CE Pharm 2023

Using Digestion by IdeS Protease to Improve Quantification of Degradants in Monoclonal Antibodies by Non-Reducing Capillary Gel Electrophoresis

27 September 2023

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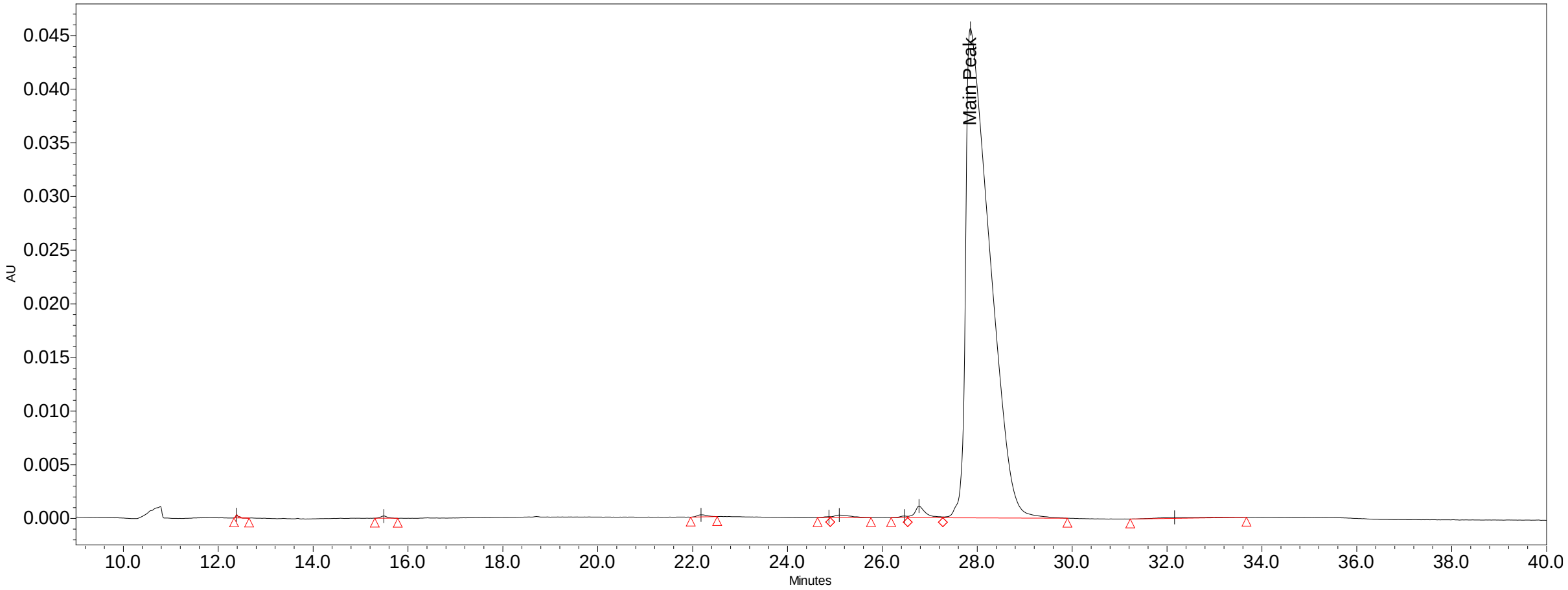
Agenda

1. Background: mAb-1 NR CGE
2. mAb-1 Molecule Structure: What was the source of the shoulder peak?
3. Solution 1: Optimization of the Integration Strategy
4. Solution 2: Method Optimization: IdeS NR Subunit Method
5. IdeS NR Subunit Method: Peak Characterization
6. Conclusions

Background: mAb-1 Non-Reducing CGE

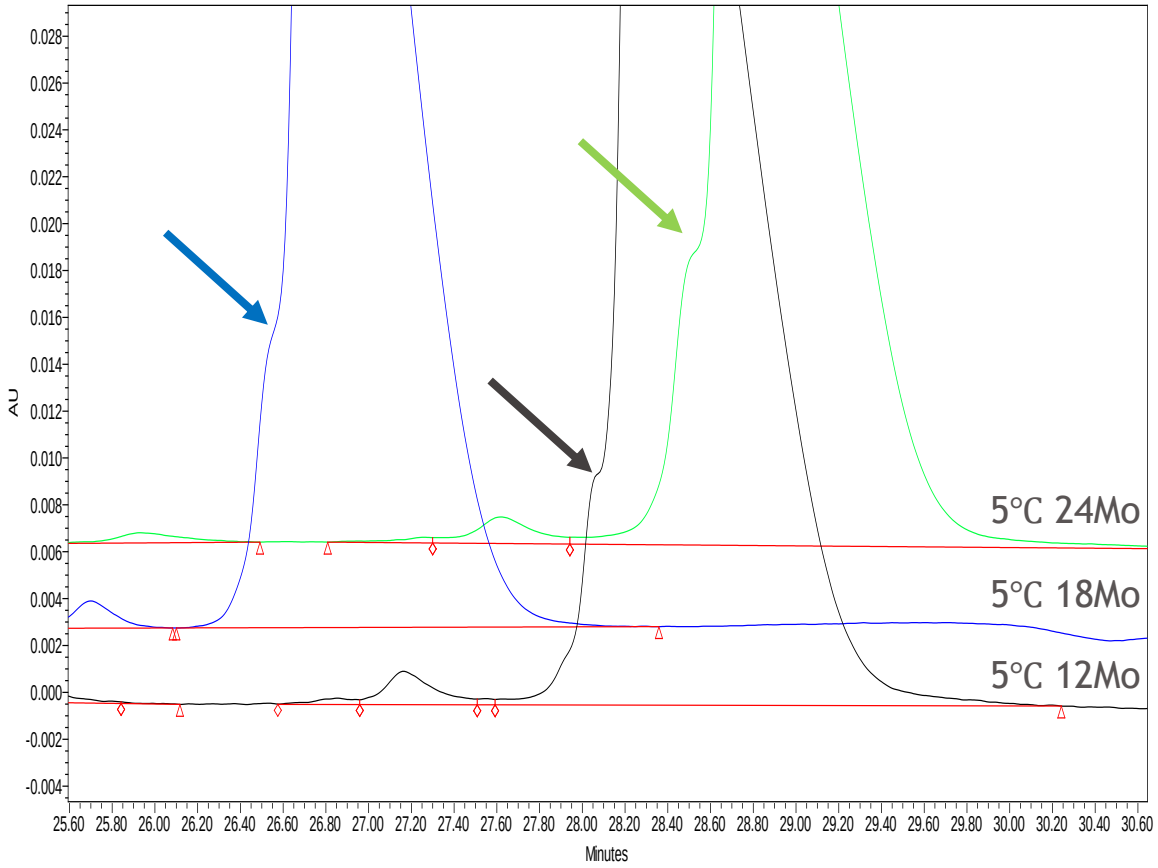
- Non-Reduced (NR) Capillary Gel Electrophoresis (CGE) is a separation technique used widely in Biopharma to quantify the Purity of mAbs and other biological molecules.
- This technique focuses on achieving high resolution of low molecular weight (LMW) species present in a sample, such as protein fragments and disulfide bond reduction impurities.
- A NR CGE Method for mAb-1 was developed for release and stability testing.
- Unstressed drug substance yielded expected profiles, with a main peak representing intact IgG and some LMWs of low levels of various reducing species

mAb-1 NR CGE: Method Introduction



Major Peak%	HMW%	LMW%
97.7%	0.4%	1.9%

Challenges Observed During Stability Testing:

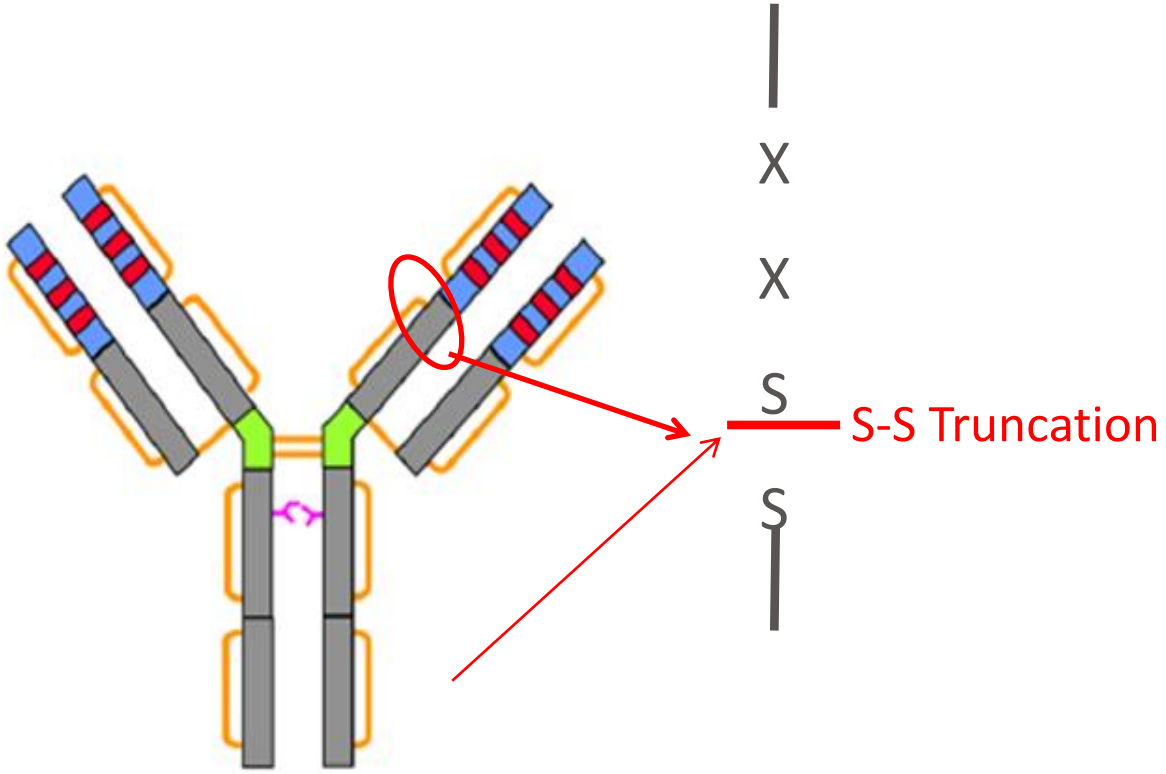


- A front shoulder off of the intact IgG peak started to appear 18-24 months into stability testing
- We needed to clarify when it was appropriate to integrate this shoulder and include in the sum of LMW impurities
- Regardless of when it was appropriate to integrate, the “sudden loss” of purity that resulted from this shoulder peak was creating shelf life uncertainty.

mAb-1 Molecule Structure: What was the source of the shoulder peak?

Slide credit to Yan Yin

What is the Shoulder?: S-S Truncation and Fragment Sizes



Higher Rate of clipping due to S-S bond[1]

- Serine-Serine peptide bonds are more easily hydrolysable[1]
- This truncation leads to a clipped species approximately 11kDa in size
- It is challenging to separate two intact IgGs differing in size by only 11kDa by non-reducing CGE

1. *Vlasak, J., & Ionescu, R. (2011). Fragmentation of monoclonal antibodies. mAbs, 3(3), 253-263. https://doi.org/10.4161/mabs.3.3.15608*

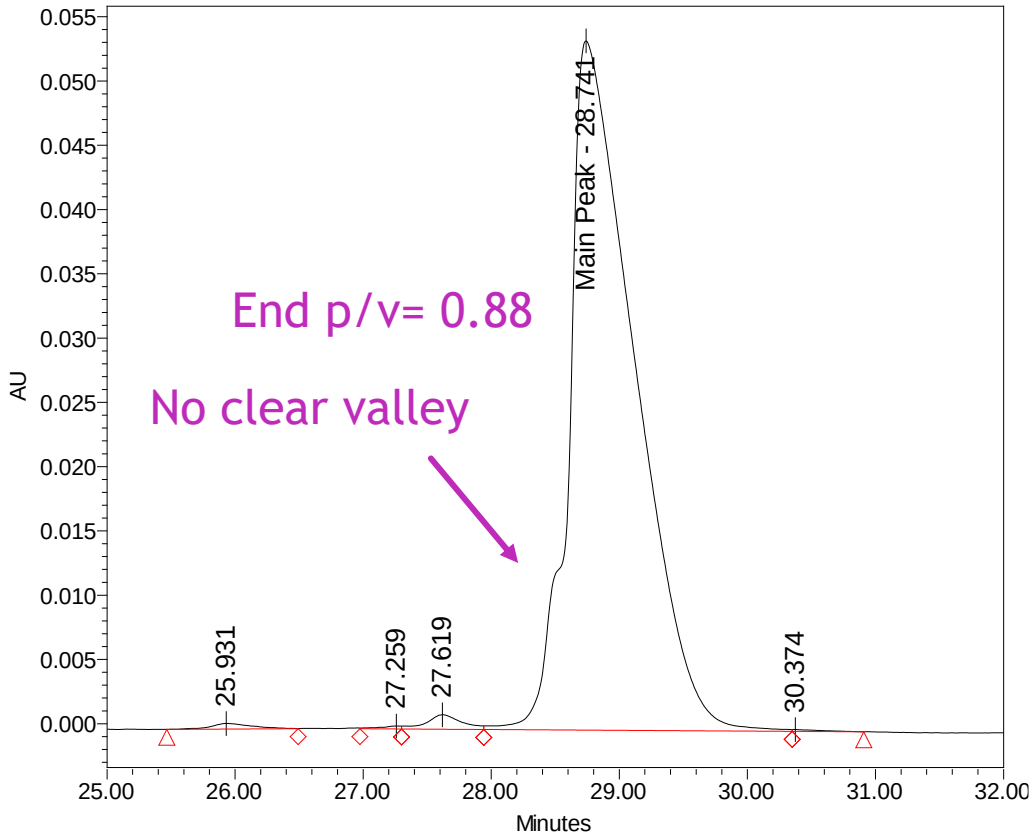
Mitigation of the 11kDa Clipped Species

- The separation potential of CGE is tested when separating the two species:
  - Main peak approx. 150 kDa
  - Clipped species (shoulder peak) approx. 138 kDa
- Separation challenges regarding the shoulder peak result in integration uncertainty
- Two approaches to reducing this uncertainty:
  1. Improve the integration approach
     - Define parameters that can be utilized consistently to eliminate analyst-to-analyst variability
  2. Better resolve the species via method modification
     - Eliminate uncertainty by fully resolving the impurity

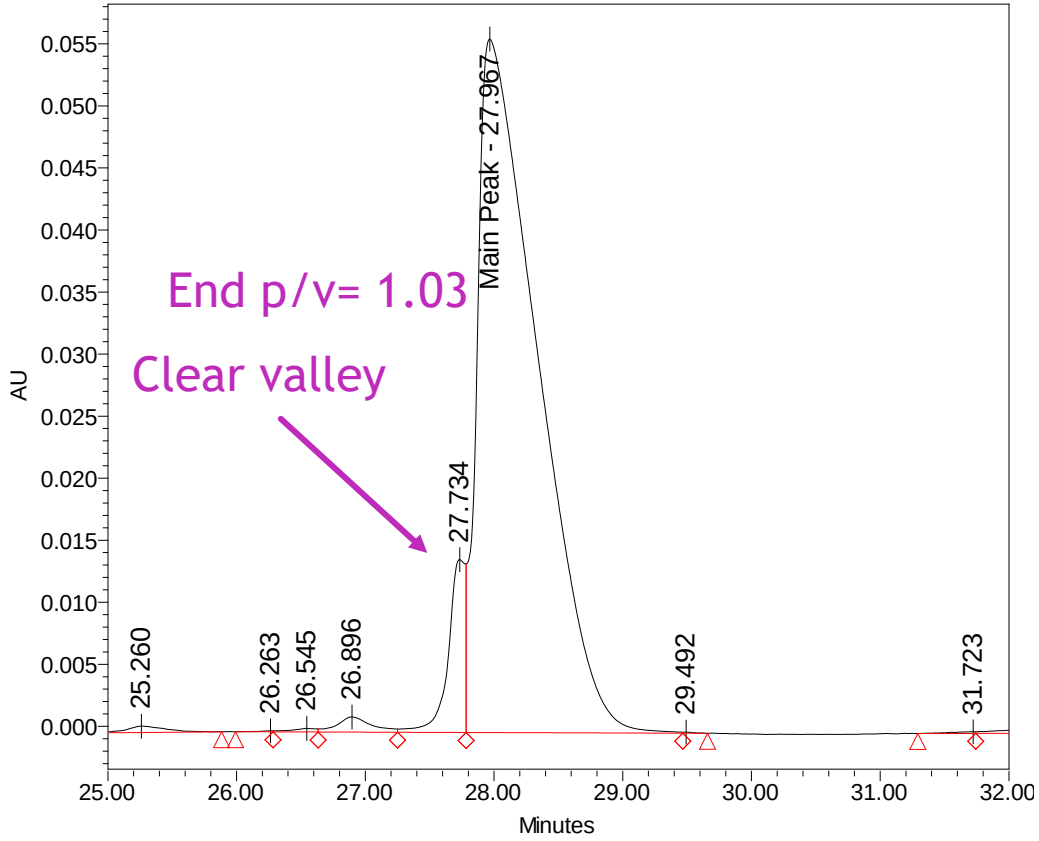
Solution 1: Optimization of the Integration Strategy

Use of Peak and Valley Parameters to Guide Integration Strategy

- 24-Month



- 30-Month



- End p/v is an Empower function that calculates the ratio of peak and valley heights. As shown above, an end p/v ratio > 1.00 shows a clear valley for integration

Slide credit: Yiting Zhang and Tara Enda

Summary – Integration Strategy

- Integration proposal: utilize dropline integration of the front shoulder peak only if the End p/v of the shoulder is ≥ 1.01.

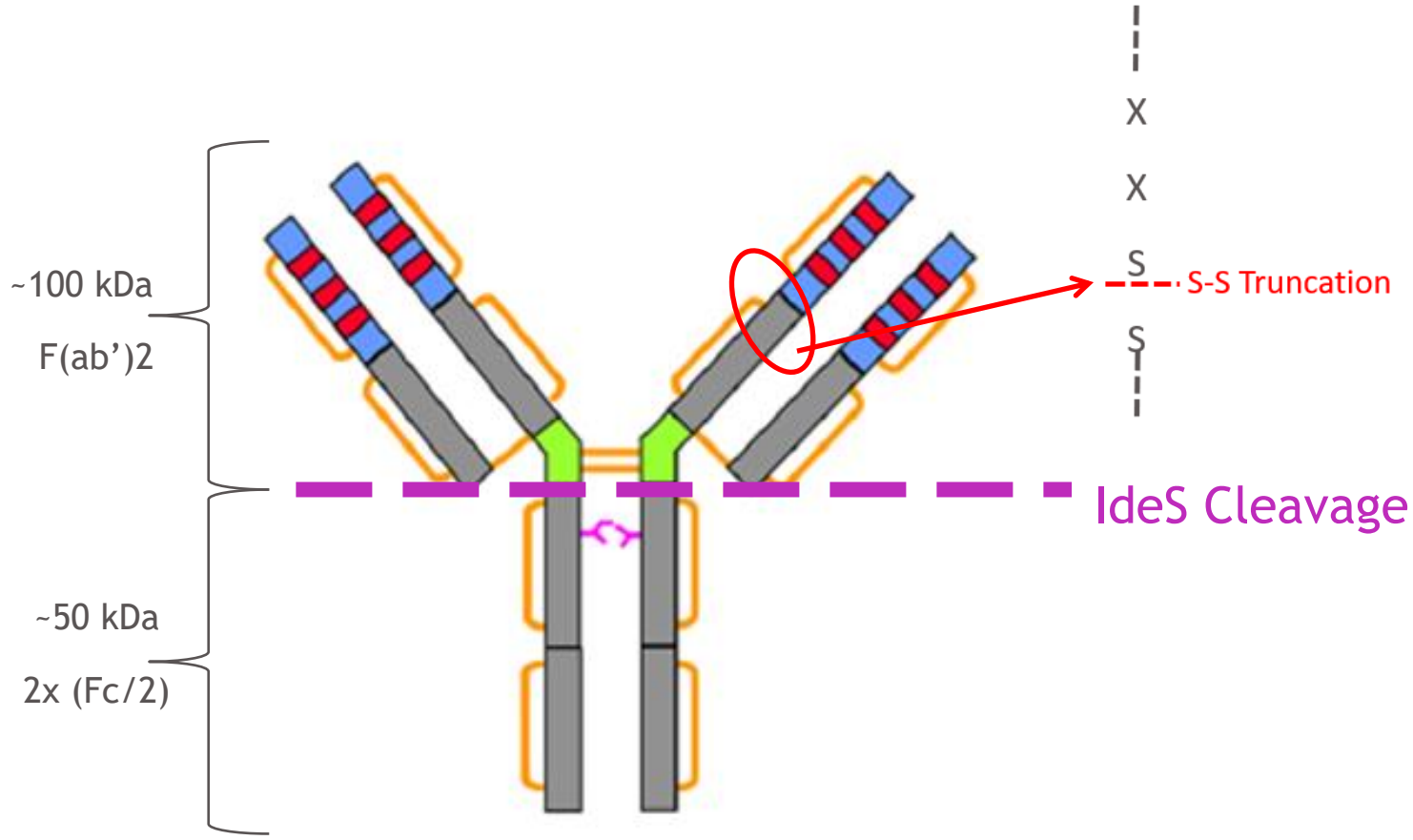
- The aim of this integration guideline is to improve consistency of results and quantification of the shoulder when integration is appropriate.

- However, **only** improving the integration strategy would not be sufficient to solve the challenges presented during stability testing.

- ***Ultimate solution to improve shoulder peak quantification is to increase the resolution of the clipped species from the main species***
  - ***Goal: baseline resolution***

Solution 2:
Method Modification:
IdeS NR Subunit Method

S-S Truncation and Fragment Sizes: Subunit Method

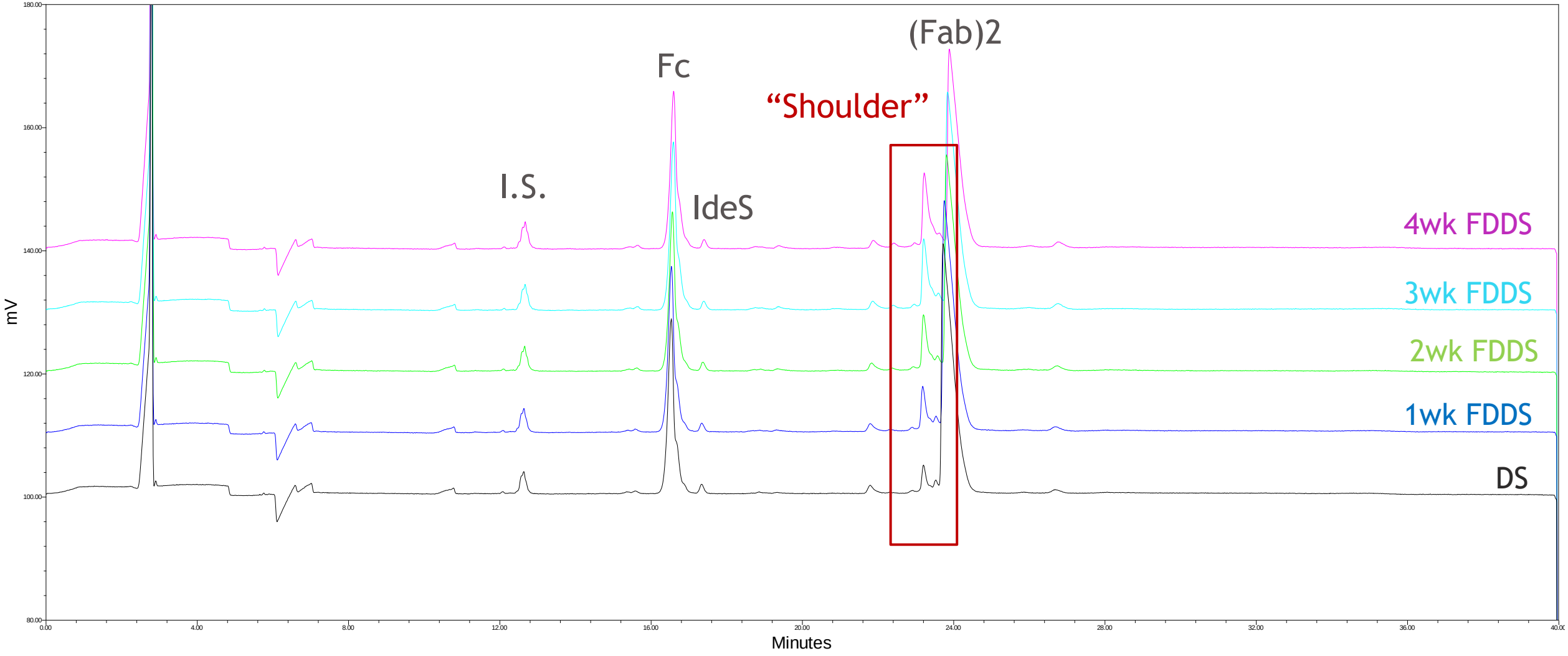


- IdeS cleavage slightly below hinge region results in ~50 kDa Fc/2 region and ~100 kDa (Fab')2 region
- After IdeS digestion, there exists ~100 kDa mAb-1 (Fab')2 region and ~88 kDa clipped species corresponding to the shoulder peak in NR
- **The increased difference in relative size allows baseline resolution of these two species**

**Note: Aglycosylated species which co-migrates with the clipped species at the intact level will be separated from the ~88kDa clipped species in the Subunit Method.*

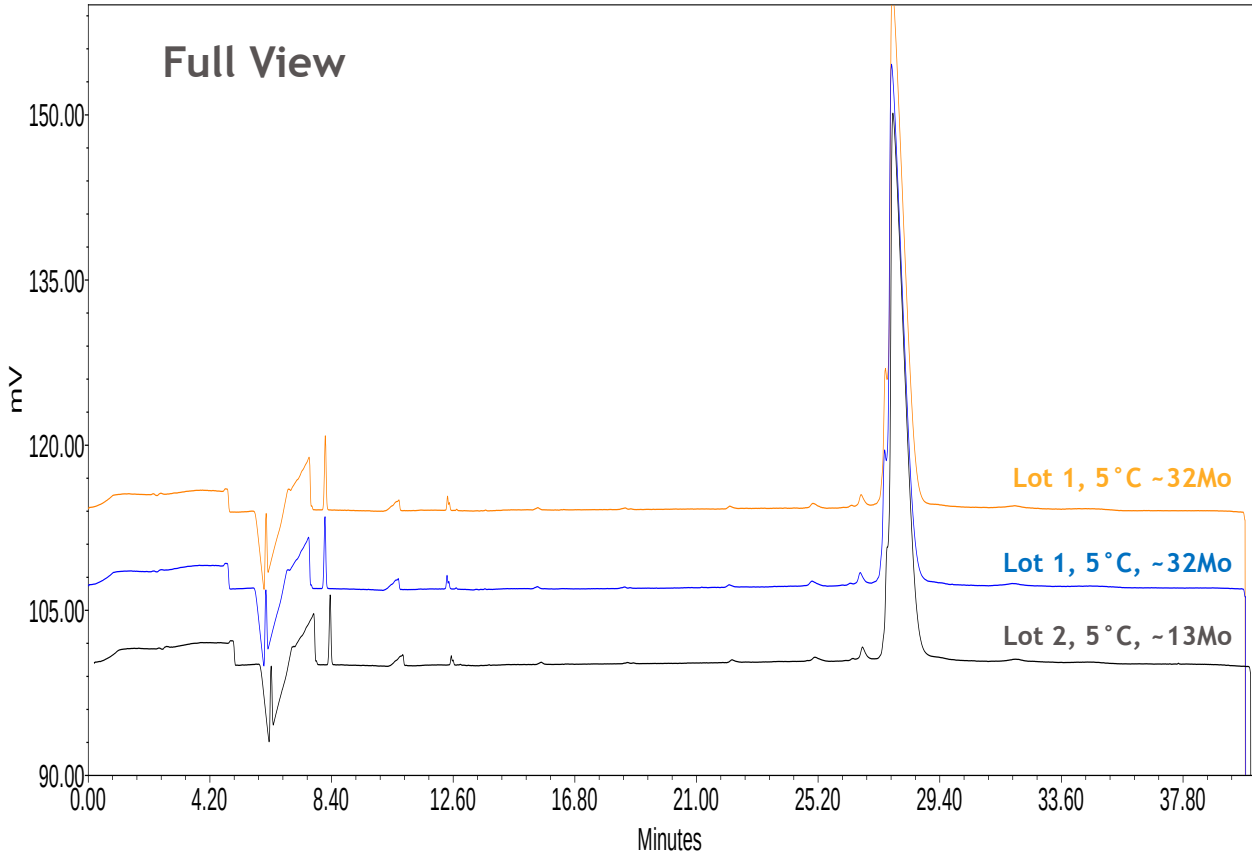
Subunit Non-Reduced Method: High Res of Shoulder

Samples: Drug Substance up to 4 weeks at 40˚C

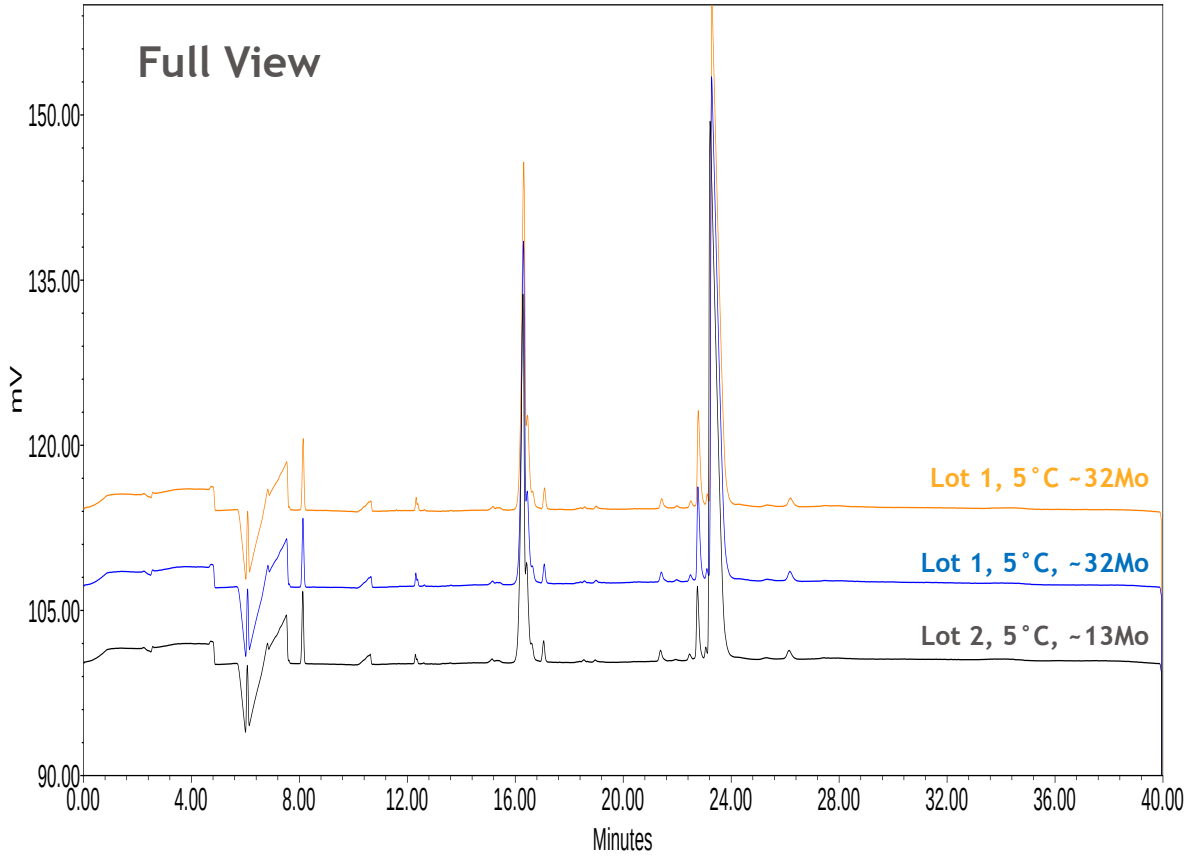


Existing Method vs. Proposed Method: Stress Samples

Initial NR Method

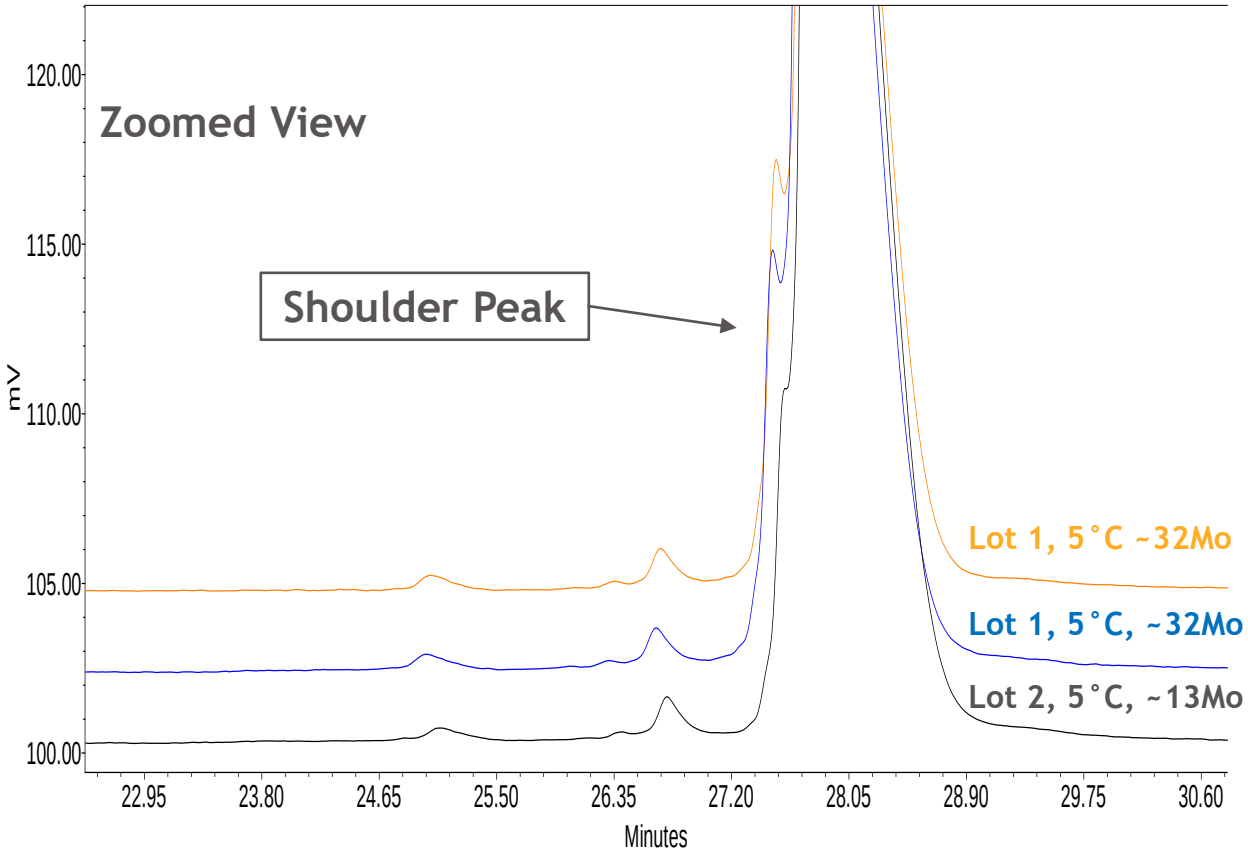


IdeS Subunit Method

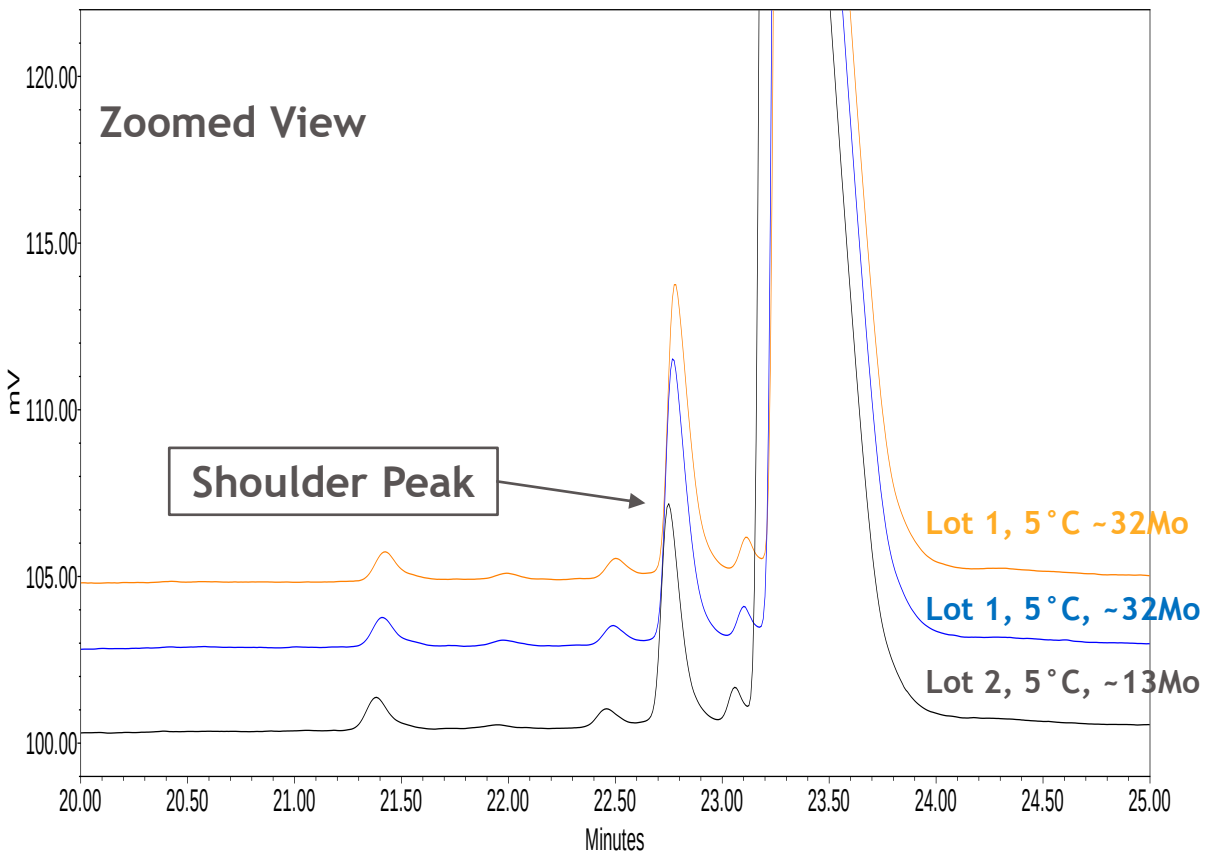


Existing Method vs. Proposed Method: Stress Samples

Initial NR Method



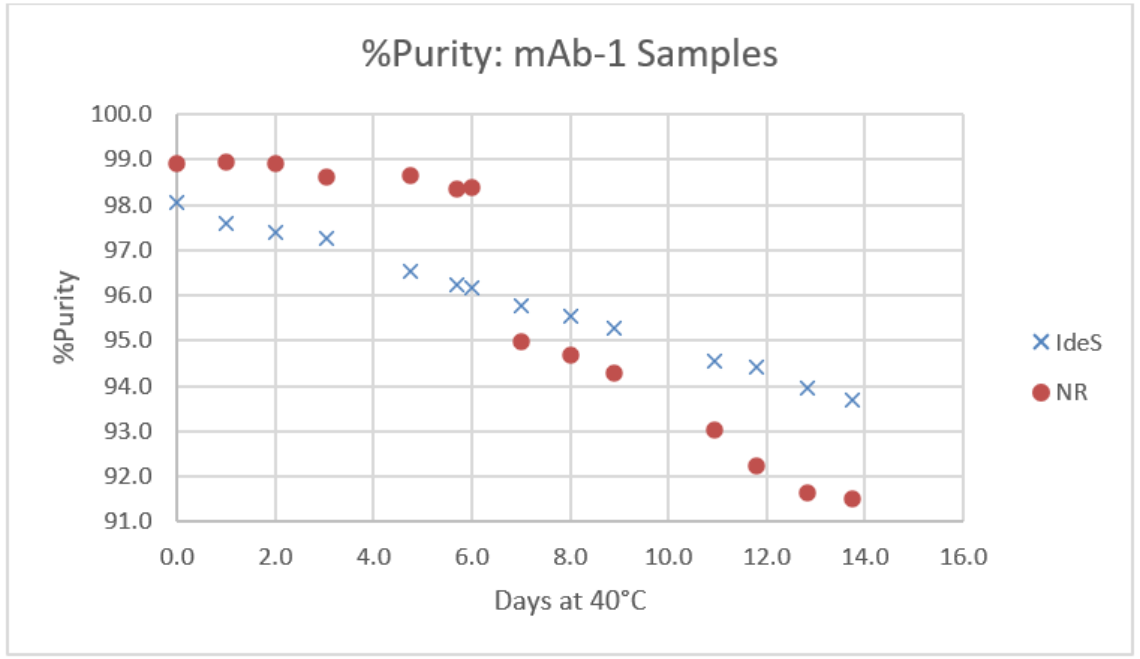
IdeS Subunit Method



Subunit Method Stability Study:
Q: What Impact Does the Method Have on Stability Assessments?

Time Point (Days) [a]	%Area: Purity		%Area: Shoulder Peak	
	IdeS Subunit NR (Sum of F(ab')2 + Fc)	Intact NR	IdeS Subunit NR	Intact NR
0.0	98.1	98.9	1.1	N/A
1.0	97.6	99.0	1.3	N/A
2.0	97.4	98.9	1.6	N/A
3.0	97.3	98.6	1.9	N/A
4.8	96.5	98.6	2.3	N/A
5.7	96.2	98.3	2.6	N/A
6.0	96.2	98.4	2.6	N/A
7.0	95.8	95.0	2.9	3.6
8.0	95.5	94.7	3.1	3.6
8.9	95.3	94.3	3.4	4.0
10.9	94.5	93.0	3.9	4.9
11.8	94.4	92.2	4.1	5.5
12.8	93.9	91.7	4.4	6.1
13.7	93.7	91.5	4.5	6.2

[a] The number of days was calculated using the time that each sample was removed from the incubator.



More accurate tracking of the shoulder peak prevents a drop in Purity that is observed in the Intact NR Method

IdeS NR Subunit Method: Peak Characterization

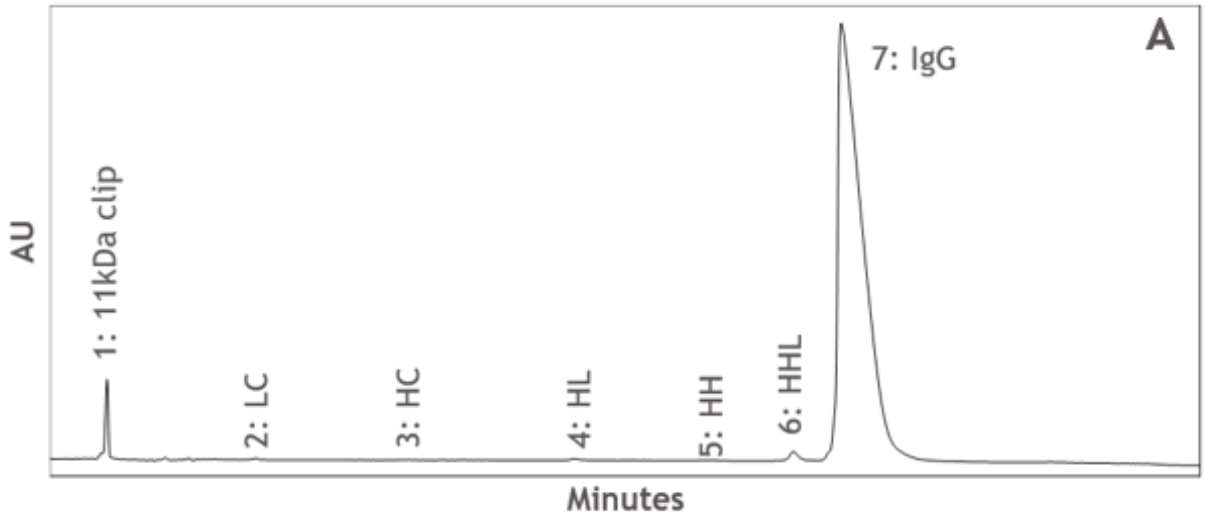
Peak Assignments

- In order to ensure a comprehensive replacement of intact NR CGE with the IdeS NR CGE, new peaks observed in IdeS NR CGE that were not present in Intact NR CGE were characterized
- Peaks were assigned using historical NR CGE knowledge, findings in the literature[2], and a series of experiments
- To assign known NR CGE peaks (ex. HHL), the relative migration times and area percentages were compared for identical samples that were ran by Intact NR CGE and IdeS Subunit CGE.
  - This is expanded further in the next slide
- New peaks, unique to the IdeS Subunit Method, were assigned by a series of experiments and investigations
  - The (Fab')2, Fc/2, and IdeS Protease peaks were assigned using the literature[2]
  - Other peaks were assigned by the following experiments:
    - Partial Reduction
    - Partial Digestion
    - Deglycosylation

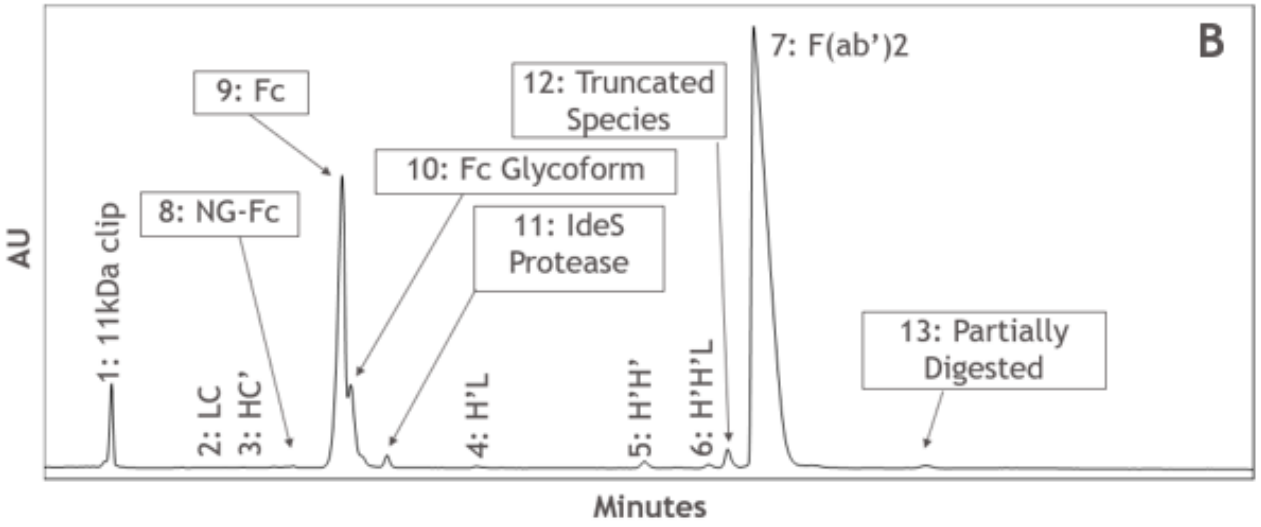
2. Duhamel, L., Gu, Y., Barnett, G., Tao, Y., Voronov, S., Ding, J., … Li, Z. J. (2019). Analytical and Bioanalytical Chemistry, 411(21), 5617-5629. https://doi.org/10.1007/s00216-019-01942-8

Peak Assignments for Intact and Subunit NR CGE

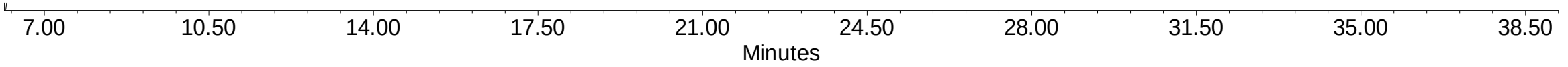
Intact NR



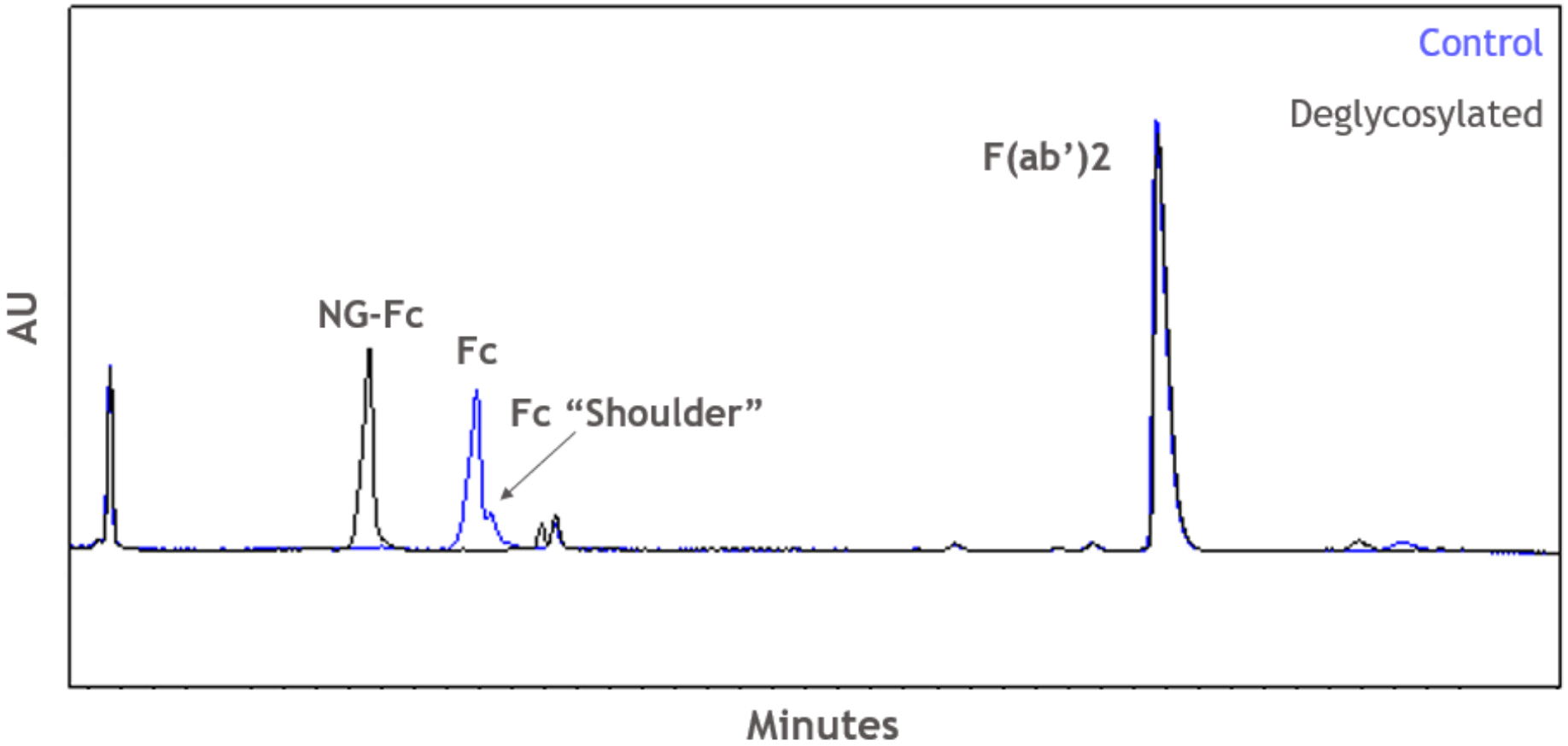
IdeS Subunit NR



Note: Peaks in IdeS Subunit NR labeled as "prime" (ex. HC in Intact vs. HC' in IdeS Subunit) indicate the loss of Fc on the heavy chain due to IdeS Digestion, correlating each Intact NR method species to its counterpart in the IdeS Subunit NR method



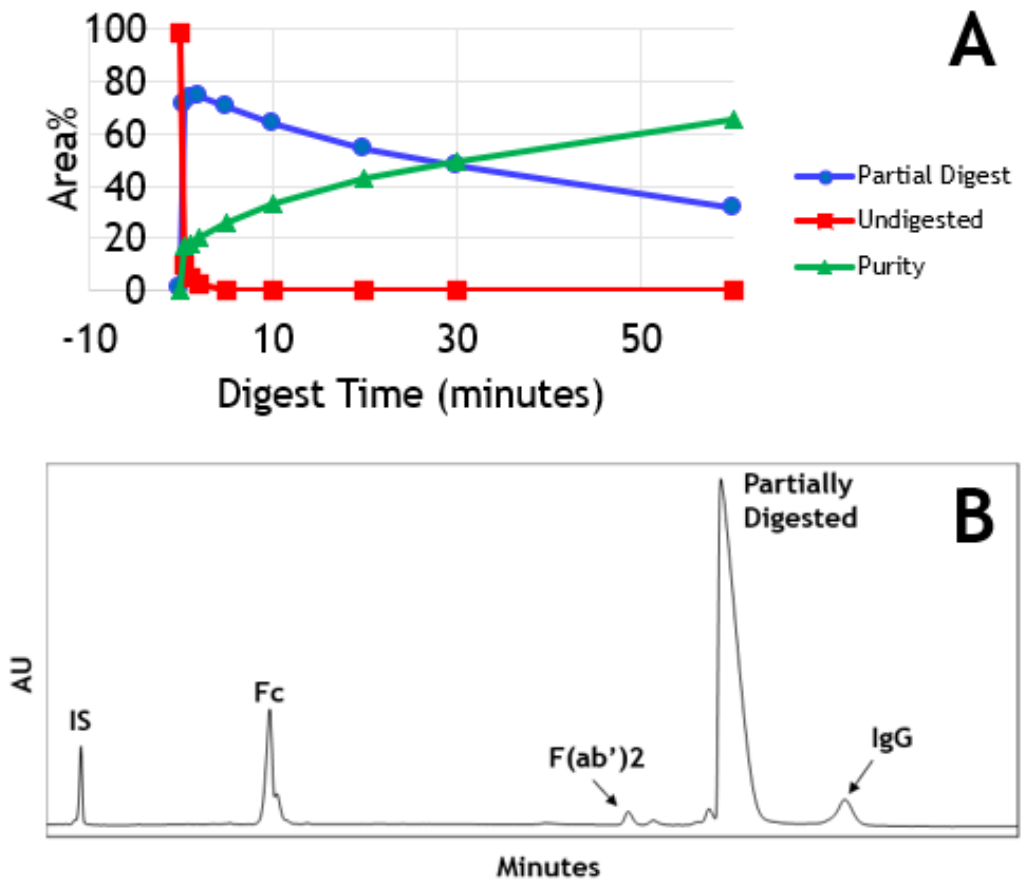
Deglycosylation Experiment: Peak 8



- NG-Fc migrates at the RRT of Peak 8
- Fc Shoulder peaks disappear in NG-Fc
- This suggests their identity as differently glycosylated Fc species
- This is also backed up by the literature[2]
- Similar results were observed using different batches with varying levels of glycosylation

2. Duhamel, L., Gu, Y., Barnett, G., Tao, Y., Voronov, S., Ding, J., … Li, Z. J. (2019). Analytical and Bioanalytical Chemistry, 411(21), 5617–5629. https://doi.org/10.1007/s00216-019-01942-8

Non-Reduced Partial IdeS Digestion: Time Course Graph



Manuscript Figure 7

- The digestion reaction was slowed to show the accumulation of the partially digested intermediate species
- Panel A shows a numerical comparison between the various species as digestion occurs
- "Purity" refers to F(ab')2 + Fc
- Panel B shows an intermediate time point to better visualize the reaction
- This lead to the confirmation of Peak 13 as the partially digested species

Under Nominal Sample Preparation Conditions “Peak 13” is less than 1%

IdeS Digestion Time (Minutes)	% Partial Digest Species (“Peak 13”)
15	1.2
30	1.0
45	0.9
60	0.8
120	0.8
1200 (20 Hours)	0.2

- The peak 13 partial digest species is not fully eliminated after 20 hours of digestion, even under nominal sample preparation conditions.
- However, the relative abundance of this species could be controlled within 1% of the total peak area if digestion occurs for at least one hour.
- Within the 1% control limit, there is no significant impact to the relative abundancies of the other peaks (data not shown)

IdeS Subunit and Intact NR CGE Peak Assignments

Peak Number	Intact	IdeS (H' = H after loss of Fc)
1	11kDa clip. (and I.S.)	11kDa clip. (and I.S.)
2	LC	LC
3	HC	HC' (from HC)
4	HL	H'L (from HL)
5	HH	H'H' (from HH)
6	HHL	H'H'L (from HHL)
7	IgG Main Peak	F(ab')2 (IgG without two Fc subunits)
8	N/A	NG Fc
9	N/A	Fc
10	N/A	Fc Glycoform
11	N/A	IdeS Enzyme peak
12	NA	88kDa Fab2 shoulder peak (without Fc, fully separated in IdeS)
13	NA	Partially Digested Species (mAb-1 with only one Fc subunit)

Summary

- **Reportable Results Summary**
  - Complete resolution of the shoulder
  - Improved resolution allowed for quantification of the 11kDa impurity under unstressed conditions (not able to do so in intact NR CGE)
    - Precise accounting of the impurities throughout stability time course
  - T=0 purities are lower as a result
    - However, there are no longer sudden drops in purity during the stability time course, and therefore no more “surprises”

- **Overall Summary: This method satisfies the request to provide a NR CGE method capable of quantitatively tracking the clipped shoulder peak.**

Conclusions and Future Directions

- Similar approaches with subunit analysis can be used across the industry with other biological molecules that have similar clipping concerns
  - Subunit Analysis helps increase the relative % difference in size between a parent molecule and its impurity to maximize resolution potential
  - Potential: Other types of biological molecules may be digested using other endopeptidases to achieve a similar effect
- The Intact NR CGE peaks have all been characterized in IdeS Subunit NR CGE such that this method can replace in the Intact NR CGE method on the testing panel.
- As with R CGE, this method can accurately track aglycosylation. However, more characterization work would need to be done to fully capture all impurities tracked by R CGE.
  - Both NR and R CGE may be able to be replaced by an IdeS NR CGE method

Acknowledgements

- Jeff Beckman
- Lih-Yueh Hwang
- Qi Wang
- Richard Ludwig
- Yan Yin
- Yan Gu
- Yiting Zhang
- Tara Enda
- Entire ADAS Team for guidance and support!

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