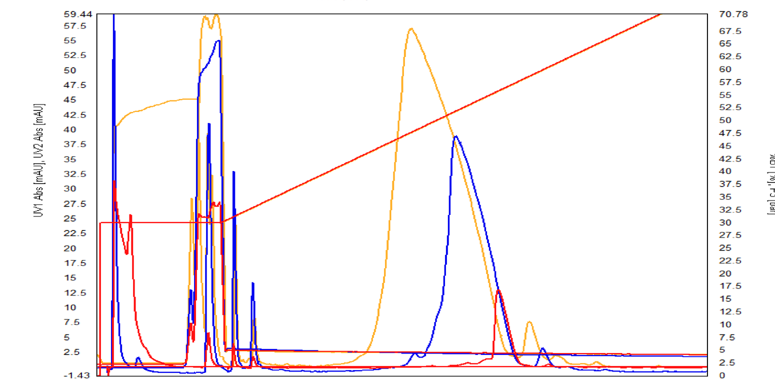
Analytical Scale: Step 1 begins with the development of suitable chromatography and determination of the desired components to be isolated. The peaks to be enriched do not need to be separated from each other. They only need to be partially separated from the main peak.



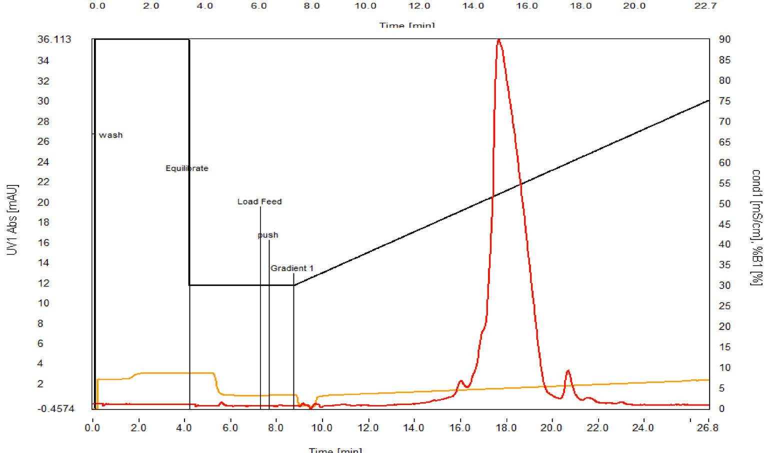
Transfer From Analytical: The methodology is transferred from the analytical system to the CUBE twin-column system.



Evaluate Loading: Several single column runs are made with more and more material being loaded. This will determine the maximum amount of material that can be loaded while maintaining suitable separations from the main peak.

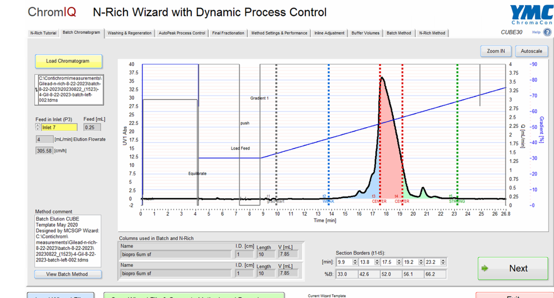


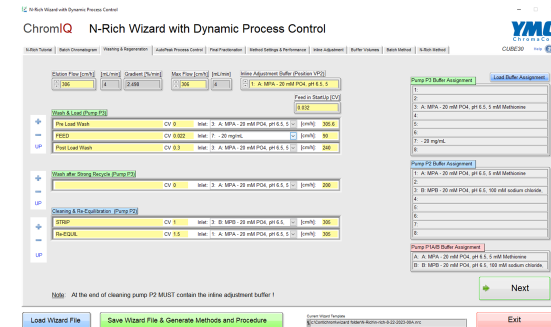
Select Conditions and the Amount to Load: To accumulate the target components, they only need to be resolved from the main peak and not need to be resolved from each other. They will be resolved from each other in the fractionation step.



N-Rich Technology Wizard

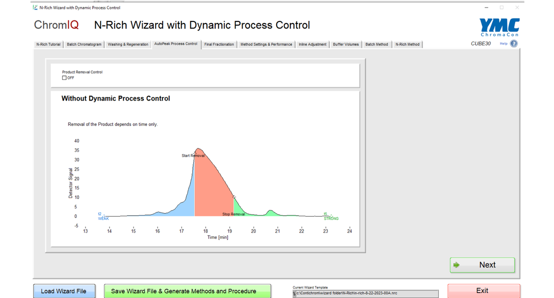
The single column batch chromatogram is loaded into the N-Rich technology Wizard. The boundaries are set for recycling the early eluting material, the material to be removed / collected, and to recycling the late eluting material.

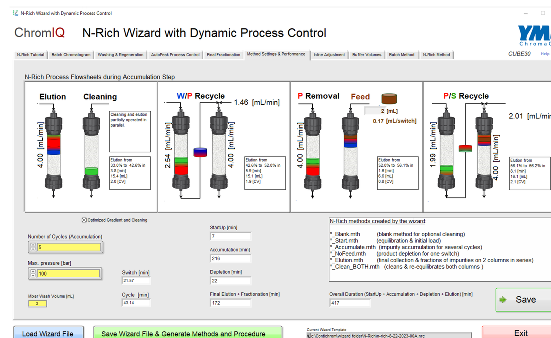




The Wizard makes an initial prediction for the chromatographic method based on the single column batch data. Adjustments can be made to sample loading, flow rates, column washes, and equilibration steps parameters.

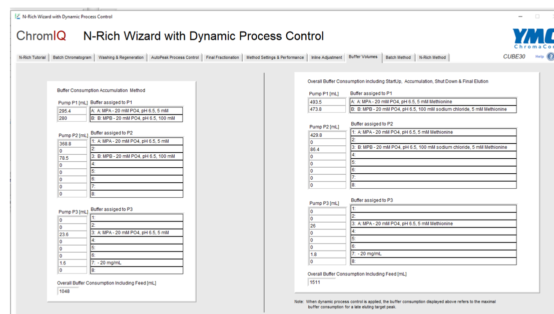
Recycling and main peak removal can be triggered by time or by detector response. The recycled solutions are diluted before entering the next column. The amount of dilution is set within the Wizard.





The number of cycles and maximum system pressure are set within the Wizard. The Wizard also makes predictions for the process duration.

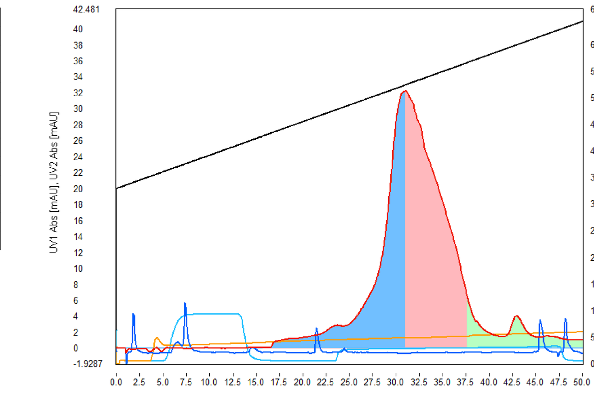
The Wizard calculates the total amount of sample feed, buffers, and eluents used for the N-Rich technology accumulation method, and for the associated startup, shutdown, and final elution methods.



Step 2: N-Rich Technology Accumulation

The accumulation step starts with a single column batch run. The early eluting components are sent to the second column (blue shaded region), the main component is removed and collected while more initial material is introduced to the second column (red shaded region), the late eluting components are sent to the second column.

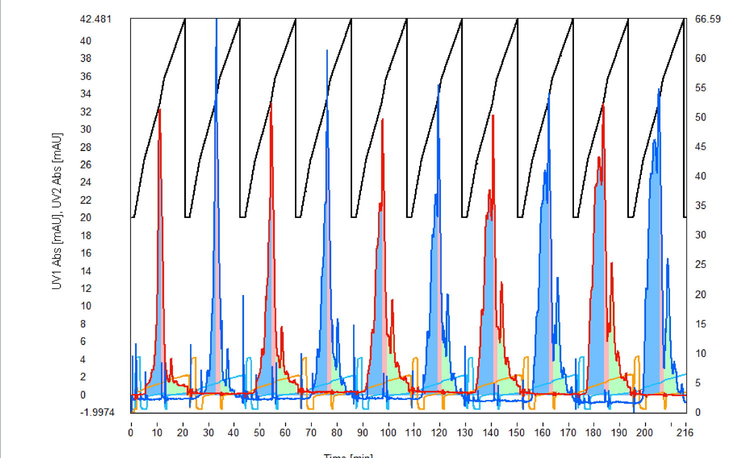
Column	BioPro IEX SP 5um		
Dimension	100x8.0mm		
Mobile Phase A	20mM Phosphate pH 6.5 5mM Methionine		
Mobile Phase B	20mM Phosphate pH 6.5 100mM NaCl 5mM Methionine		
Gradient	Time (min)	% MPA	% MPB
	0	70	30
	18	25	75
Flow Rate	4 mL/min		
Detection	UV @ 280nm		
Temperature	Ambient		



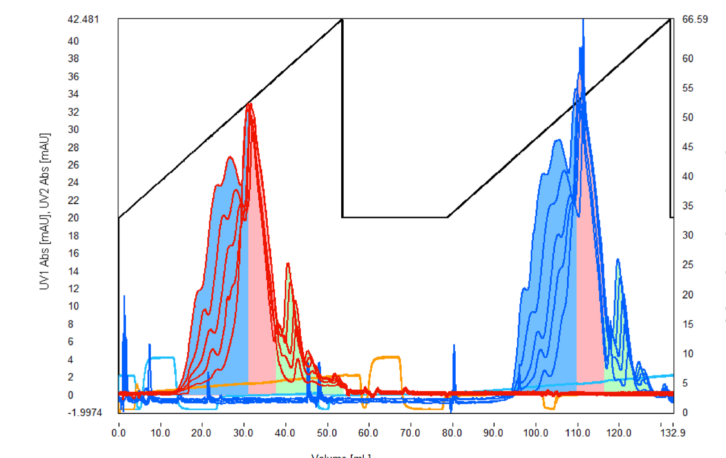
1st Switch: This is the same as running the crude on a single column run

A single column run is called a "switch." A "cycle" is when a switch is completed on both columns.

In this experiment, 5 cycles (10 switches: 5 on column 1 and 5 on column 2) were run to accumulate the desired minor components.



5 Cycles of accumulation: Column 1 and Column 2 are both run 5 times

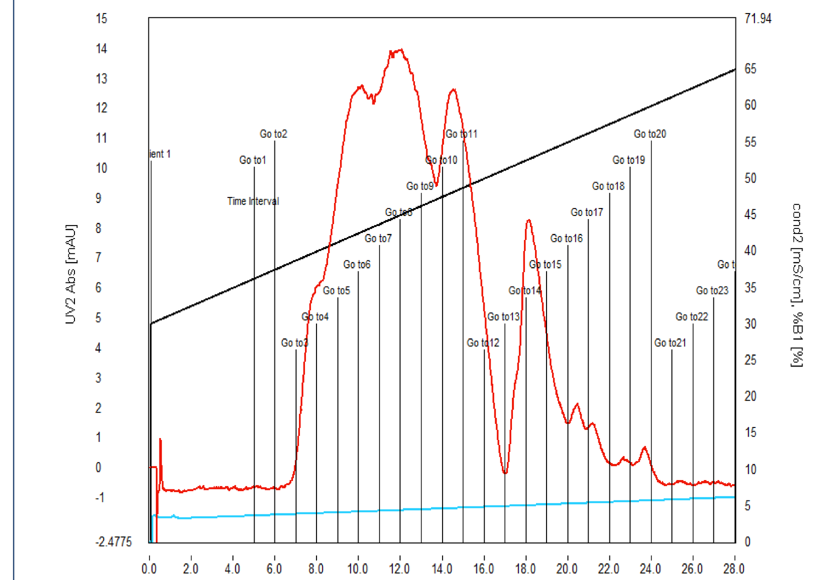


Overlay of the same 5 cycles

Step 3: Fractionation

Fractionation can be done with different methodology than was used for accumulation. This experiment used a fractionation method with a gradient slope that was ½ of the one used for accumulation (the gradient time was doubled but the initial and final compositions remained the same).

Column	BioPro IEX SP 5um		
Dimension	100x8.0mm		
Mobile Phase A	20mM Phosphate pH 6.5 5mM Methionine		
Mobile Phase B	20mM Phosphate pH 6.5 100mM NaCl 5mM Methionine		
Gradient	Time (min)	% MPA	% MPB
	0	70	30
	36	25	75
Flow Rate	4 mL/min		
Detection	UV @ 280nm		
Temperature	Ambient		

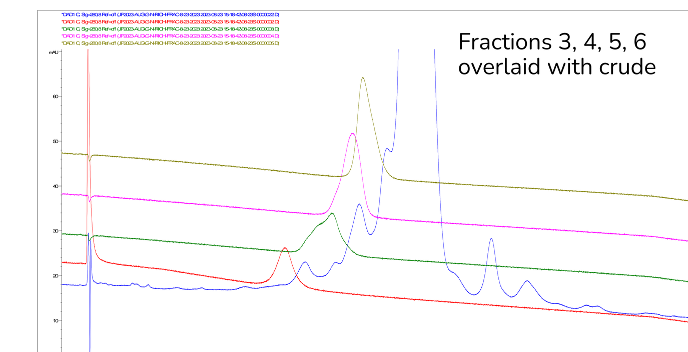


Fractions are collected across the entire elution profile

Analytical Analysis of Fractions

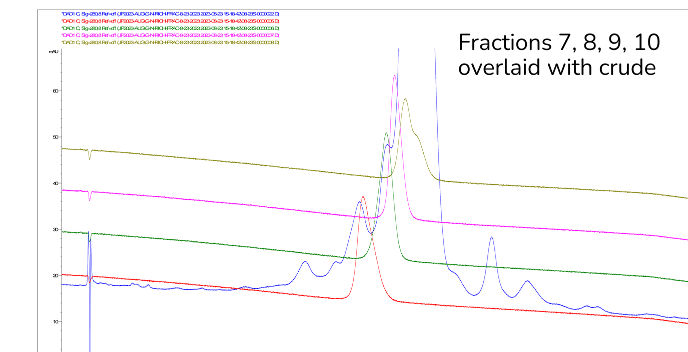
Presented here are chromatograms of the collected fractions that contained relevant material.

Each figure displays 4 or 5 fractions that are overlaid with a chromatogram of the crude for comparison. The crude chromatogram is always in blue. The earliest eluting fraction in the figure, is always displayed as the lowest one and the latest eluting fraction is displayed as the top one.



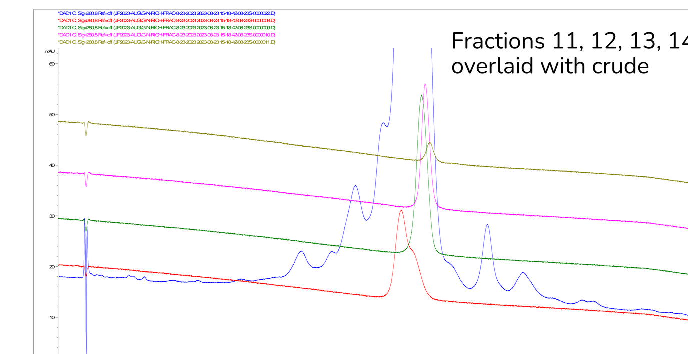
Fractions 3, 4, 5, 6 overlaid with crude

Fractions 3, 4, 5 and 6 contain at least 5 components that elute on the front side of the main peak. Fractions 3, 5 and 6 appear to be mainly a single component while fraction 4 seems to be a mixture of 2 components. The peaks in Fractions 4 and 6 overlay with significant peaks in the crude.



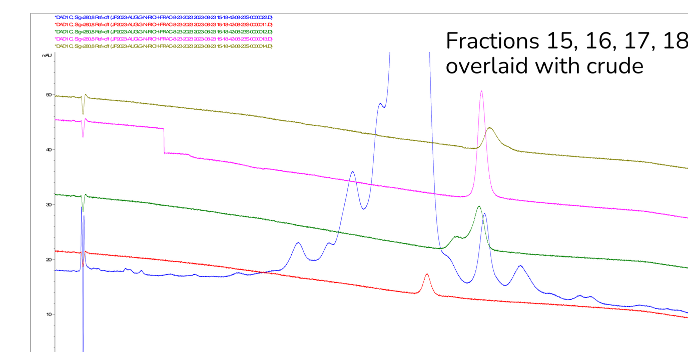
Fractions 7, 8, 9, 10 overlaid with crude

Fractions 7, 8, 9 and 10 contain several different components that elute at the very front edge of the main peak. Fractions 9 and 10 contain the same significant peak in the crude.



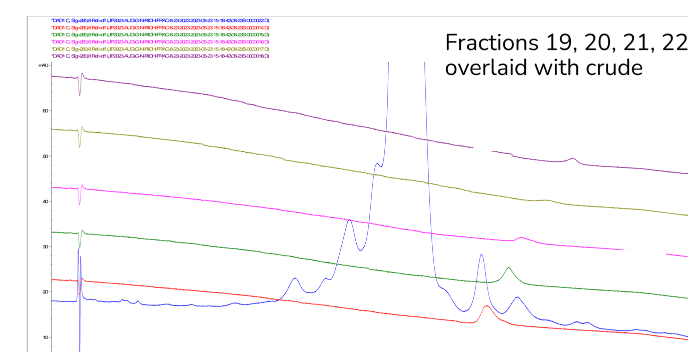
Fractions 11, 12, 13, 14 overlaid with crude

Fractions 11 contains a component that elutes completely under the main peak while fraction 12, 13 and 14 contain primarily the main peak.



Fractions 15, 16, 17, 18 overlaid with crude

Fractions 15, 16, 17 and 18 contain peaks that eluted on the tail of the main peak. Fractions 15 and 16 contain peaks that are large and late eluting in the crude.



Fractions 19, 20, 21, 22 overlaid with crude

Fractions 19, 20, 21 and 22 contain small peaks that are later eluting. Fraction 20 contains a significant late eluting peak.

Column	BioPro IEX SF 5 μm		
Dimension	100 x 4.6 mm		
Mobile Phase A	20 mM Phosphate pH 6.5 5 mM Methionine		
Mobile Phase B	20 mM Phosphate pH 6.5 100 mM NaCl 5 mM Methionine		
Gradient	Time (min)	% MPA	% MPB
	0	65	35
	12	35	65
Flow Rate	1 mL/min		
Detection	UV @ 260 nm		
Temperature	Ambient		

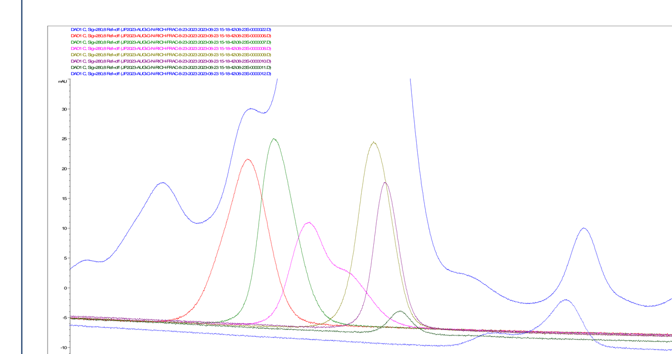
Results and Discussion

By comparing the first switch chromatogram with the last switch chromatogram, it is clear that the early eluting and late eluting variants were successfully enriched. The enriched material was chromatographed with a long, shallow gradient and the eluent was collected as a series of fractions.

First Switch

Last Switch

These fractions were evaluated without concentration using a typical analytical UV-HPLC system. The concentrations were sufficient that even minor impurities, barely detectable in the crude, could easily be detected as an isolated fraction. Several variants that eluted under the early part of the main peak and were not discernable with the accumulation chromatography, were sufficiently concentrated.



Overlay of UV-HPLC chromatograms for isolated variants that eluted under the early part of the main peak

The entire experiment with N-Rich technology took approximately 7 hours to complete.

Batch Chromatography Run	45 min*
N-Rich Wizard	30 min
Start Method	15 min**
5 cycles N-Rich	4 hrs
No load Run	30 min
Total:	**7 hrs**

* After the chromatography has been worked out
** Not including the HPLC fraction analysis (~ 6hrs)

Conclusions

N-Rich is a very effective technique for the isolation of related substances in pharmaceutical materials. It is particularly advantageous for mAb and protein work since they are not synthesized compounds and isolation is the only way to obtain related material for characterization and standards. N-Rich saves significant amounts of time and effort by automating the accumulation of isolated materials and producing fewer fractions to be analyzed. The fractions from the N-Rich technique are significantly more concentrated than typically produced by prep chromatography. This technique can easily be applied to peptides and oligos. Some small molecules can work with the N-Rich technique. The main factor that determines if a small molecule will work with N-Rich is if the components eluting from one column can be diluted to an eluent composition where they will stack on the head of the next column.

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