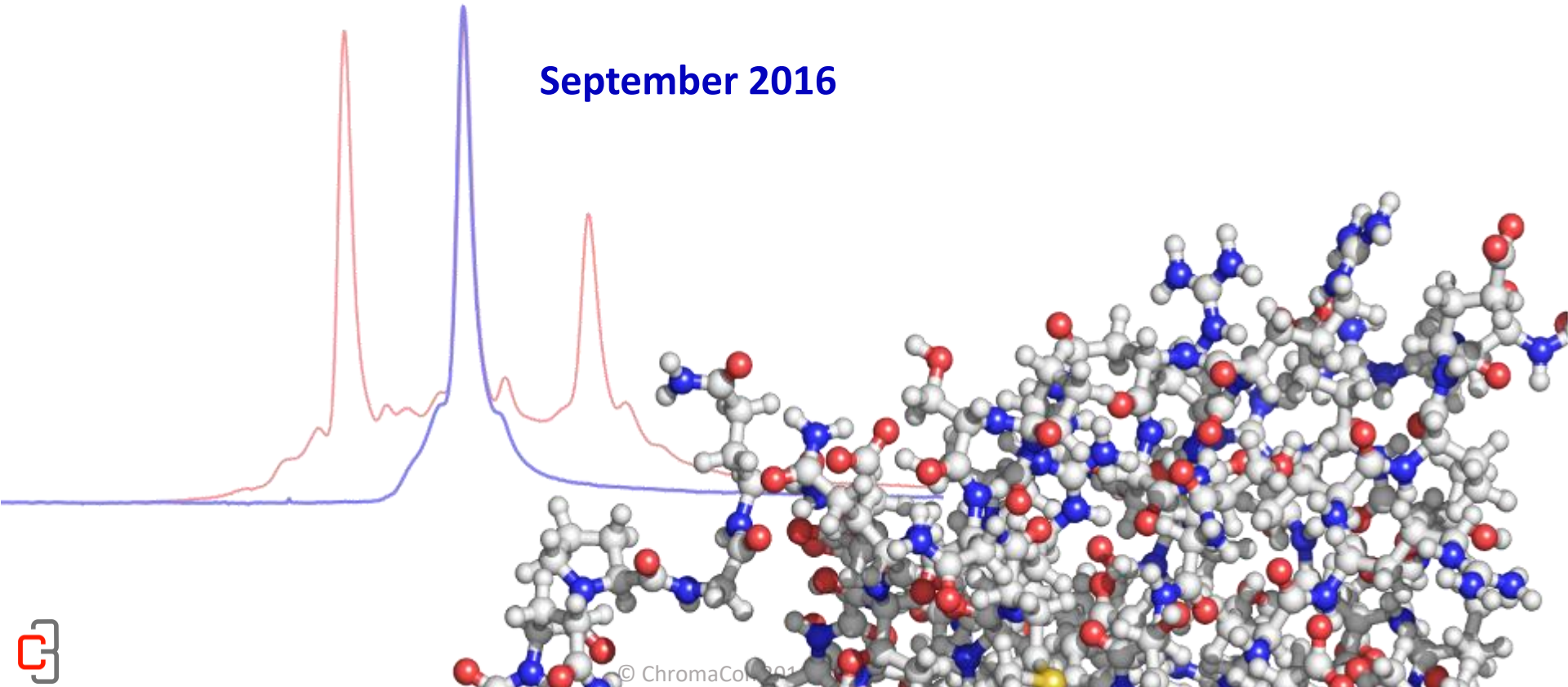
ChromaCon®
A new dimension in purification

N-Rich®: A tool for efficient isolation of minor compounds from complex matrices

September 2016



Outlook

N-Rich® has a wide range of applications, including:

- rapid isolation of product-related compounds for pre-clinical research, stability and formulation studies
  - Monoclonal antibody isoforms
  - Antibody conjugates
  - Biosimilar isoforms
  - Blood plasma proteins
  - Other API, natural products
- preparation of analytical standards
- mining and isolation of natural products with therapeutic potential
- mining of biomarkers, proteomes or metabolomes:
  as general sample preparation tool with MS/MS detection
- isolation of therapeutic targets for development of bioassays

N-Rich Introduction

- N-Rich is a powerful preparative chromatographic periodic countercurrent process allowing to enrich minor components from complex mixtures and at the same time deplete major components of the mixture that may interfere in the isolation of the minor component of interest
- N-Rich achieves unparalleled enrichment and separation capabilities only comparable to high resolution analytical HPLC but with the advantage of isolating preparative amounts of compounds within a short time
- A twin-column HPLC/FPLC system configuration (Contichrom®) allows to run N-Rich efficiently
- Conventional resin material such as IEX, HIC, RP-C8, etc. can be used. No need for any type of specific affinity step or pre-fractionation
- N-Rich applications can be grouped for:
  - ✓ Isolation of product-related impurities for pre-clinical research, stability studies and formulation studies (when the product is known)
  - ✓ Discovery applications: screening for biomarkers, of proteomes or metabolomes (when the compounds of interest are not yet known)

Use of N-Rich for Isolation of Protein Isoforms

- Regulatory requirements (ICH Q6B and ICH Q3A (R2) guidelines) require the isolation and characterization of product-related impurities
- Follow-on biologics (Biosimilars) need to be as close as possible to their originator product in order to be eligible for biosimilarity and for product interchangeability claims. The analytical identity to a standard is an important starting point
- Biological products contain isoforms, based on post-translational events, ageing and stress. Those isoforms may change the safety and efficacy profile and they therefore need to be isolated and characterized.
- Isoforms being closely related to the product are difficult to isolate in substantial amounts for the required characterization assays
- Repetitive analytical injections (several hundred) are frequently used to isolate product-related impurities. The process is tedious and may stress the isolated impurity during the isolation process

Use of N-Rich for Isolation of Protein Isoforms (cont'd)

- A long impurity isolation process may change the nature of the impurity itself during the isolation process, making the isolated impurity non-representative

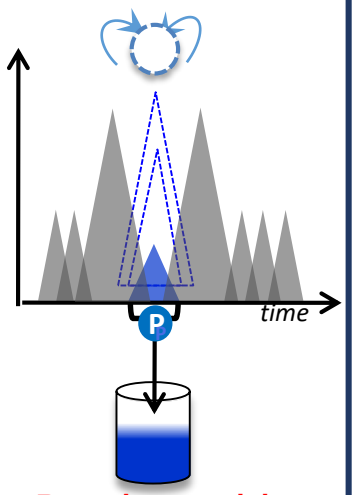
- N-Rich is a twin-column chromatography process providing highly enriched material from complex samples without the use of any specific resin material
  - ✓ in a short period of time (overnight)
  - ✓ automatically with minimal handling effort

N-Rich for Biosimilars

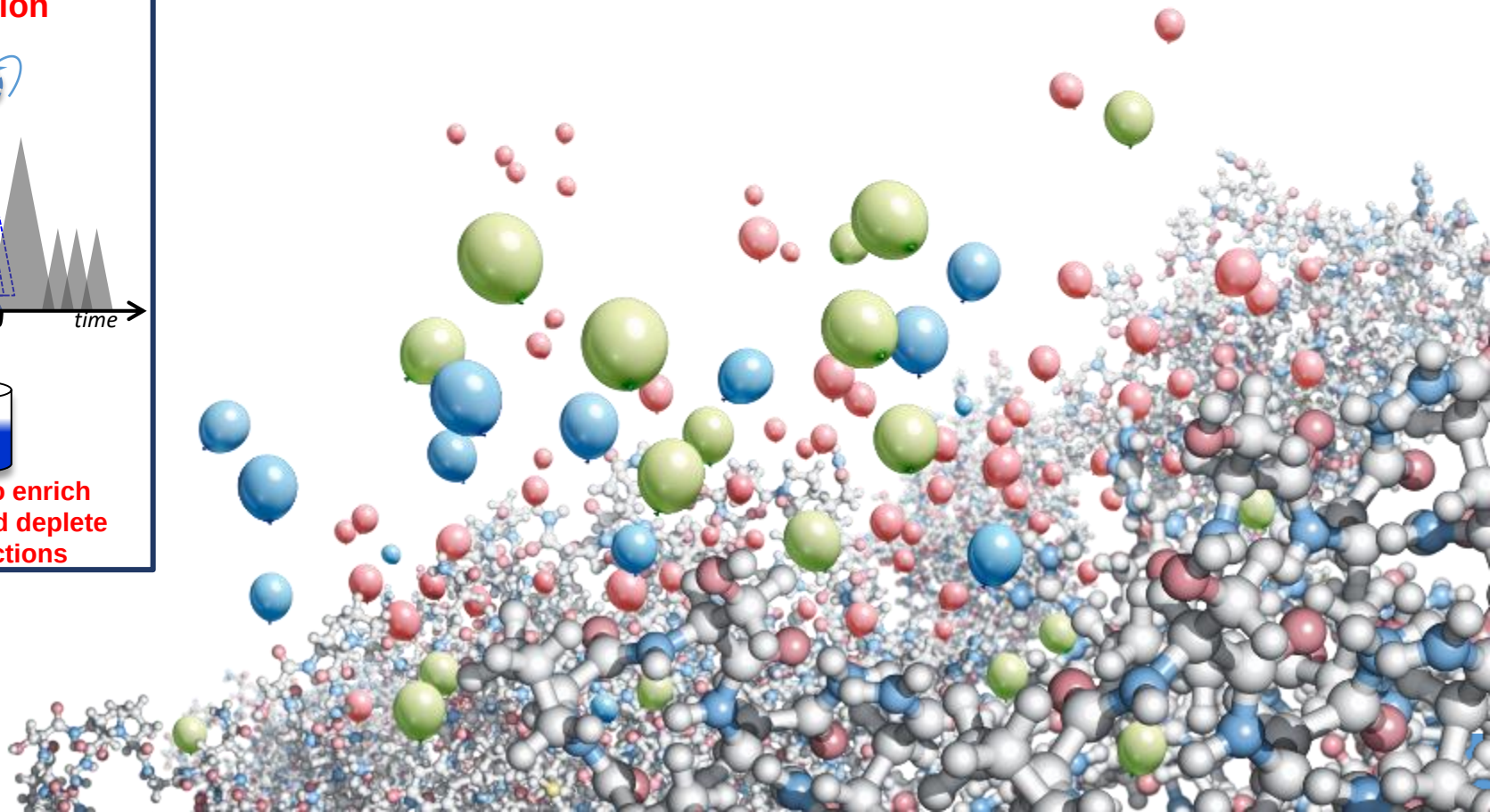
- A biosimilar has to be defined stringently as a product that is very similar to an originator product
- Analytical similarity data is the foundation of biosimilar development – understanding the relationship between quality attributes and the clinical safety & efficacy profile aids to determine residual uncertainty about biosimilarity and to predict expected “clinical similarity” from the biosimilar product quality data.
- Biosimilarity is thus defined initially through the product composition compared to a standard and only later confirmed through safety data
- Separation of the product composition is the main task before product component characterization and comparison to the originator product
- ChromaCon has used N-Rich successfully to isolate Biosimilar product-related impurities for industrial customers, satisfying requests from regulatory bodies including FDA

The N-Rich® Process and its Applications

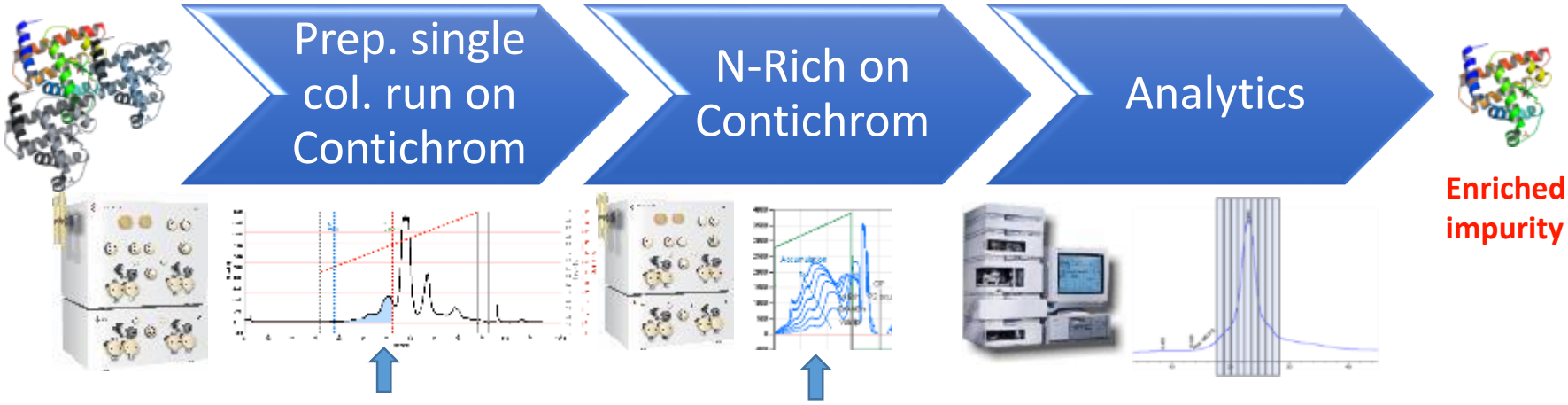
Cyclic enrichment before gradient elution



Recycle to enrich product and deplete side fractions



N-Rich process using Contichrom® CUBE equipment

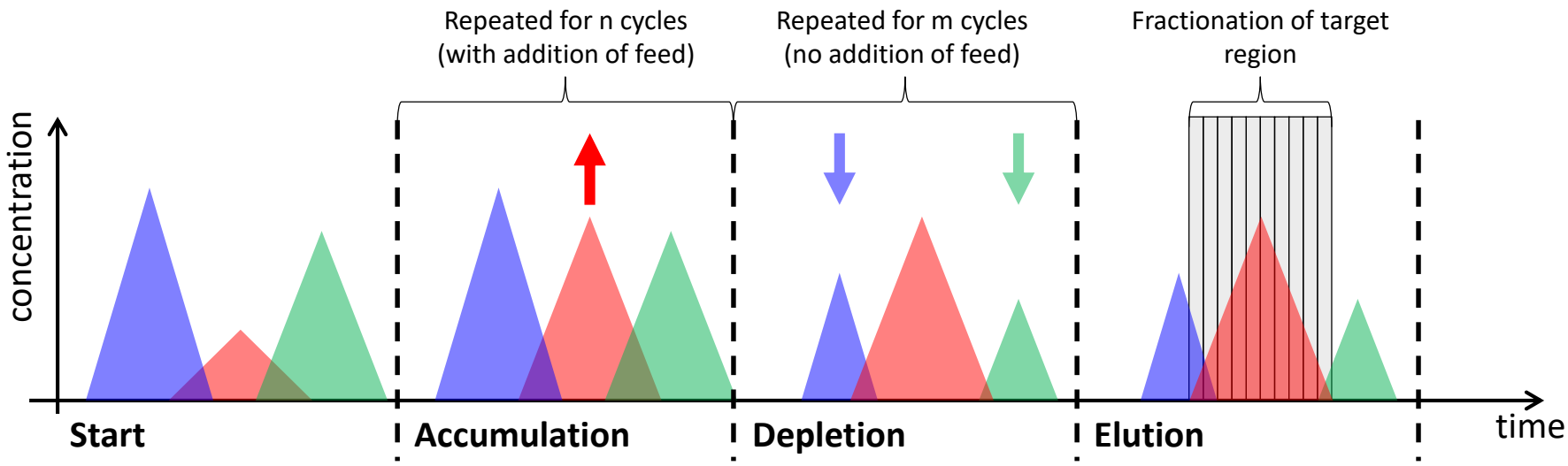


Contichrom runs multiple cycles automatically to enrich side compounds

- ❑ Avoid tedious collection of fractions from analytical runs
- ❑ N-Rich® single run to enrich side components or isoforms AND deplete undesired main component overnight
- ❑ Save weeks of tedious repetitive work when using analytical HPLC for preparative isolation tasks
- ❑ Preserve integrity of desired component through fast isolation

How does the N-Rich process work?

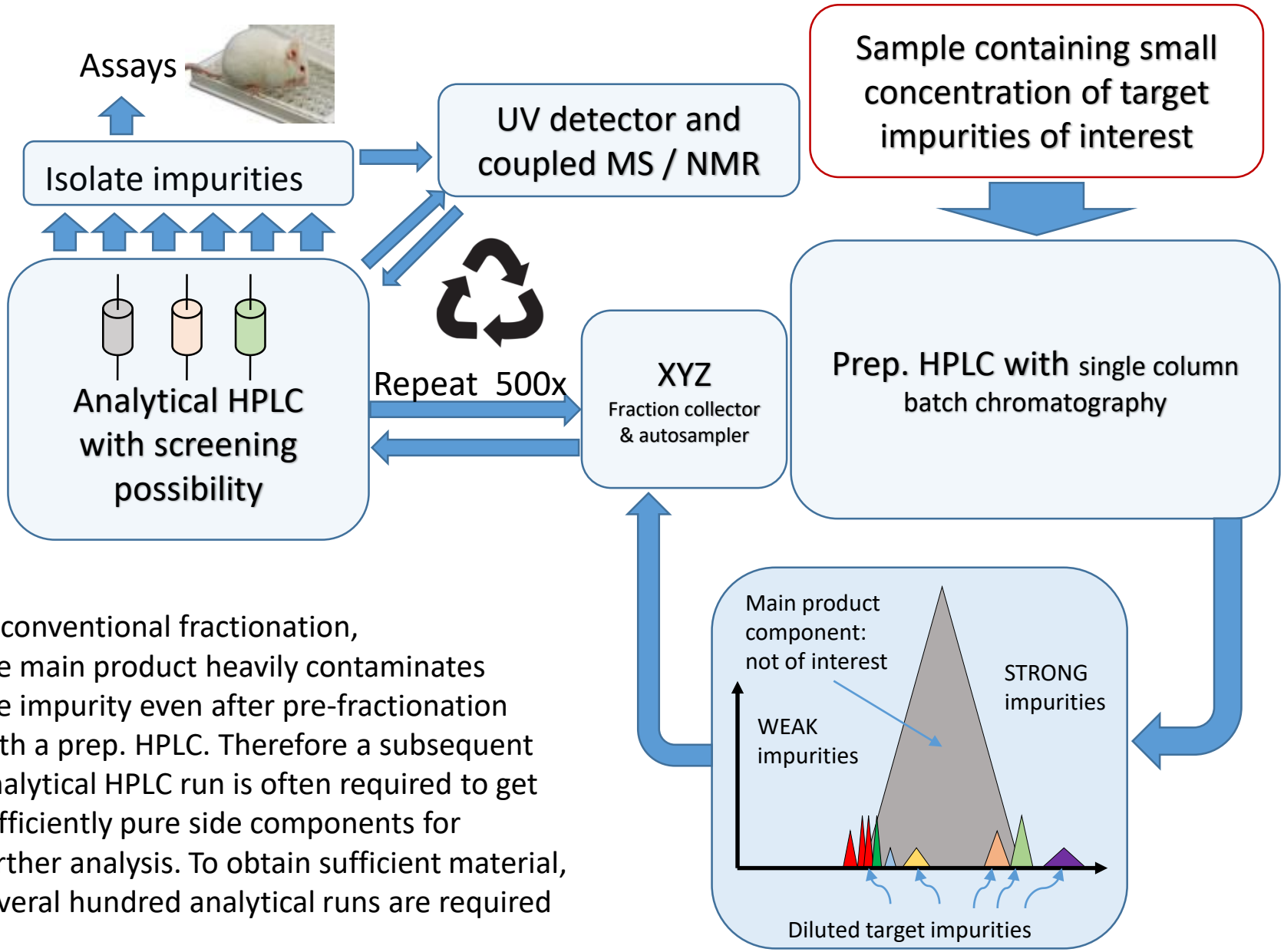
- Process scheme: N-Rich consists of 3 sub-processes



Start	Accumulation	Depletion	Elution
Small amount of **T**arget between **W**eakly and **S**trongly absorbing other components	Increases concentration of **T** →Relative decrease of concentrations of **W** and **S**	Decreases concen-trations of **W** and **S** →Relative increase of concentration of **T**	With shallow gradient and fine fractionation

- The process operates with two identical columns packed with the same resin

Discovery Applications: Conventional Fractionation



In conventional fractionation,
the main product heavily contaminates the impurity even after pre-fractionation with a prep. HPLC. Therefore a subsequent Analytical HPLC run is often required to get sufficiently pure side components for further analysis. To obtain sufficient material, several hundred analytical runs are required

Discovery Applications: N-Rich Fractionation Automated

Sample containing small concentration of target impurities of interest

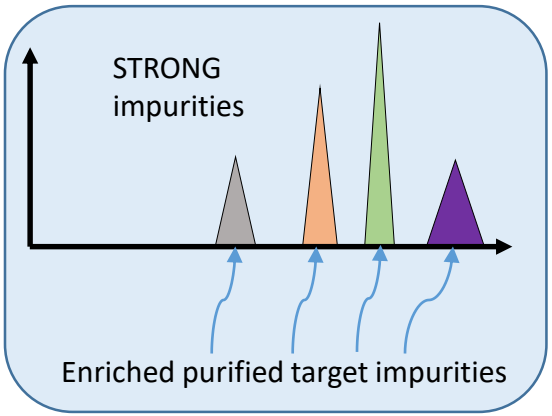
N-Rich® Process

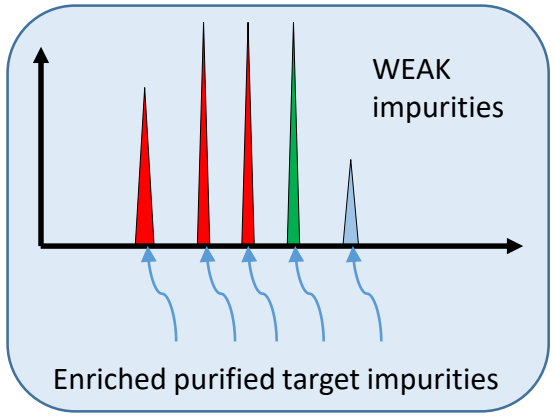
UV detector and coupled MS / NMR

XYZ
Fraction collector & autosampler

Cell/ Animal Assays

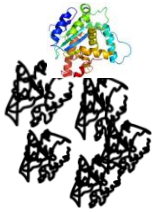
With N-Rich we have a generic sample preparation tool that provides a preparative high resolution separation
Using only one instrument allowing to obtain sufficient purified material with one run





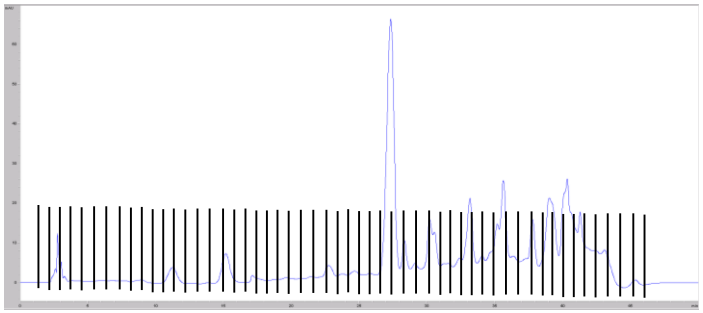
N-Rich for Discovery Applications

Automated Proteome screening with N-Rich®



Proteomic
protein mixture

Iteratively enrich selectively for
unknown lead in each fraction using N-Rich

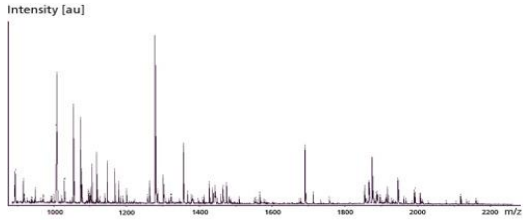


peptide fragments

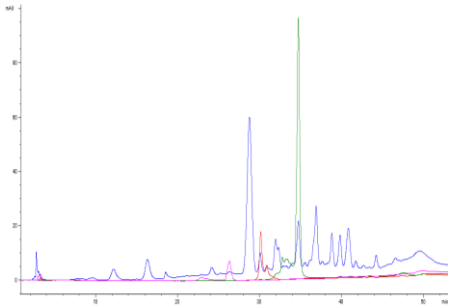
Bottom-up
approach

whole protein

Top-down
approach

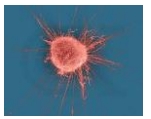


MS/MS Spectrum



Repeat 1D LC with online selective
enrichment of another rare species
until all peaks sequenced

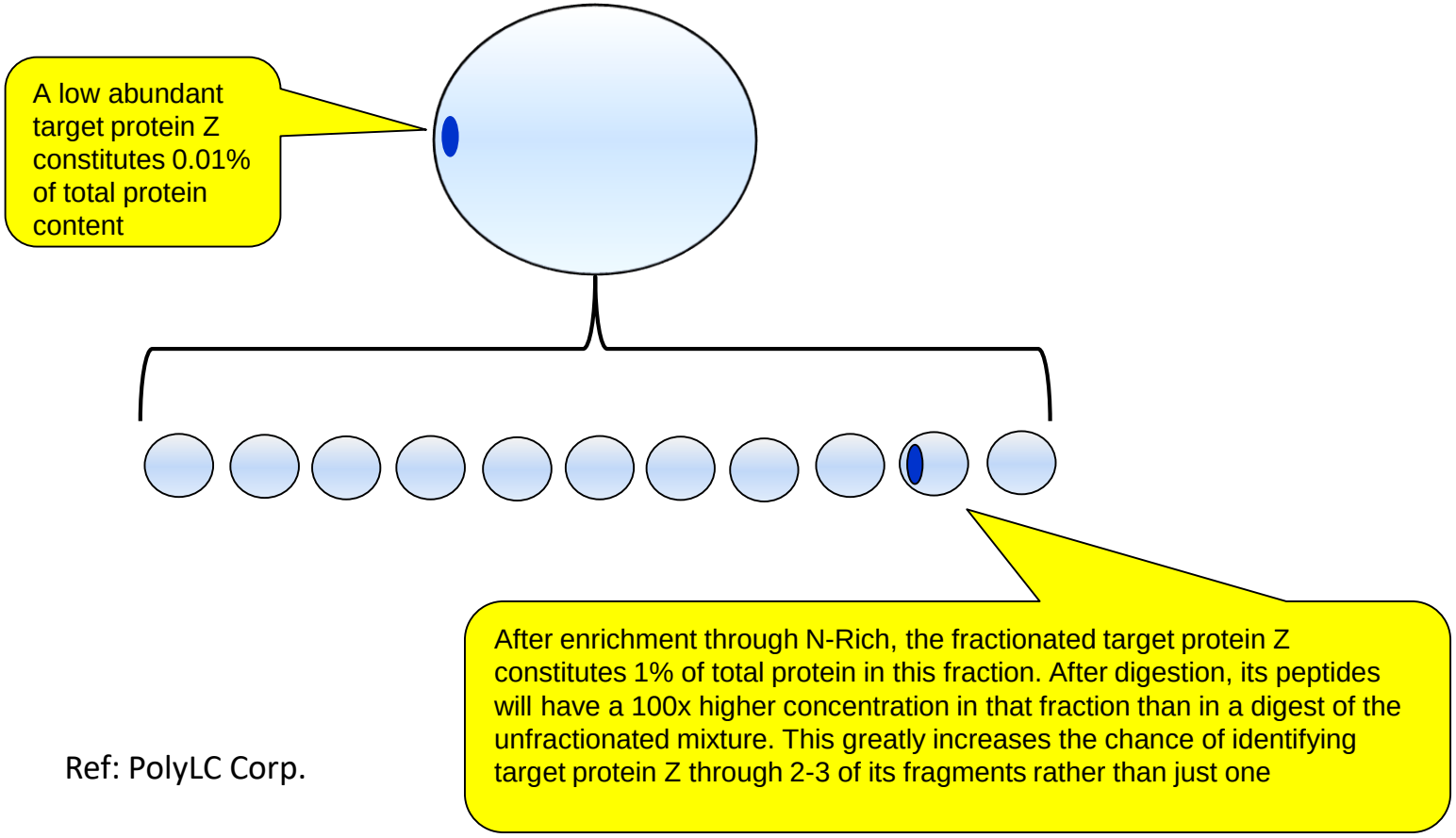
Match against database



Isolate rare species
for functional
characterization

Example: Fractionation of a proteome

- The fractionation of intact proteins of a proteome is necessary because it results in an increased likeliness of detection of proteins of low abundance



Ref: PolyLC Corp.

Example: Fractionation of a proteome

ASSUMPTIONS:

A: 5'000 proteins in the mixture yielding 40'000 tryptic fragments

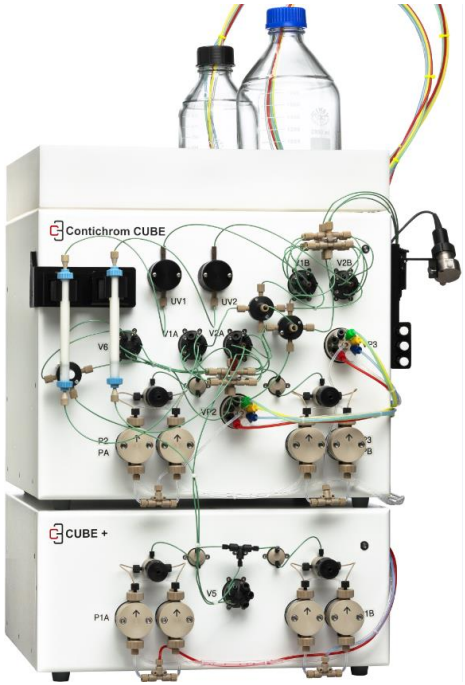
B: the average M_w of the proteins is 30 kDa

C: at least 15 fmol are needed to obtain a peptide sequence by MS/MS

- This means 450 pg of each protein x 5000 proteins = 2.25 µg of total protein needed
- Unfortunately proteins differ by 10^6 in relative abundance and a few proteins dominate the sample. Therefore in order to get 15 fmol of the least abundant protein you need to have at least 10^5 more sample
- 2.25 µg x 10^5 = **225 mg TOTAL PROTEIN NEEDED**
- This means that you need to collect and process 500+ fractions with a strong cation exchanger (SCX) step using analytical HPLC
- With N-Rich the same task can be achieved in a much shorter time!

Ref: Andrew J Alpert PolyLC Inc.

Hardware and Software



The Contichrom® CUBE Combined FPLC/ and HPLC System

Specifications:
CUBE/HPLC 30: 0.1-36 mL/min
CUBE/HPLC 100: 0.1-100 mL/min
UV-LED Detectors at 280 & 300 nm, optional 260 nm, optional variable wavelength detectors 190-500 nm
Pressure rating:
50 bar (FPLC)
100 bar (HPLC)

Cooling the feed and the fractions during operations

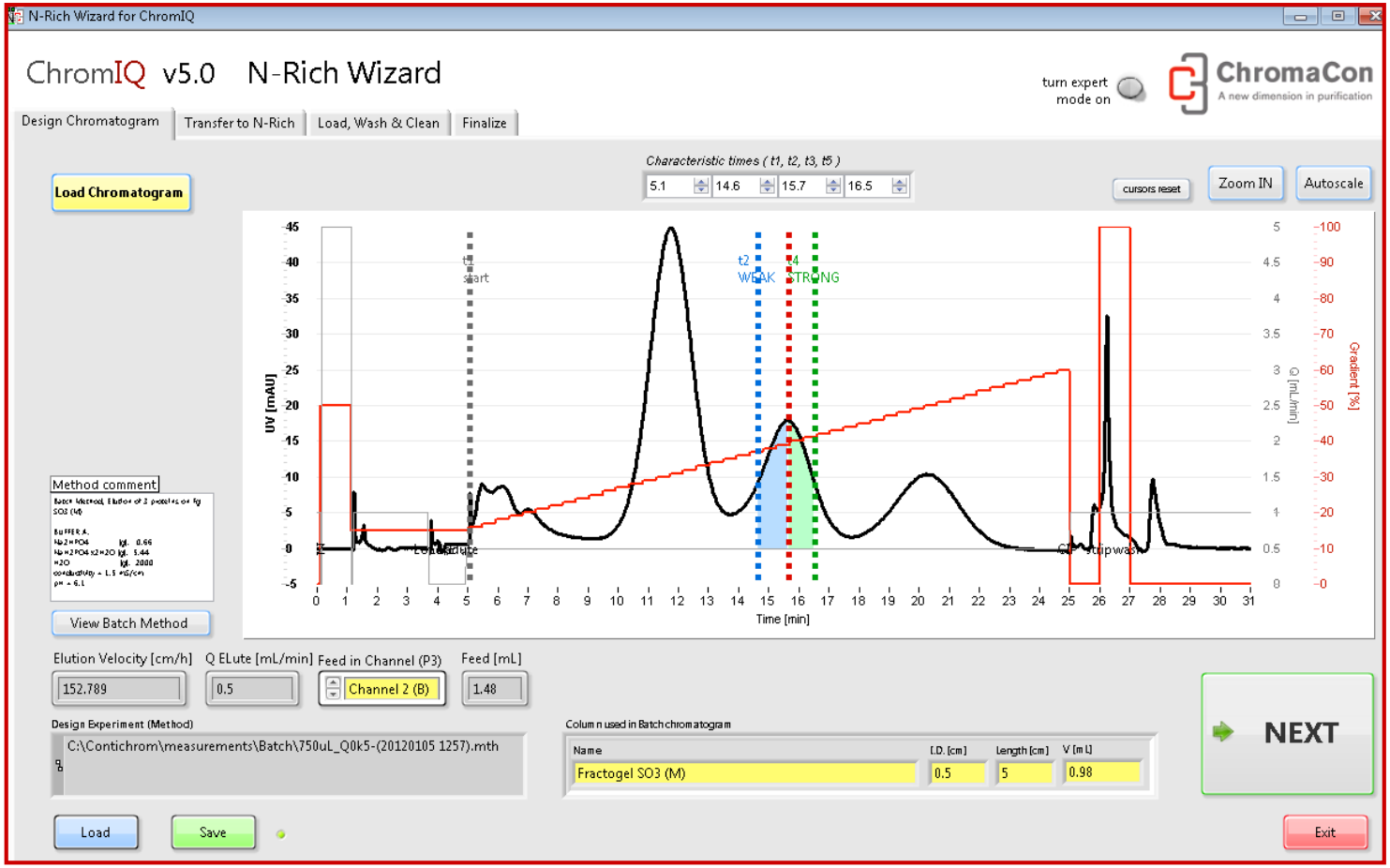
N-Rich Wizard: Process design in 4 easy steps

STEP 1:
Load chromatogram of batch run and select region to enrich by Drag & Drop

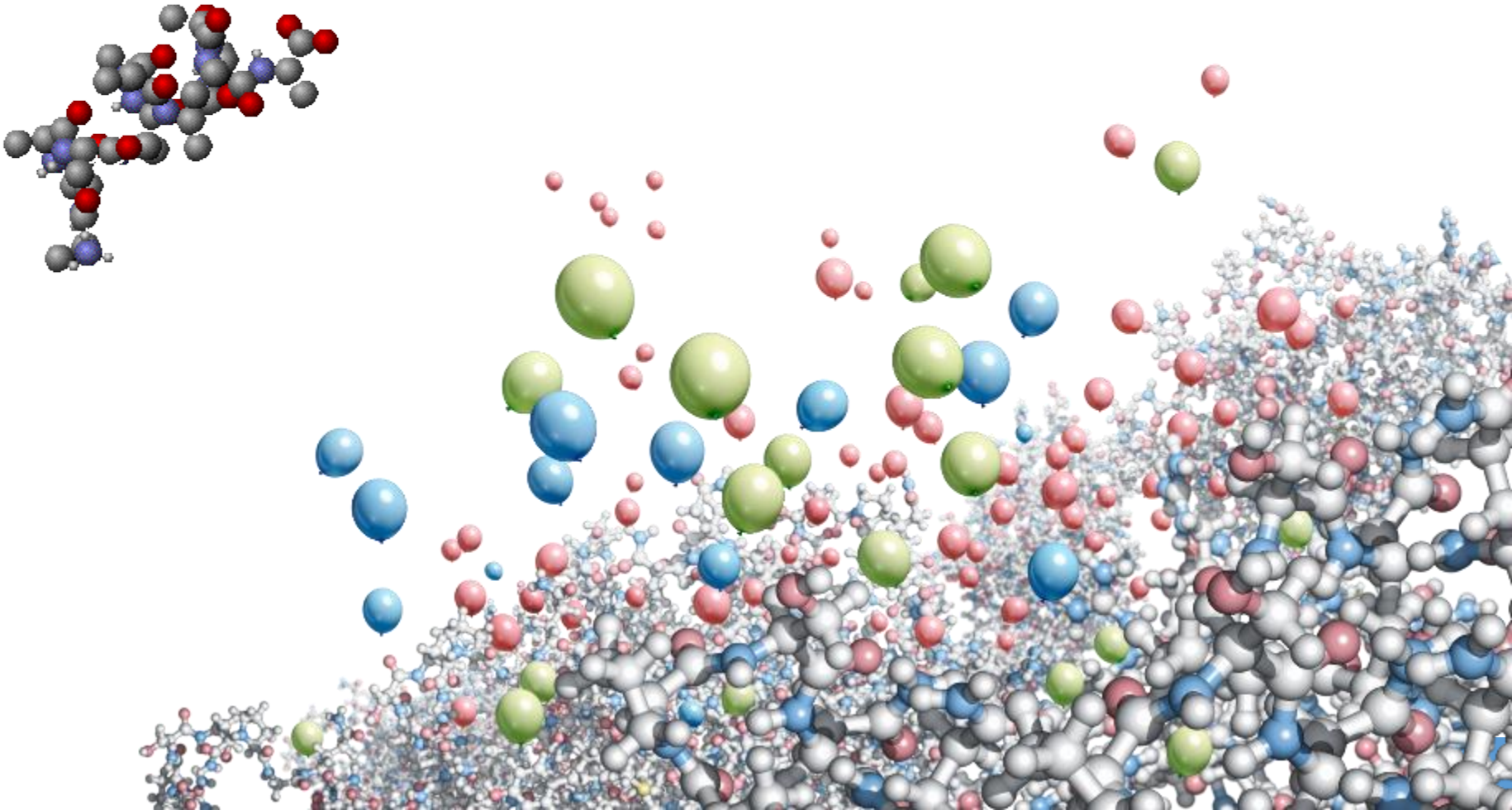
STEP 2:
Set column size

STEP 3:
Set desired washing and cleaning protocol

STEP 4:
Finalize method: Set number of cycles and fractionation

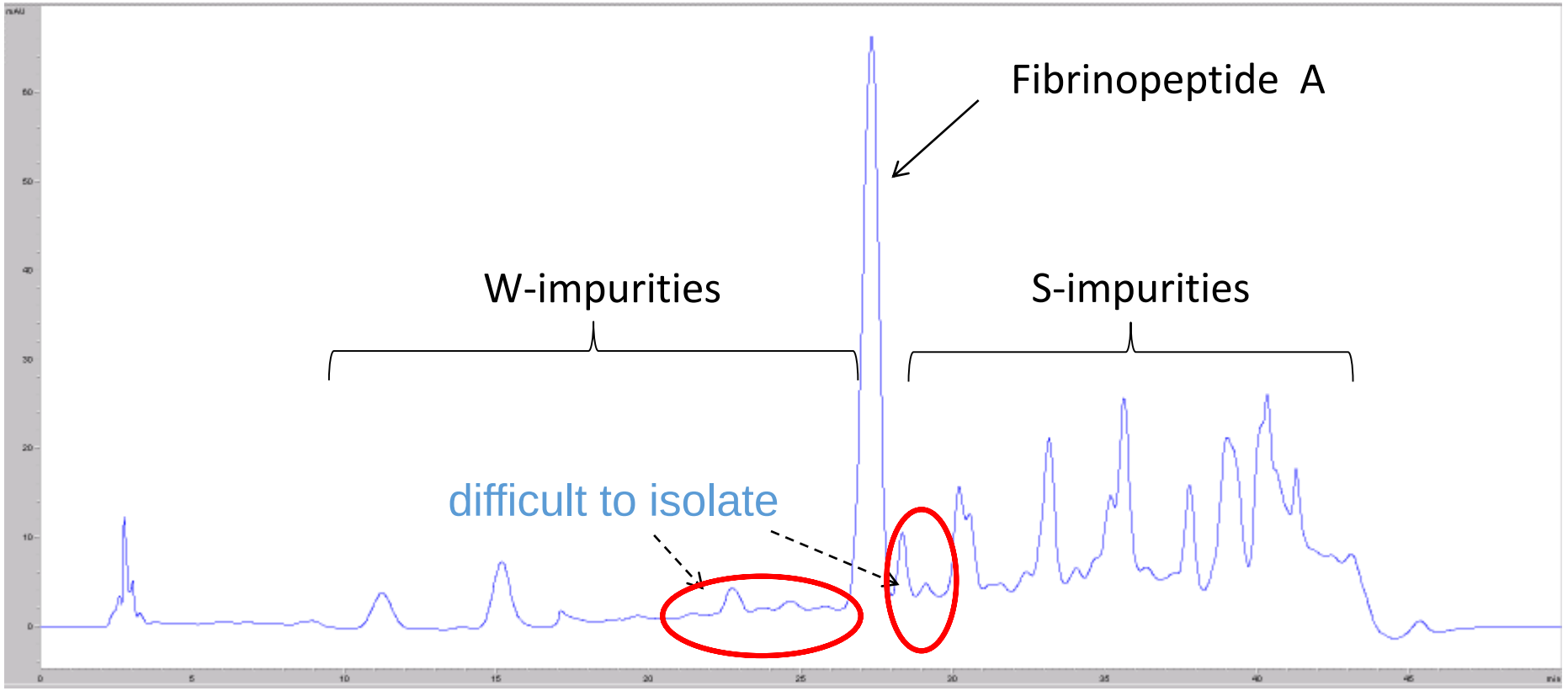


Case Study: N-Rich for isolation of a synthetic peptide impurity



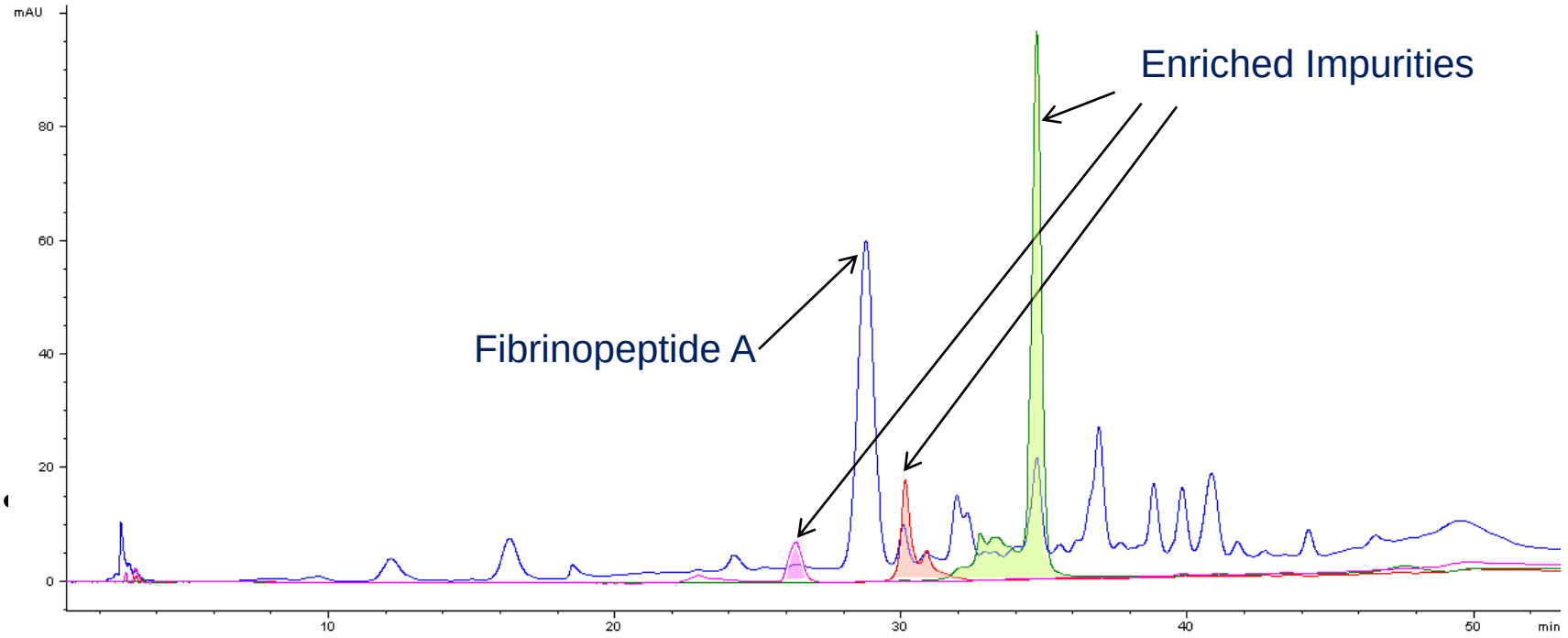
Case Study: Product-Related Impurity Isolation

- Fibrinopeptide A: Analytical injection 100 µL of Feed (3.0 g/L Fibrinopeptide) onto preparative RP-HPLC column



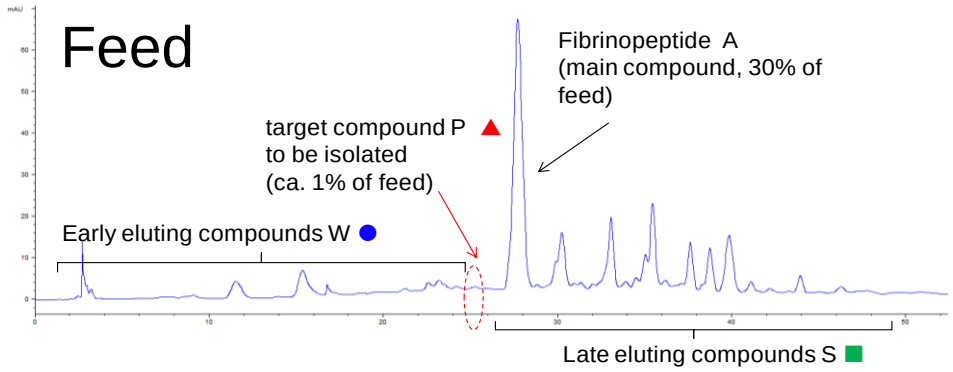
Case Study: Product-Related Impurity Isolation

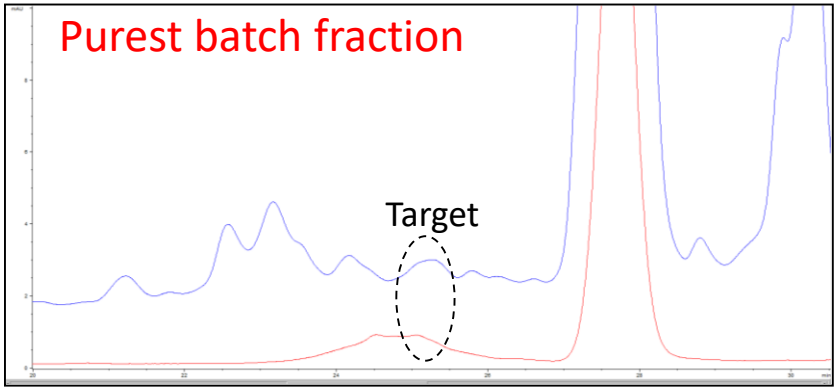
- Overlay of chromatogram of final gradient elutions (1min/ fraction)

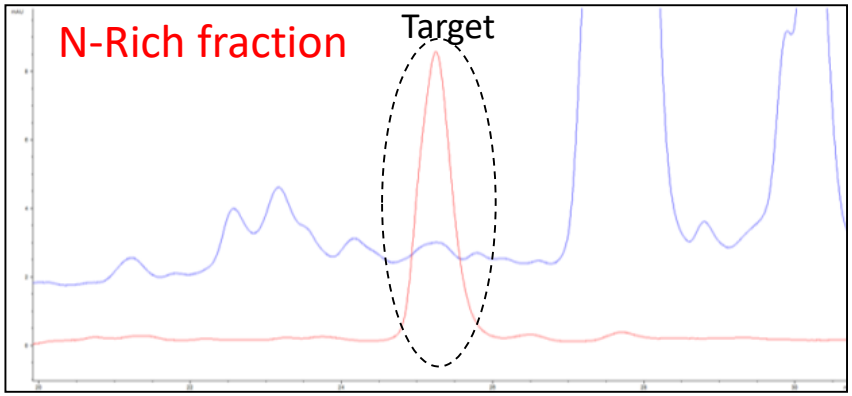


- Blue: feed
- Pink, red, green: product related impurities isolated with N-Rich

Case Study: Product-Related Impurity Isolation



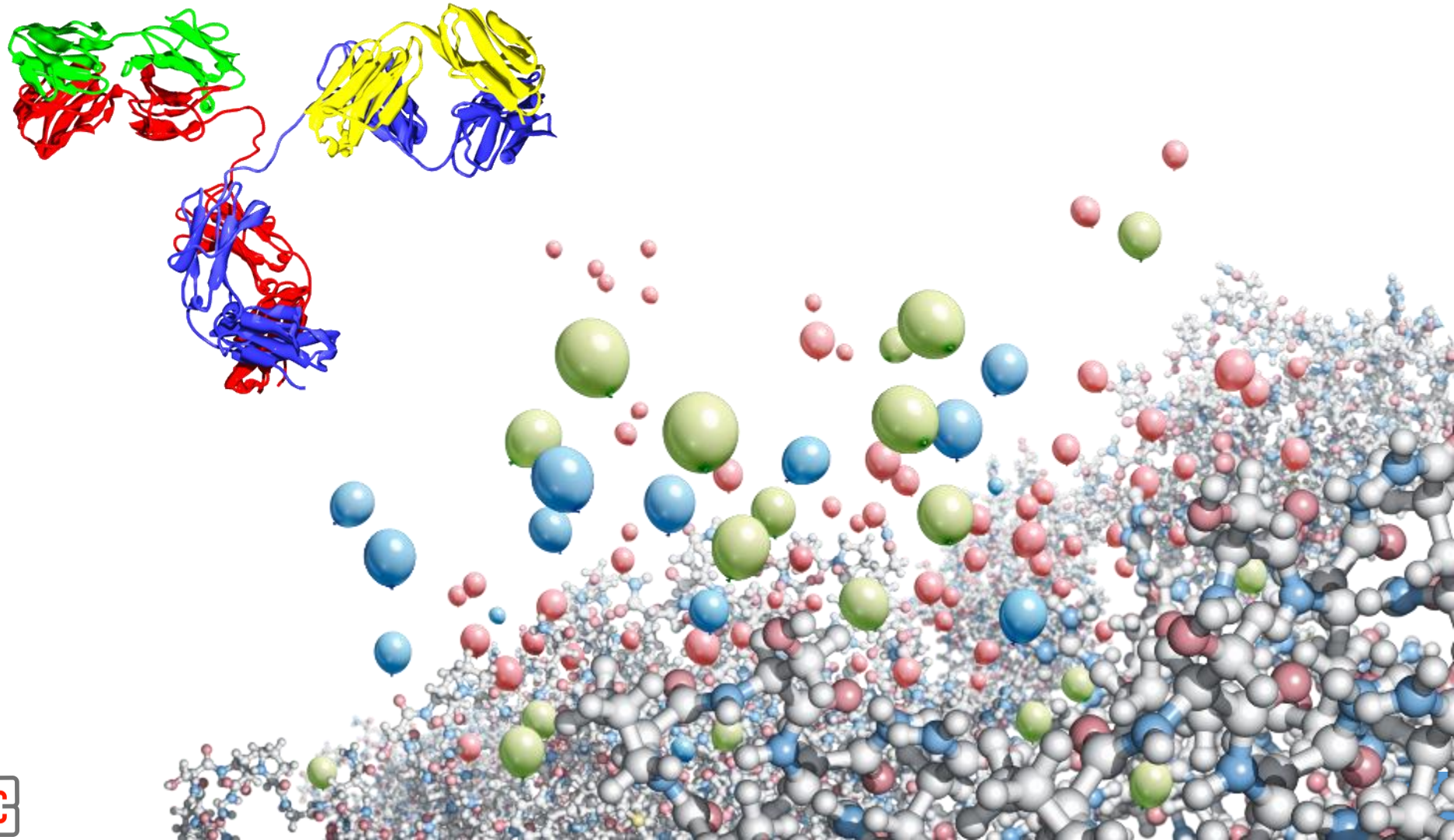




Process	Purity	Concentration improvement	Enrichment factor*
Batch	< 20%	1x	n.a.**
N-Rich	> 80%	10x	> 600x

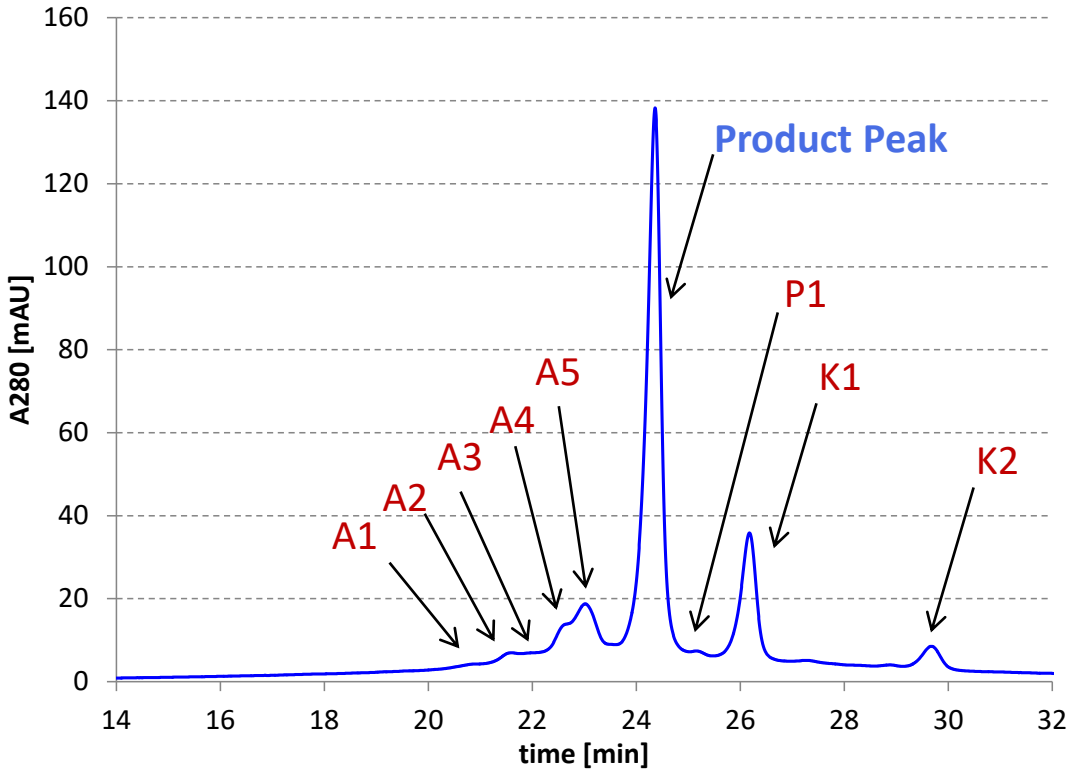
*with respect to main compound; **not available, because purity is too low.

Case study – Isolation of Biosimilar Antibody Isoforms



Case Study: Isolation of biosimilar mAb isoforms

- Industrial customer project: regulatory authority request for pre-clinical biosimilar impurity characterization
- Produce > 1 mg of 9 isoforms of a biosimilar mAb with > 90% purity

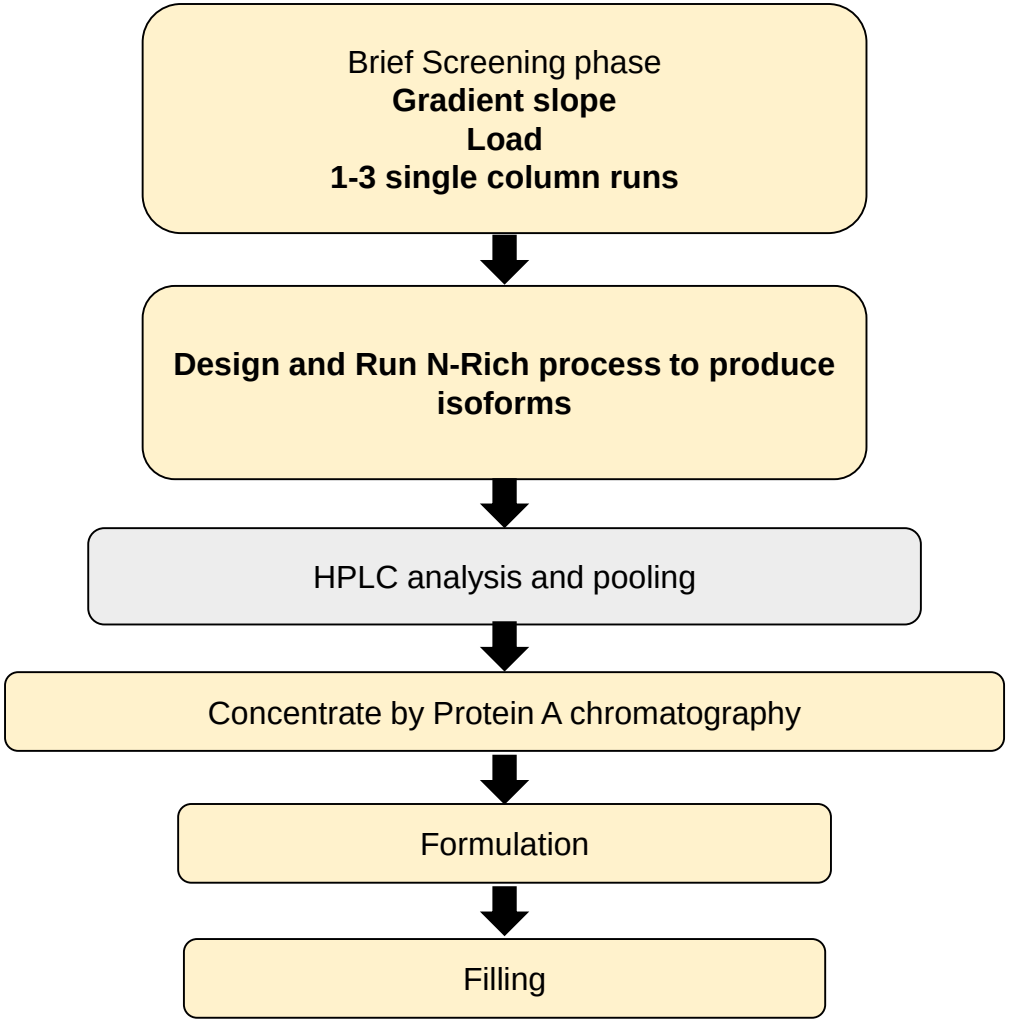


Isoform	in Feed
	[%]
A1	1.5%
A2	2.5%
A3	1.5%
A4	5.0%
A5	8.5%
K0	56.0%
K1	14.5%
P2	1.5%
K2	3.5%

Analytical
IEX chromatogram

Case Study: Isolation of biosimilar mAb isoforms

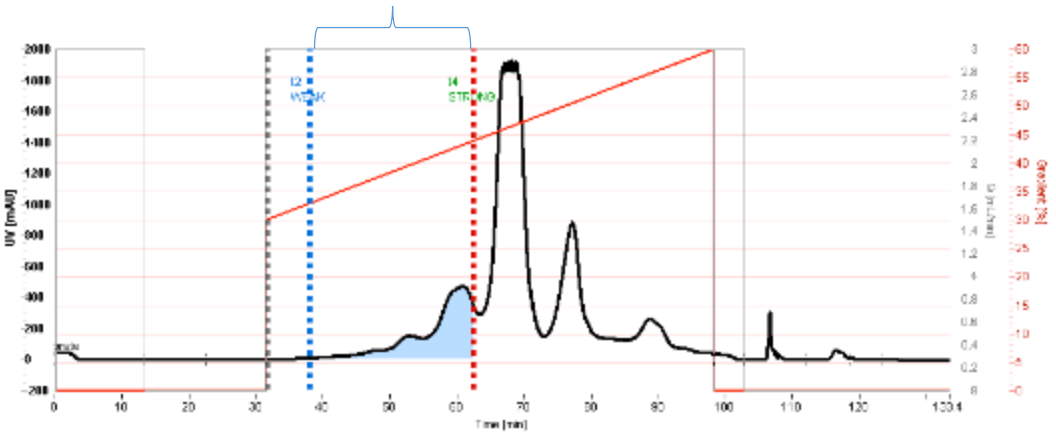
Workflow for isoform isolation



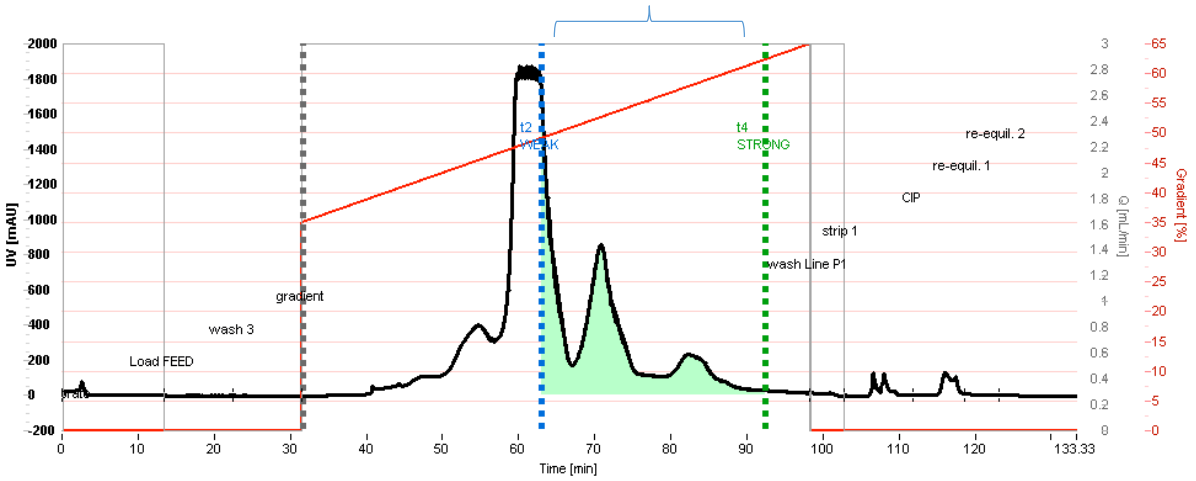
Case Study: Isolation of biosimilar mAb isoforms (cont'd)

Step 1: run a preparative CUBE FPLC run with a CIEX resin to identify early (left) and late (right) eluting isoforms)

Left region for enrichment

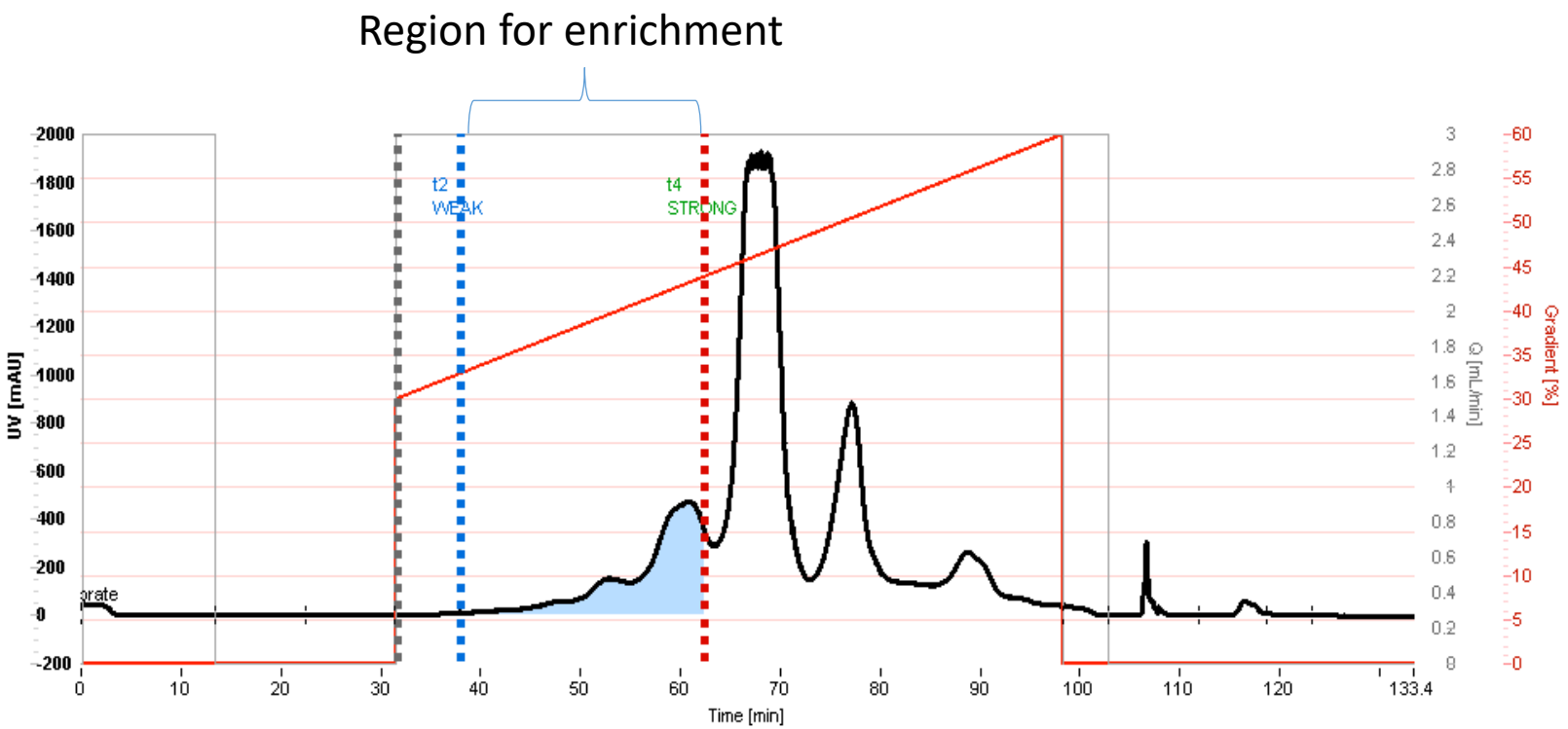


Right region for enrichment



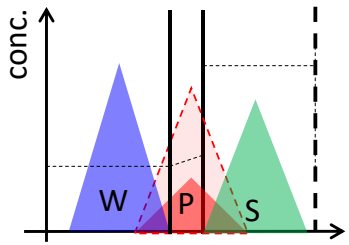
Case study: mAb isoform enrichment (cont'd)

Step 2: Upload the preparative batch from Step 1 and convert to N-Rich process in a guided way using the N-Rich wizard. Then execute pH gradient elution using a small particle CIEX resin.
The left region for enrichment chosen → corresponds to weakly adsorbing acidic isoforms

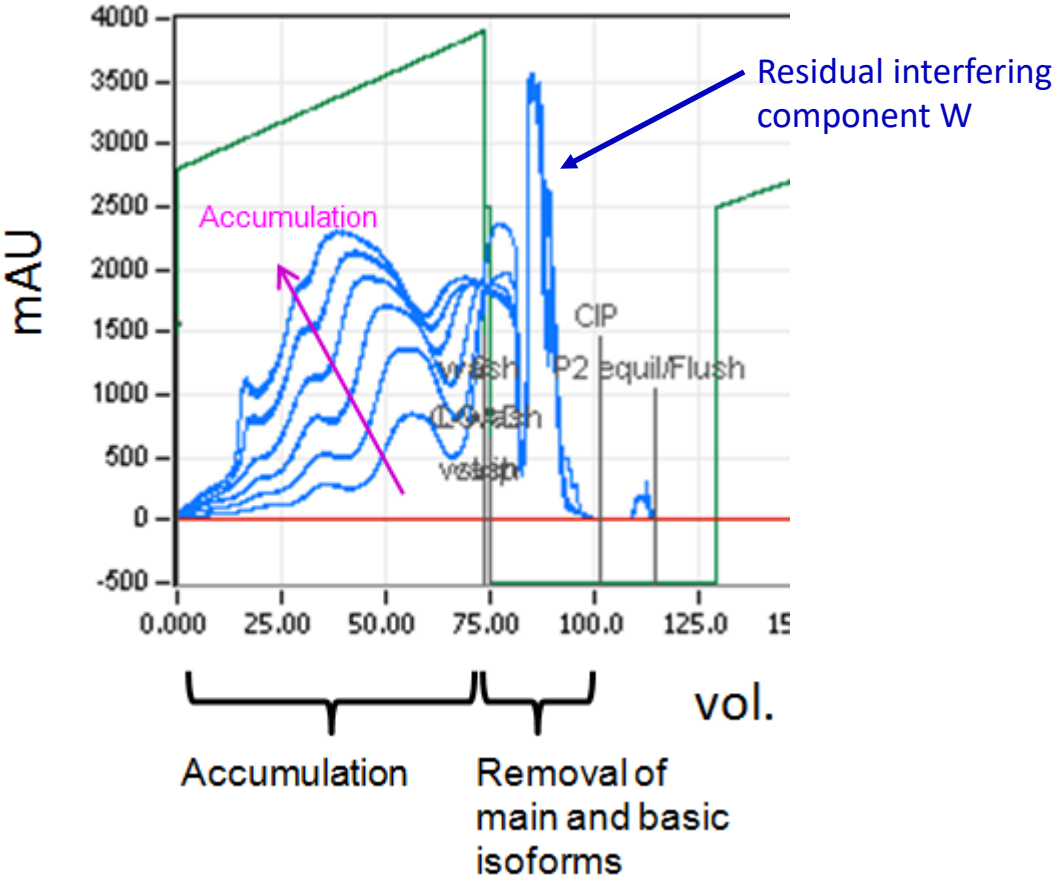


Case study: mAb isoform enrichment (cont'd)

Step 3 (automatic): Run N-Rich process for isolation of acidic isoforms overnight. Cycle overlay shows automated accumulation and enrichment of acidic isoforms P

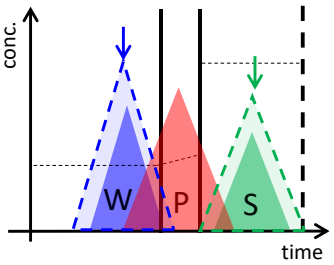


Accumulation
Increase concentration of P, absolute and relative to W and S.



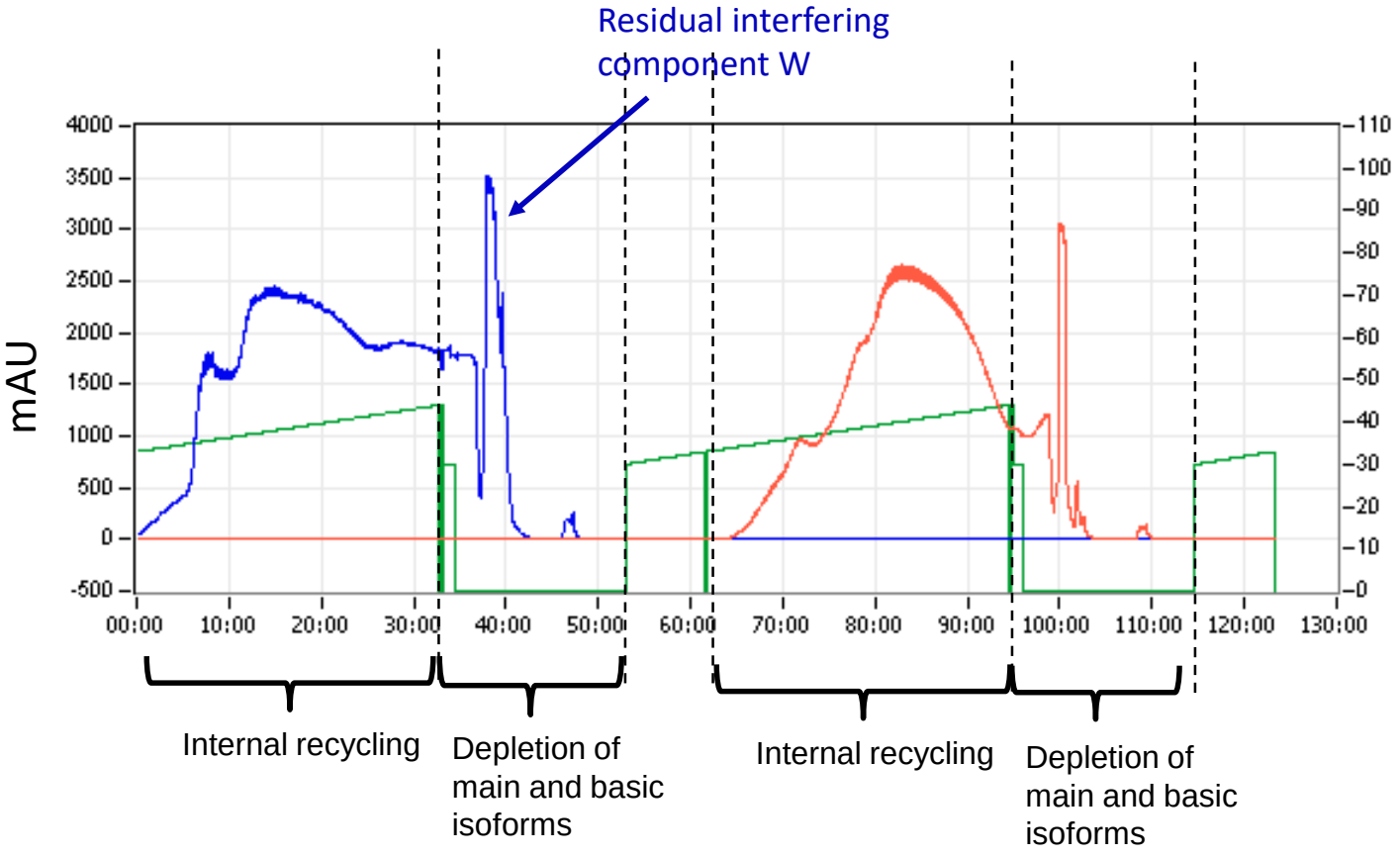
Accumulation phase: overnight

Case study: mAb isoform enrichment (cont'd)



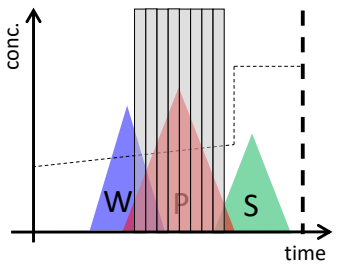
Separation
Increase conc. of P, absolute and relative to W and S.
Different gradient possible.

Step 4 (automatic): The residual interfering component W is depleted. In this phase no feed is added. The objective of this step is to get the accumulated P components pre-purified



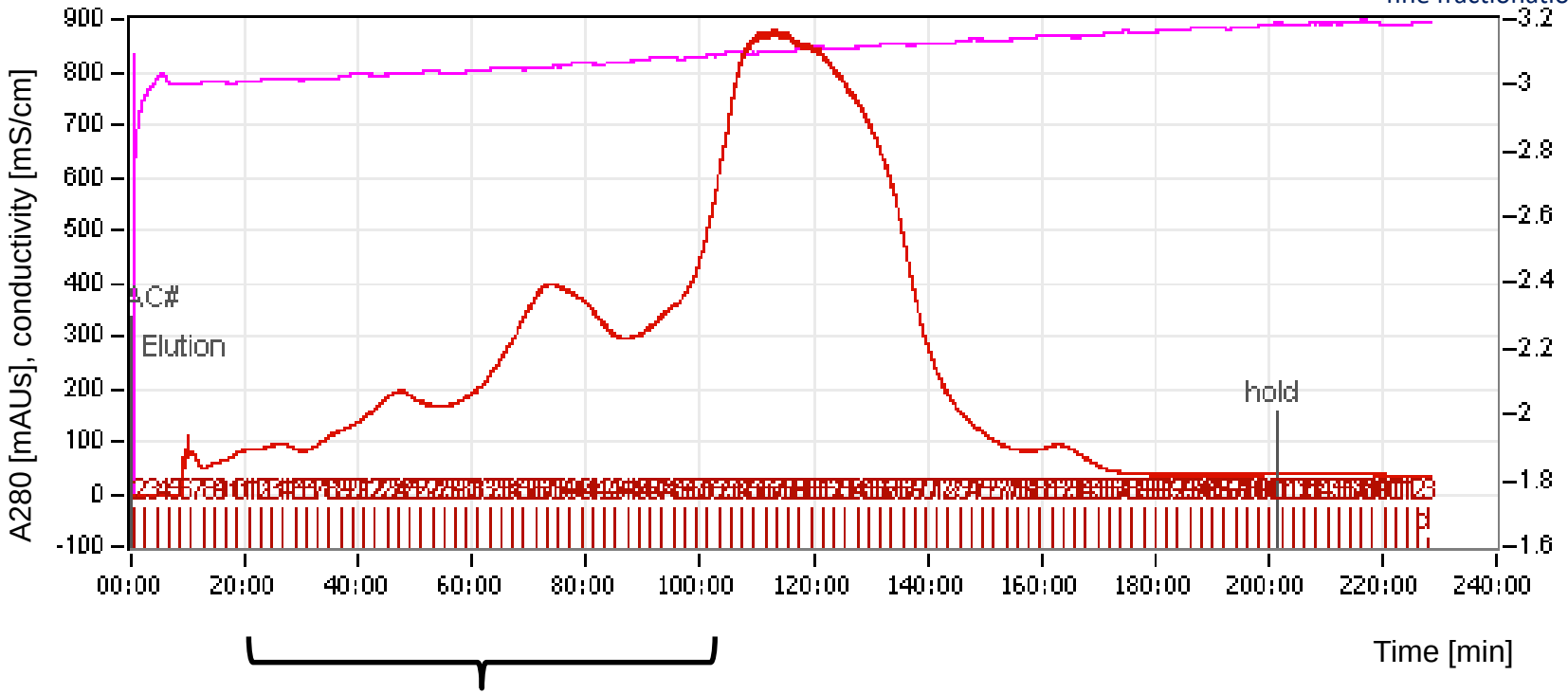
Total duration of sub-process: 3 h

Case study: mAb isoform enrichment (cont'd)



Elution
Final elution with shallow gradient and fine fractionation.

Step 5 (automatic): The pre-purified, accumulated component is further purified using a flat gradient obtaining the purified acidic isoforms



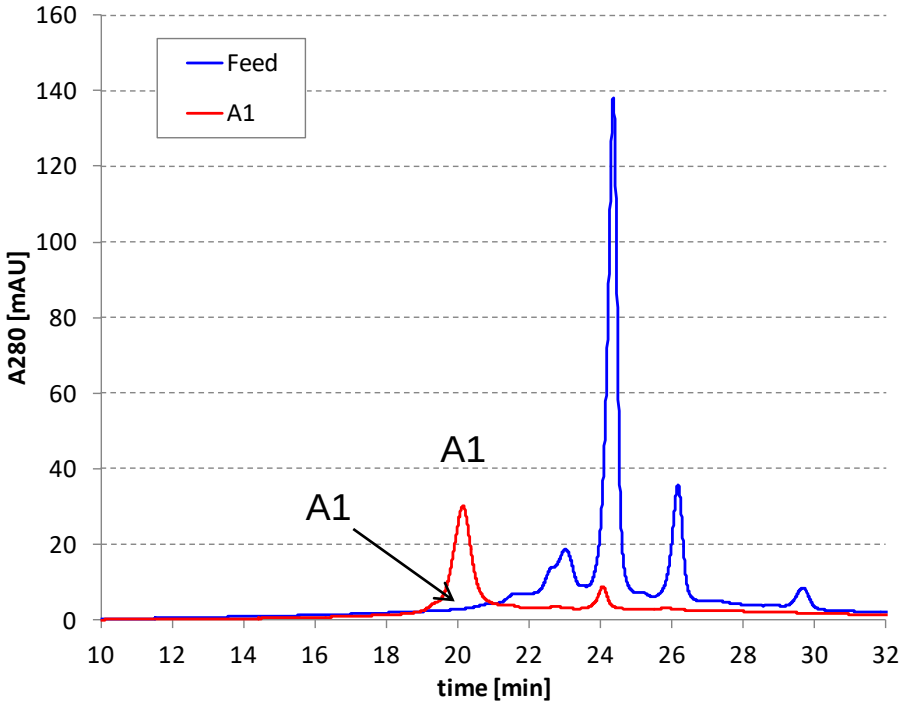
Enriched acidic Isoforms → Sample analysis, pooling and formulation

Total duration of sub-process: 4 h

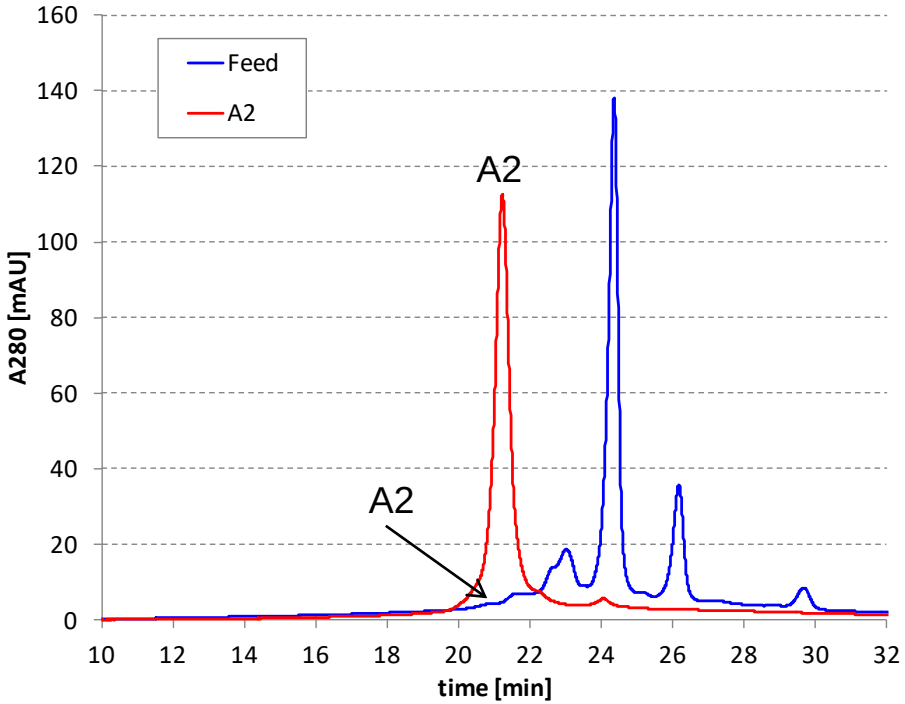
Case Study: Results of acidic isoform isolation using N-Rich

- Isoforms A1 and A2 enriched and isolated

Analytical
IEX chromatogram

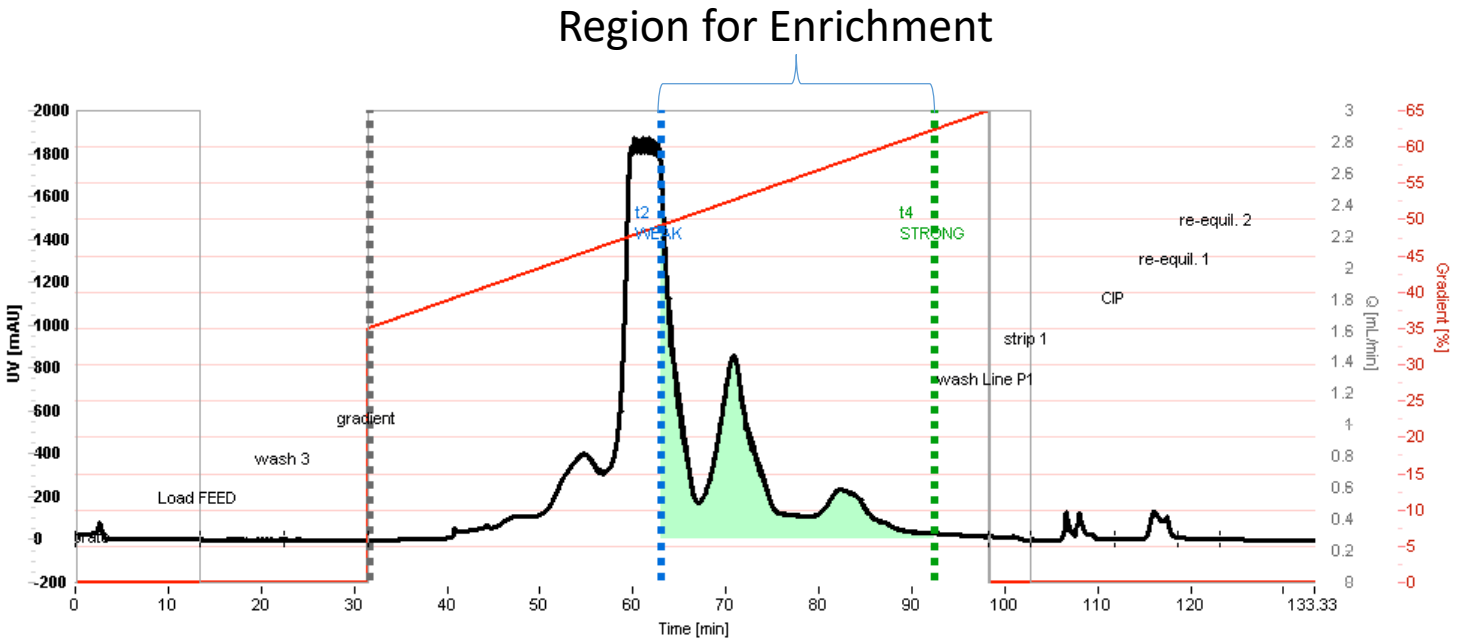


Analytical
IEX chromatogram

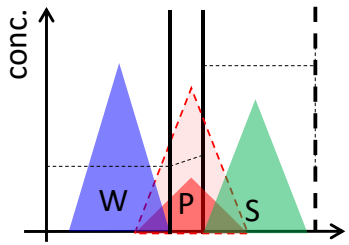


Case study: mAb isoform enrichment (cont'd)

Step 2 repeated for basic isoforms: Upload the preparative batch from Step 1 and convert to N-Rich process in a guided way using the N-Rich wizard. Then execute pH gradient elution using a small particle CIEX resin.
Theright region for enrichment chosen → corresponds to weakly adsorbing basic isoforms

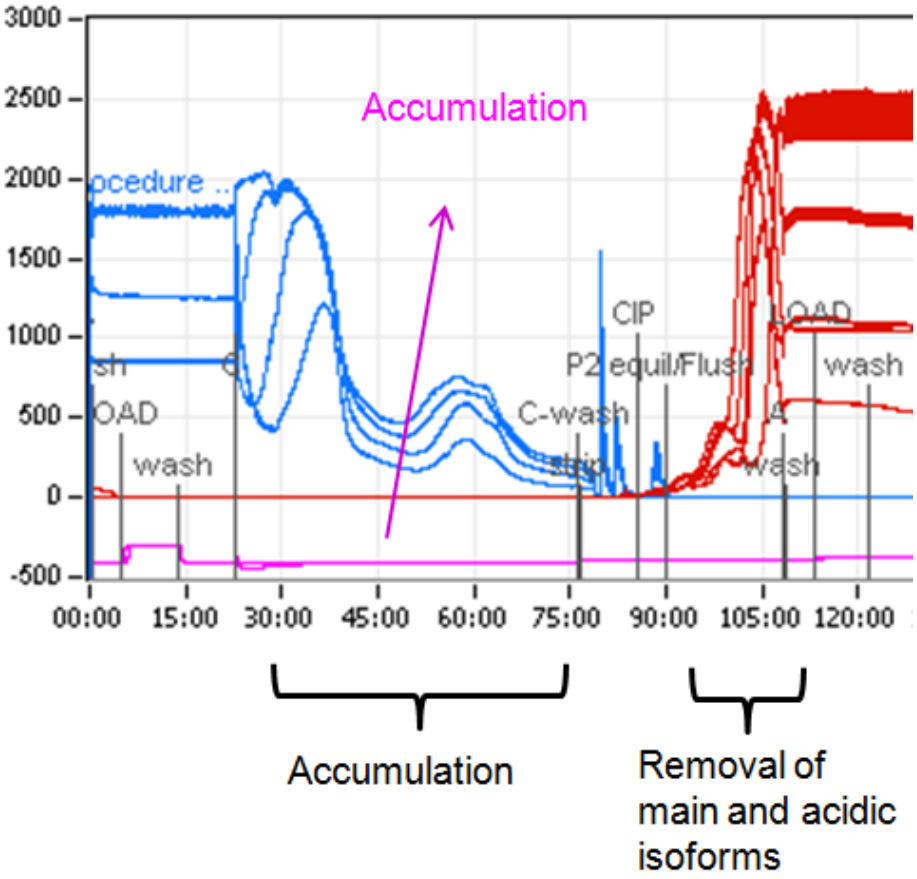


Case study: mAb isoform enrichment (cont'd)



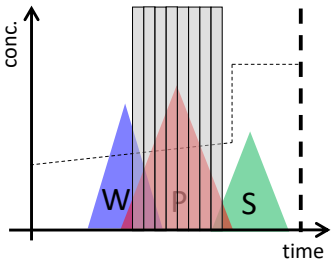
Accumulation
Increase concentration of P, absolute and relative to W and S.

Step 3 repeated for basic isoforms (automatic): Run N-Rich process for isolation of basic isoforms overnight. Cycle overlay shows automated accumulation and enrichment of basic isoforms P



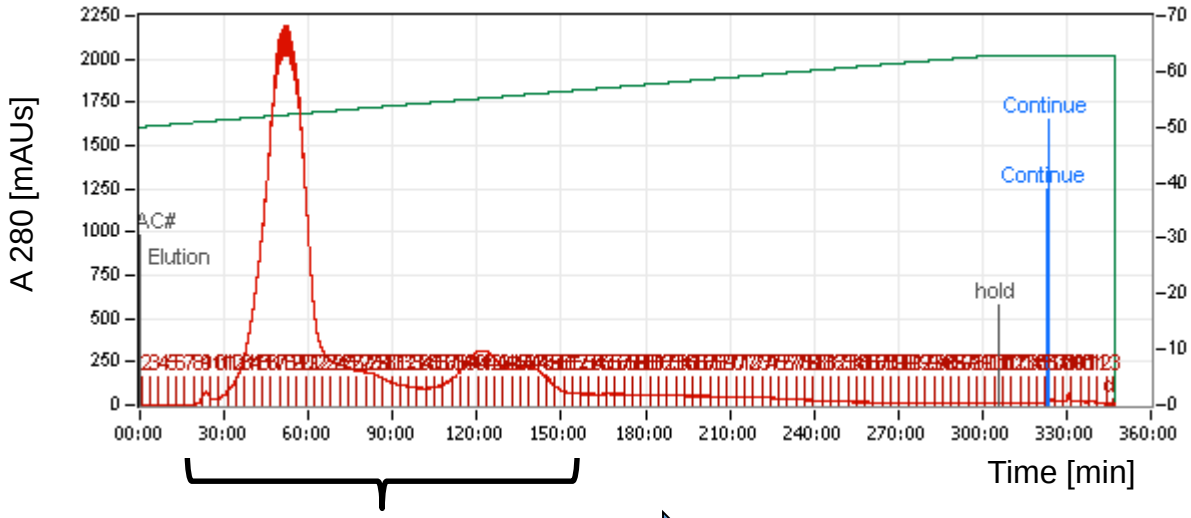
Duration of sub-process: overnight

Case study: mAb isoform enrichment (cont'd)



Elution
Final elution with shallow gradient and fine fractionation.

Step 5 repeated for basic isoforms (automatic): The pre-purified, accumulated component is further purified using a flat gradient obtaining the purified basic isoforms

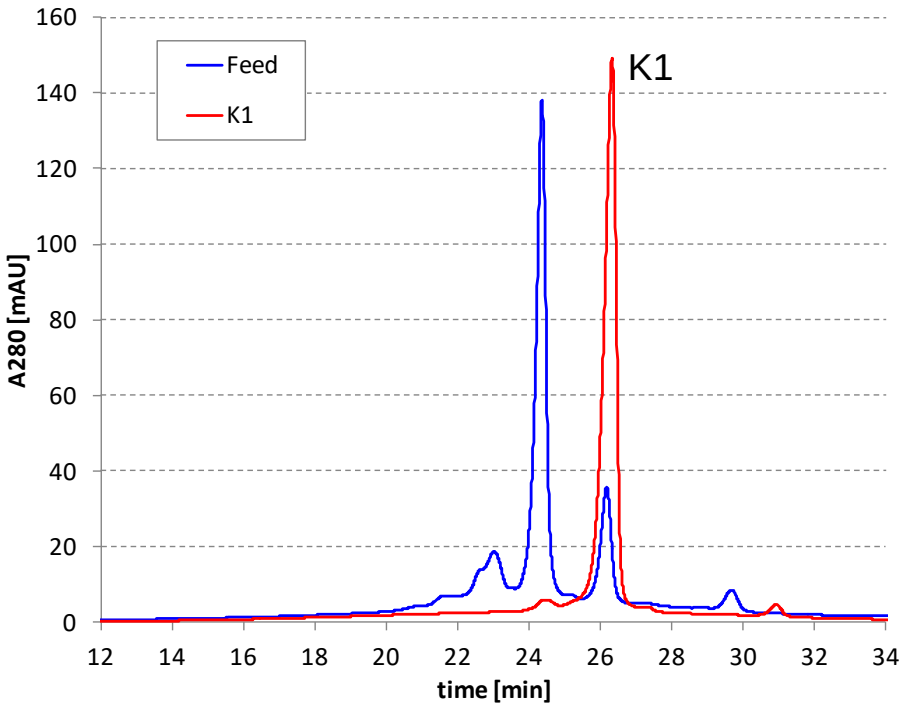


Enriched basic Isoforms → Sample analysis, pooling and formulation

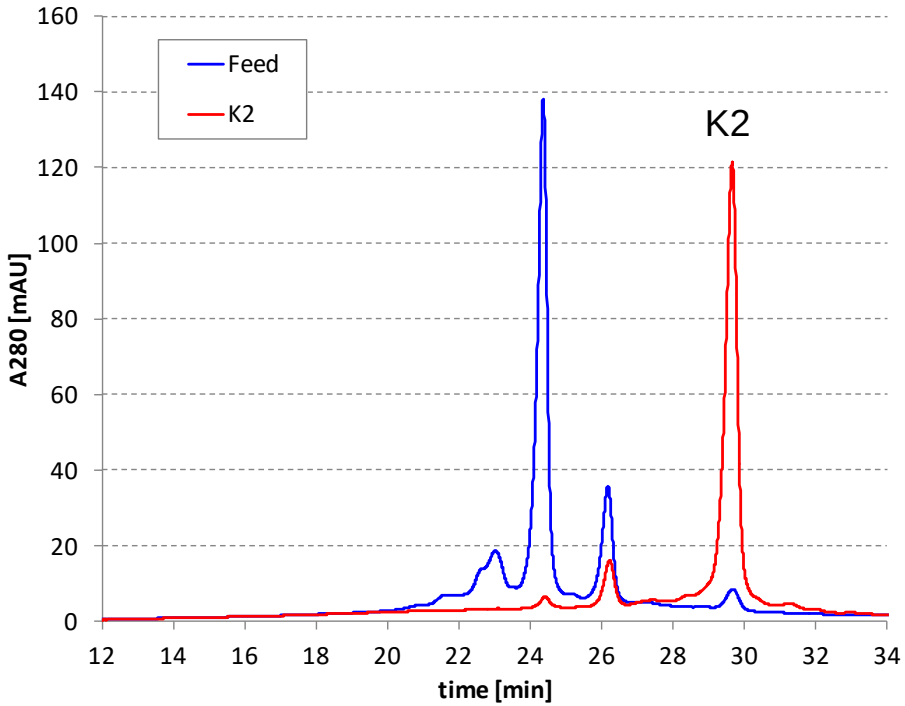
Case Study: Results of basic isoform isolation using N-Rich

- Isoforms K1 and K2 isolated

Analytical
IEX chromatogram



Analytical
IEX chromatogram



Enrichment through N-Rich process

- Final results: 5 acidic, 3 basic isoforms and the main isoform were isolated.

Isoform	in Feed	in N-Rich product	mass produced	enrichment
	[%]	[%]	[mg]	(by N-Rich)
A1	1.5%	80%	> 1.5	53x
A2	2.5%	85%	> 4.0	32x
A3	1.5%	90%	> 1.5	69x
A4	5.0%	> 90%	> 7.0	18x
A5	8.5%	> 90%	> 3.0	10x
K0	56.0%	> 90%	> 6.0	2x
K1	14.5%	90%	> 8.0	6x
P2	1.5%	50%	> 1.0	29x
K2	3.5%	75%	> 8.0	21x

Time savings through N-Rich process

- mAb isoform isolation example:
  - Single isoform to isolate
  - Target amount 10 mg
  - 2.5% content in feed
  - 50 mL resin

N-Rich can reduce:

✓ the time for isolating and analyzing mAb isoforms from 32 to 3 days

✓ the number of samples to analyze from >600 to 50

→ 10x increase in efficiency

Assumptions

Target amount	[mg]	10
Average content of isoform in feed	[%]	2.5%
Protein concentration in Feed	[mg/mL]	5.0
time for sample analysis	[hrs]	1
Resin volume	[mL]	50

Isoform Isolation		**Batch**	**N-Rich**
yield	[%]	5%	15%
load (total protein)	[g/L]	5	10
batch run / cycle duration	[hrs]	3	3
Number of cycles (N-Rich)	[-]		6
time for final elution N-Rich	[h]		6
amount of isoform produced per run	[mg]	0.3	11.3
cycles / runs needed	[-]	32	1
time for isoform isolation	[hrs]	96	24

Analytics

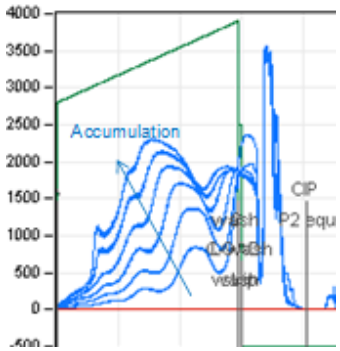
fractions for run	[-]	20	50
time for run fraction analysis	[h]	20	50
time for sample handling per run	[h]	0.7	1.7

Summary

total time for isolation	**[d]**	32	3
thereof analytics time	**[d]**	27	2

Summary

- Isolation and analysis time of mAb isoforms could be reduced 10-fold through
  - N-Rich process
  - Reduced sample number / analytical burden
- mAb isoform isolation becomes a matter of days rather than weeks
- Using UPLC will lead to further reduction in overall time



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