

# Direct Protein Analysis Using Size Exclusion Chromatography Zenix™ SEC and Mass Spectrometry

ISPPP P14

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## INTRODUCTION

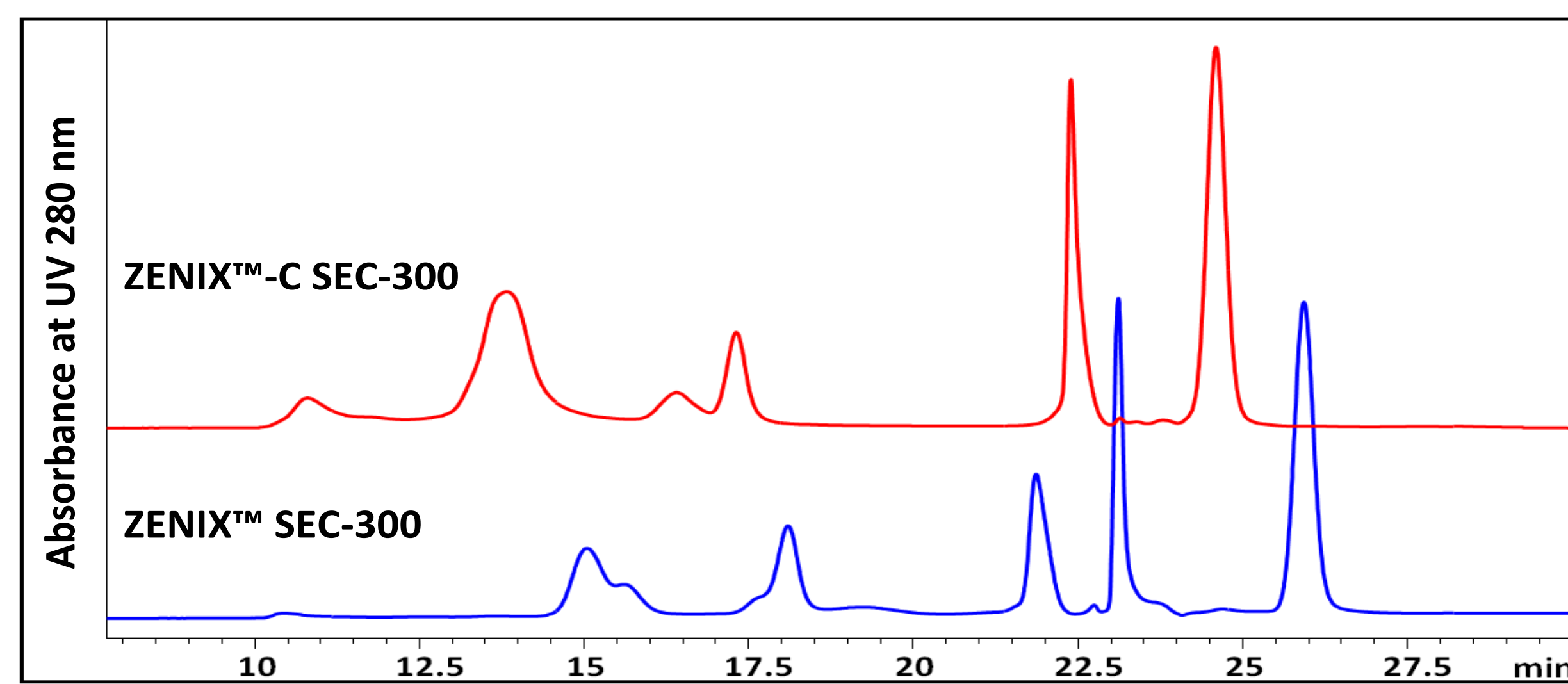
Size exclusion chromatography has been widely used in protein analysis. Aggregates, monomers and degradation products can be separated on size exclusion columns based on their molecular weights under native conditions. In general protein native buffer conditions such as salts pH are not mass spectrometry friendly. In this study, we investigated monoclonal antibody (MAb) subunits with SEC separation coupled with mass spectrometry. An intact monoclonal antibody was reduced to generate heavy and light chain subunits. Then the heavy and light chains were separated on Zenix™ SEC, and the mass of each chain was obtained with mass spectrometry under an optimized mobile phase condition. The combination of Zenix™ SEC and mass spectrometry achieves the separation of proteins and protein subunits on SEC according to their sizes and the determination of their molecular weight by mass spectrometry.

## EXPERIMENTAL

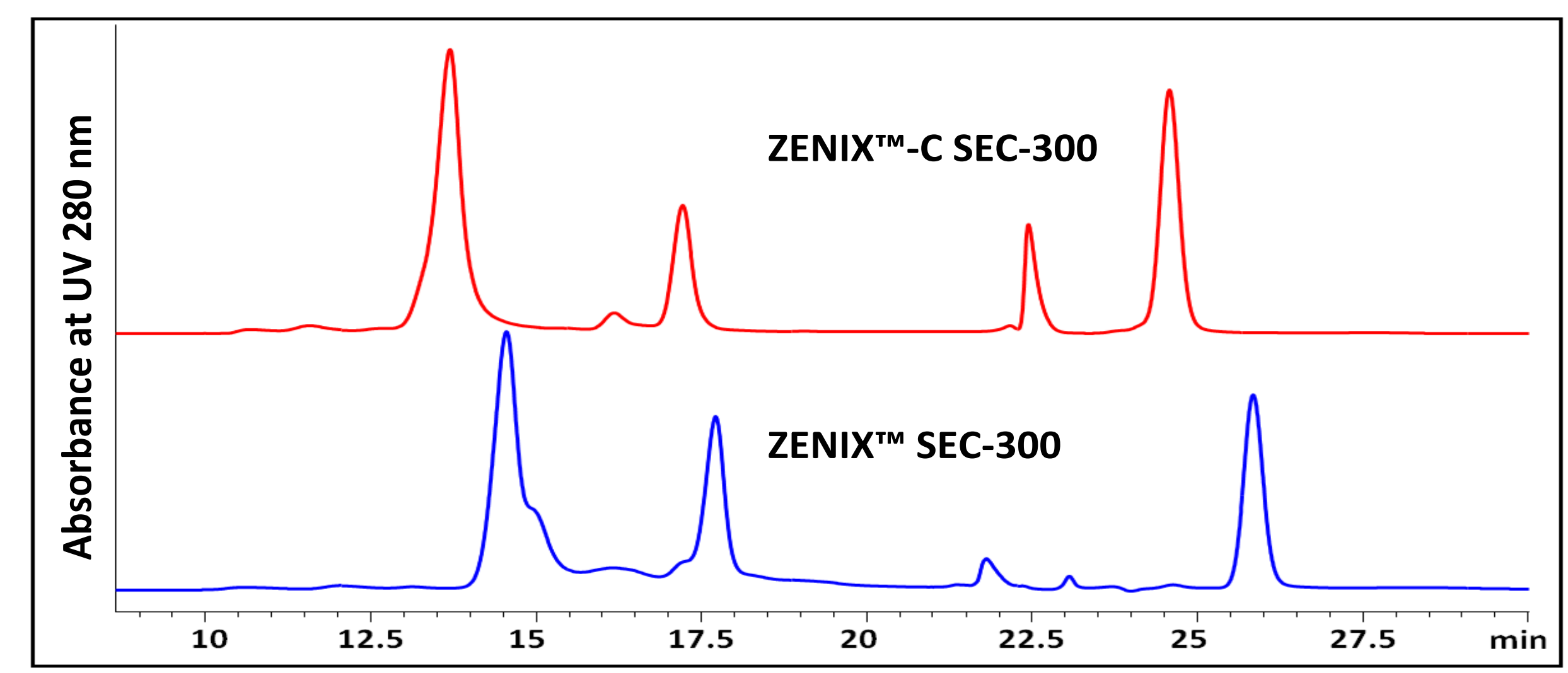
SEC Column: Zenix™ SEC-300 (3 μm, 300 Å, 7.8x300 mm)  
Mobile Phase: 0.1% trifluoroacetic acid (TFA) and 0.1% formic acid in 20% acetonitrile (ACN)  
HPLC System: Agilent 1200 HPLC with binary pump  
Detection: UV 280 nm  
Mass Spectrometer: Waters Q-ToF Ultima  
Scan Range: 350 - 3000 amu  
Source Temperature: 80 °C  
Desolvation Temperature: 150 °C  
Capillary Voltage: 4.44 kV

Sample preparation: For dithiothreitol (DTT) reduction, a 5 mg/mL stock solution of MAb 321 was diluted to 1 mg/mL with 0.5 M DTT and water. The diluted sample was then incubated at 65°C for 20 minutes and centrifuged for 10 seconds.

## ANALYSIS OF TWO DIFFERENT REDUCED MONOCLONAL ANTIBODIES ON ZENIX™ SEC-300 AND ZENIX™-C SEC-300

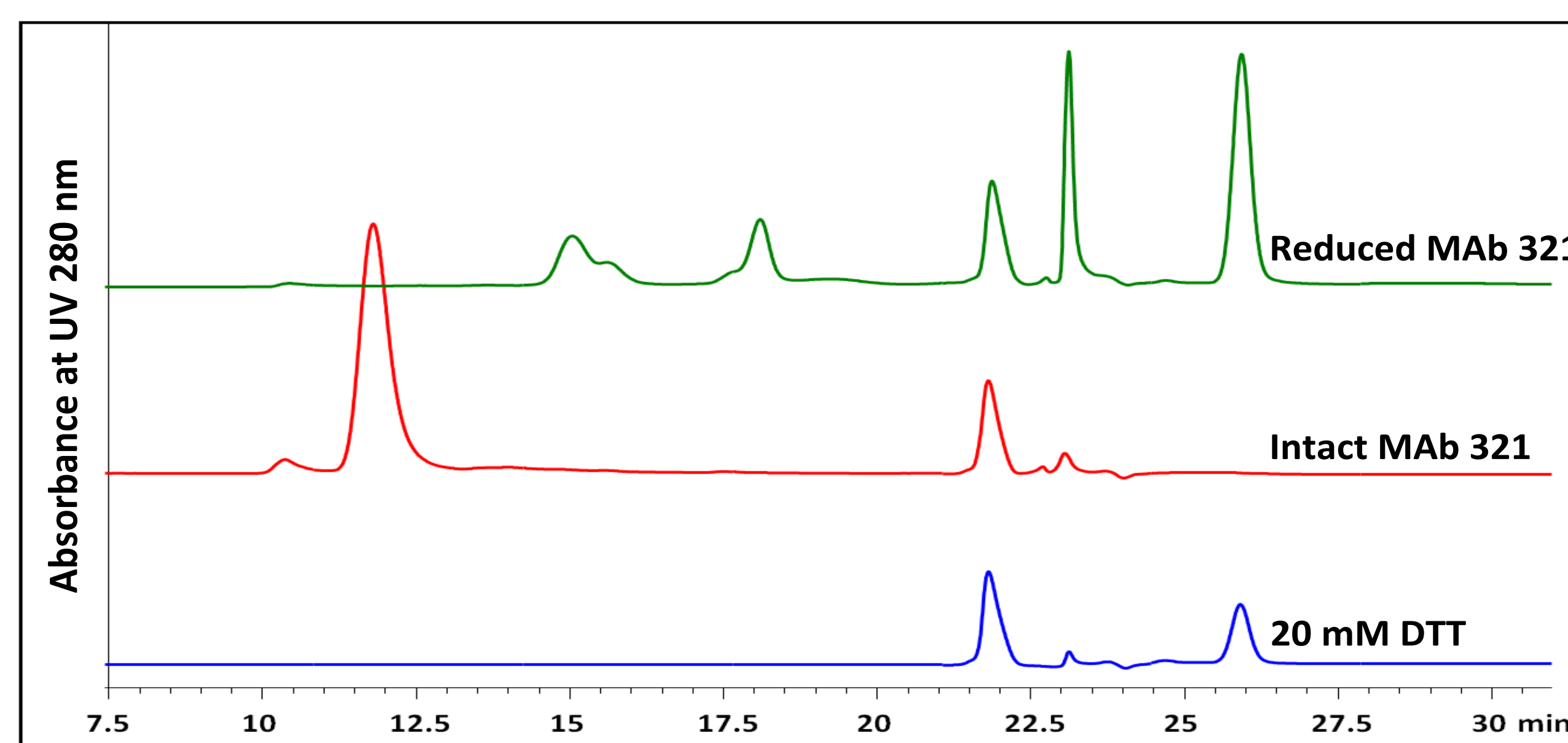


Overlay of reduced MAb 321 injections on Zenix™ SEC-300 and Zenix™-C SEC-300 running at a flow of 0.5 mL/min with 0.1% TFA and 0.1% formic acid in 20% ACN as the mobile phase.



Overlay of reduced Creative Lab Humanized MAb injections on Zenix™ SEC-300 and Zenix™-C SEC-300 running at a flow of 0.5 mL/min with 0.1% TFA and 0.1% formic acid in 20% ACN as the mobile phase.

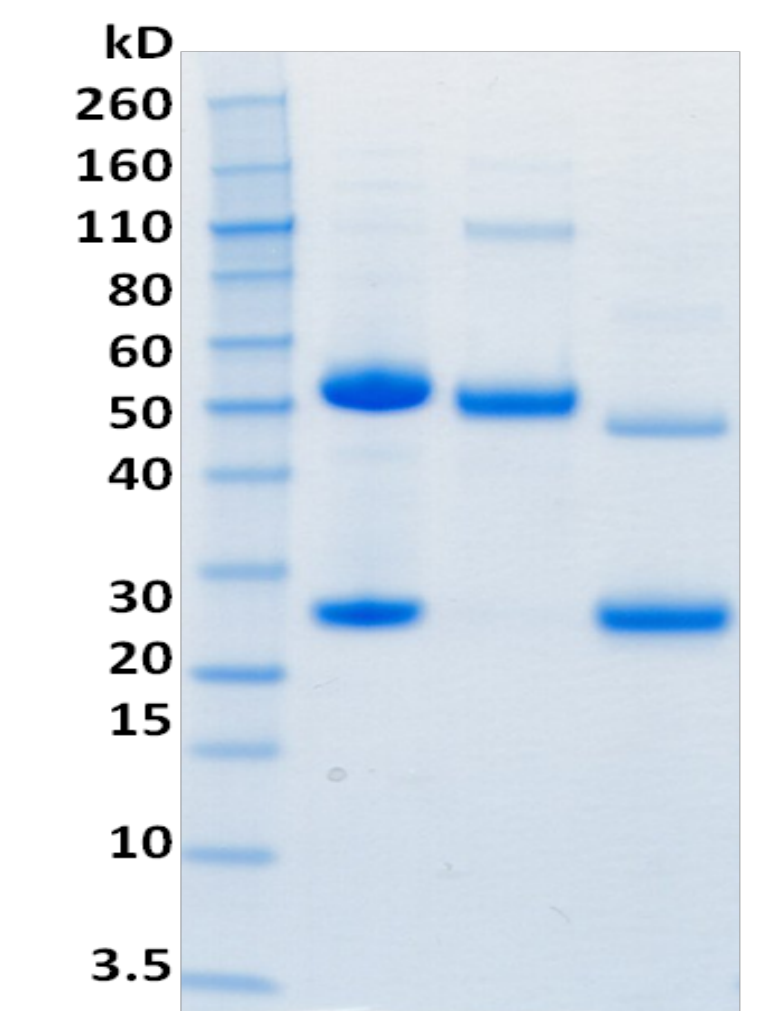
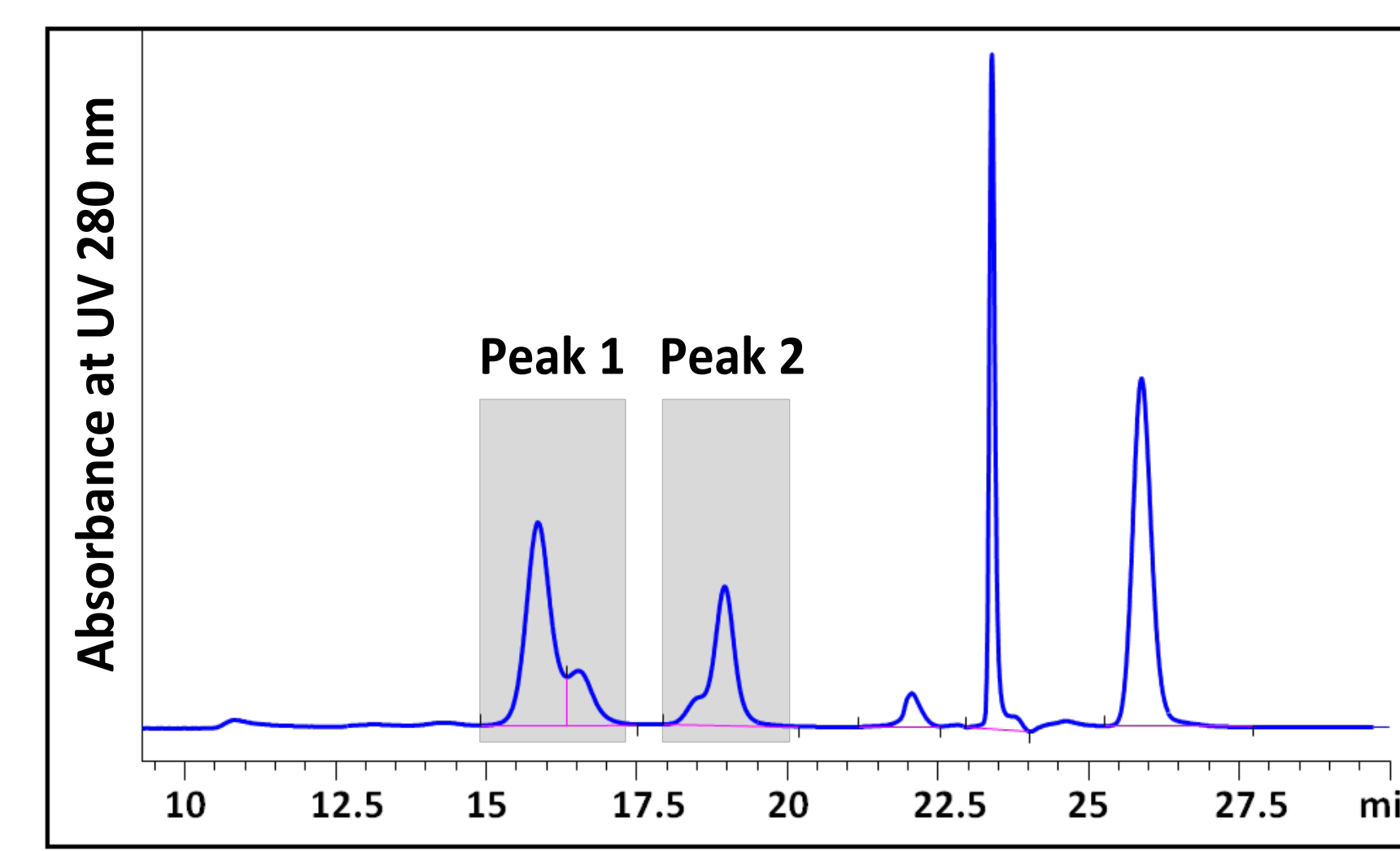
## ANALYSIS OF MAb 321 AGGREGATION ON ZENIX™ SEC-300



Overlay of three different runs on Zenix™ SEC-300 at a flow rate of 0.5 mL/min. The blue trace is the chromatogram of a 20 μL blank injection of 20 mM DTT. The red trace is a 30 μg injection of intact MAb 321 and the green trace is a 30 μg injection of MAb 321 that has been reduced using DTT.

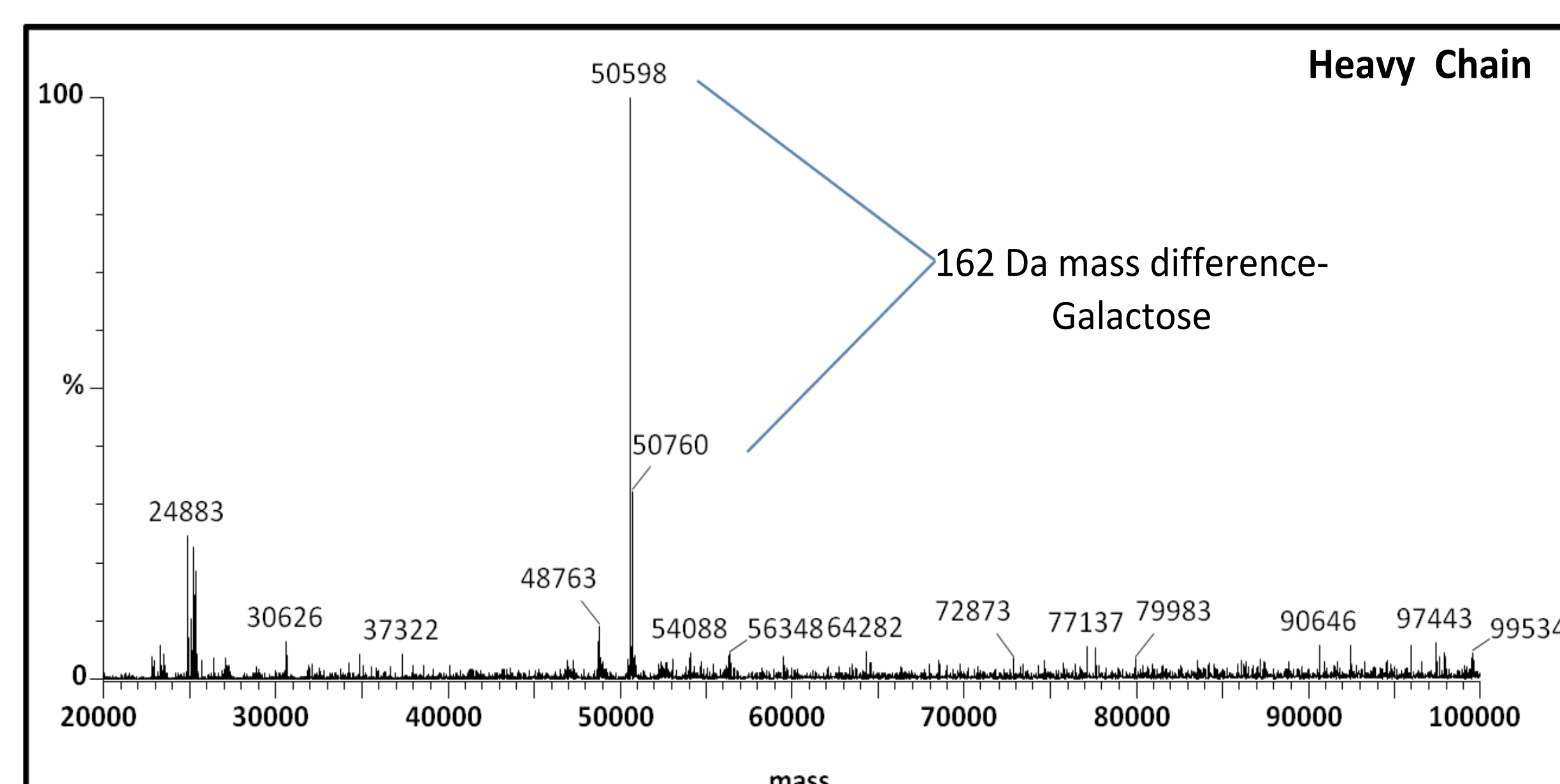
## HEAVY AND LIGHT CHAIN OF MAb 321 ON ZENIX™ SEC-300

Analysis of 30 μg of reduced MAb321 injected on Zenix™ SEC-300 running at 0.5 mL/min with 0.1% TFA and 0.1% formic acid in 20% ACN. Peak 1 and Peak 2 were collected and dried using a speed vac before being re-dissolved in SDS-PAGE gel sample buffer and ran on a gel.

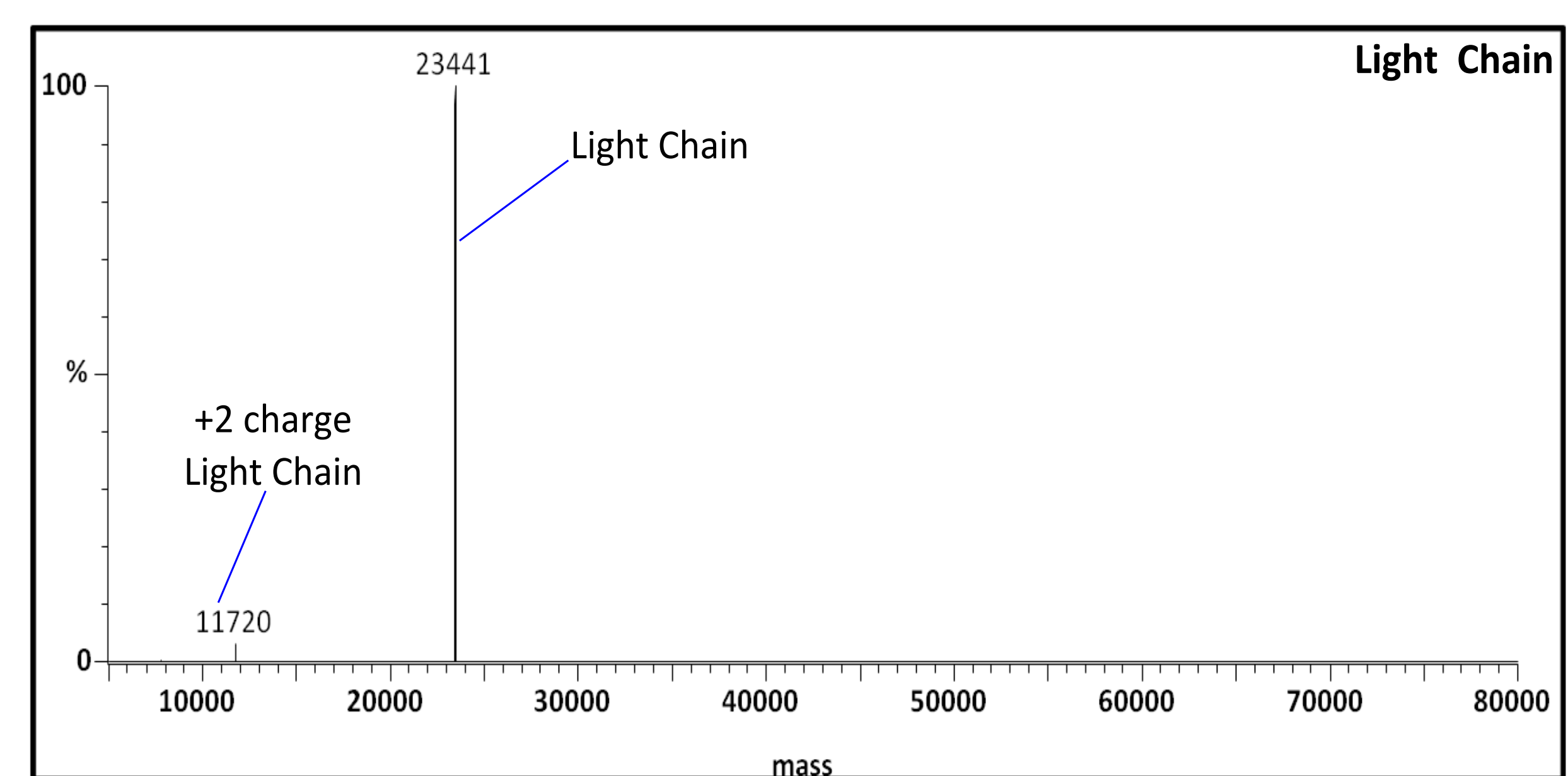


1. Protein marker
2. Reduced MAb321
3. Peak 1 heavy chain
4. Peak 2 light chain

## MASS SPECTRA FOR THE HEAVY AND LIGHT CHAINS OF MAb 321

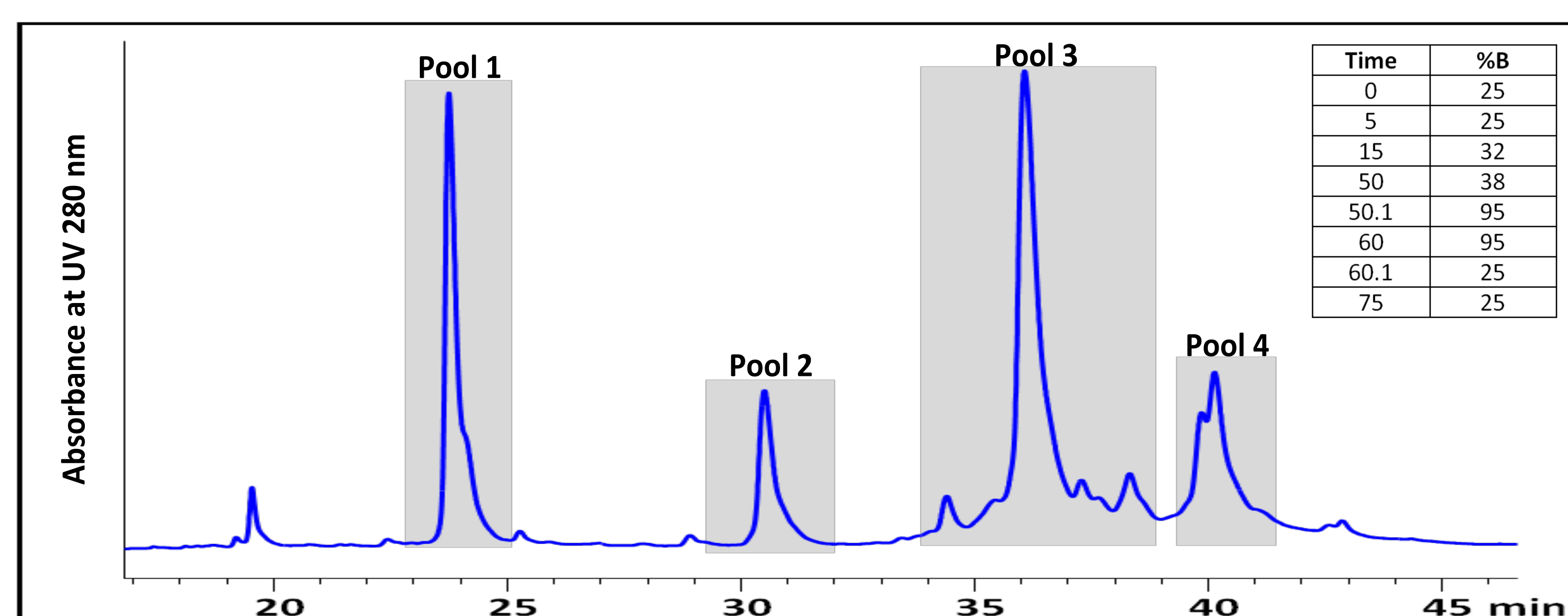


Mass Spectra of Reduced MAb 321 heavy chain fraction collected after separation on a Zenix™ SEC-300.

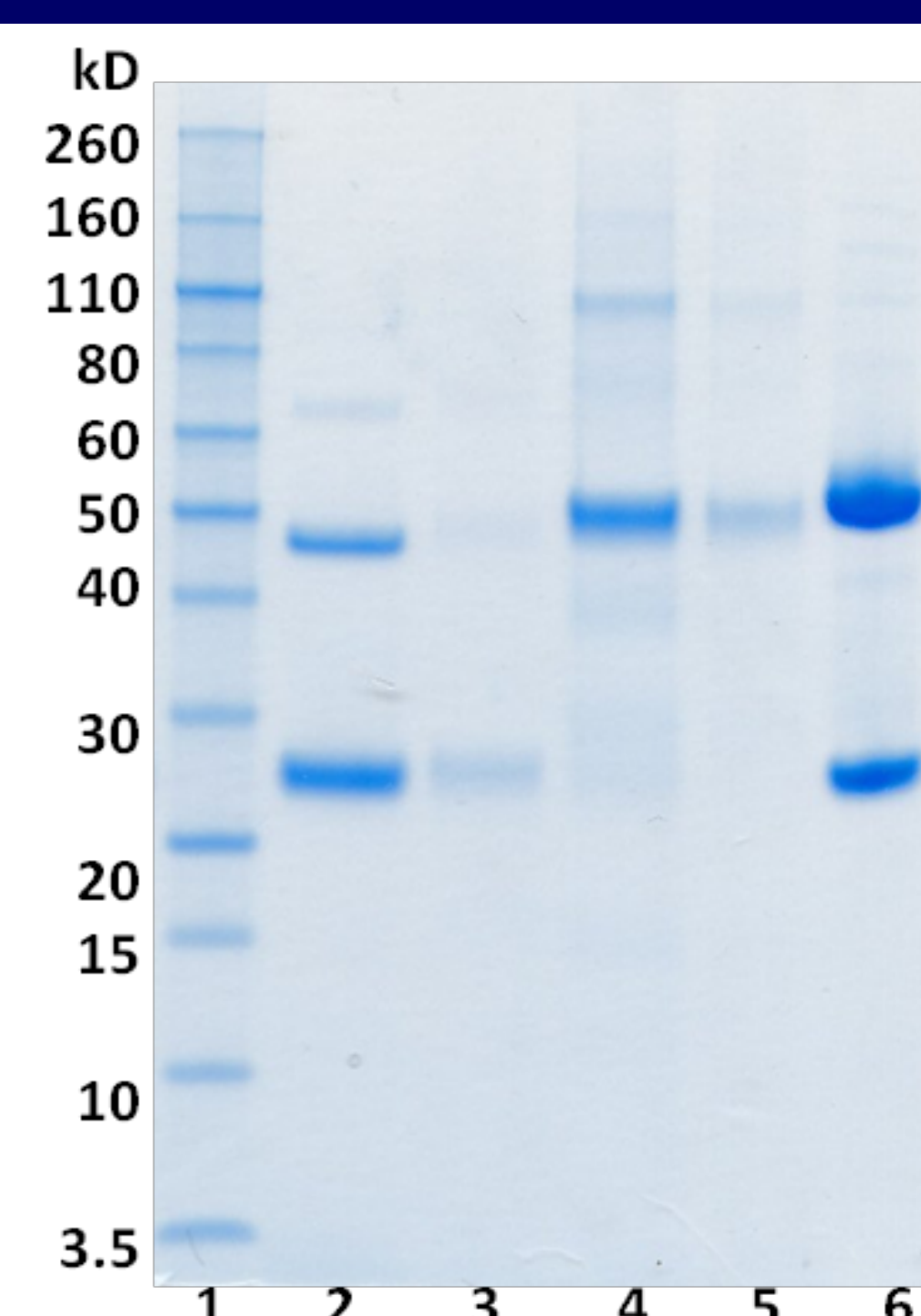


Mass Spectra of Reduced MAb 321 light chain fraction collected after separation on a Zenix™ SEC-300.

## MAb 321 HEAVY AND LIGHT CHAIN SEPARATION ON BIO-C8



Column: Bio C8 (3 μm, 300 Å, 4.6 x 100 mm), Mobile Phase A: 0.11% TFA in water, Mobile Phase B: 0.09% TFA in ACN, Flow: 0.5 mL/min, Column Temperature: 75°C, Detection: UV 280nm, Sample: 30 μg MAb 321. Heavy chain 50 kD, light chain 25 kD.



1. Protein marker
2. Pool 1 (light chain)
3. Pool 2 (light chain)
4. Pool 3 (heavy chain)
5. Pool 4 (heavy chain)
6. Reduced MAb 321

## CONCLUSION

- Sepax size exclusion chromatography Zenix™ SEC-300 (3 μm) successfully separated reduced monoclonal antibody into heavy and light chains according to their molecular weights using volatile buffer 0.1% TFA, 0.1% formic acid and 20% acetonitrile.
- Based on the chromatogram overlays, intact monoclonal antibody was separated from the heavy and light chains.
- With a reduced flow rate, on-line SEC (Zenix™ SEC-300)-MS generated accurate masses for heavy and light chains.
- On-line SEC-MS with volatile buffers can be applied to general protein separation and mass detection, a complementary work flow to RP-HPLC-MS.

## REFERENCES

1. Liu H.; Gaza-Bulseco G.; and Chumsae C. Analysis of Reduced Monoclonal Antibodies Using Size Exclusion chromatography Coupled with mass Spectrometry. J. Am. Soc. Mass Spectrom (2009) 20, 2258-264
2. Yan B.; Valliere-Douglass J.; Brady L.; Steen S.; Han M.; Pace D.; Elliott S.; Yates Z.; Han Y.; Balland A.; Wang W.; Pettit D. Analysis of post-translational modifications in recombinant monoclonal antibody IgG1 by reversed-phase liquid chromatography/mass spectrometry. J. Chromatogr. A 1164 (2007) 153-161.

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