Application Note 1133

SEC-MS with Volatile Buffers for Characterization of Biopharmaceuticals

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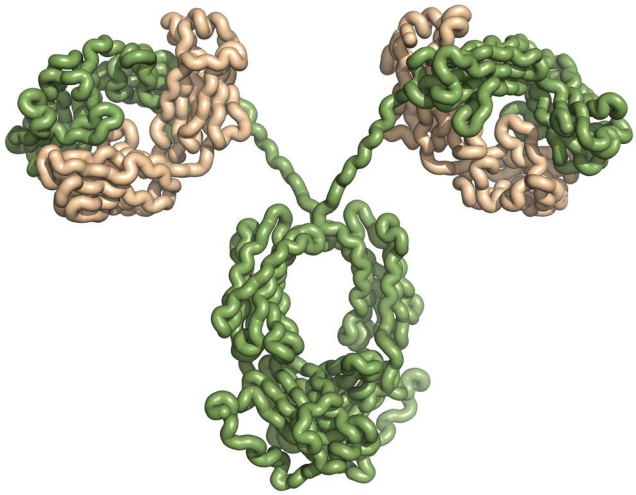
Key Words
Monoclonal Antibodies, MAbPac SEC-1, Biocompatible UHPLC, Biotherapeutics Characterization, Biopharma

Goal
Prove the suitability of the biocompatible Thermo Scientific™ Vanquish™ Flex system for size exclusion chromatography separations with mass spectrometry compatible volatile buffers.

Introduction
Size exclusion chromatography (SEC) is a routine method for the characterization of biopharmaceuticals. The conventional SEC setup uses UV detection, due to high salt concentrations in commonly used buffers. In this study, the separation of proteins by denaturing SEC with direct coupling to a mass spectrometer (MS) was investigated. A mobile phase containing acetonitrile, trifluoroacetic acid, and formic acid was used, which also allowed a direct molecular weight measurement by mass spectrometry.

The denaturing SEC method with a separation based on size differences provides an alternative to the commonly used (ion pair) reversed-phase chromatography (IP-RPC). Especially for large proteins, IP-RPC peaks often have significant tailing, due to secondary interactions with the column. This effect can be reduced with elevated column temperatures (>70 °C), but can be problematic for temperature-sensitive proteins. SEC can be used at room temperature with satisfying peak shape also for large proteins. Trifluoroacetic acid (TFA) is a common ion-pairing reagent that is often used to increase the separation efficiency for proteins in reversed-phase separations.[1] In SEC buffers, the addition of TFA reduces the protein-protein and protein-column interactions and is used instead of salts to avoid these negative effects.[2] However, it has been known that TFA suppresses the mass spectrometry signal; therefore, a very low TFA concentration of 0.05% was used.



Experimental
System evaluation was performed using the HPLC protein standard mixture (Sigma-Aldrich Chemie GmbH). The commercially available monoclonal antibodies rituximab (F. Hoffmann-La Roche, Ltd) and denosumab (Amgen) were used for this study, and disulfide bonds were reduced by incubation for 30 minutes at 60 °C with 5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) to separate light and heavy chain.

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Equipment

Vanquish Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

Experimental Conditions - HPLC	
Column	Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 300 Å (4 x 300 mm (P/N 074696)
Mobile Phase	A: 0.1% FA and 0.05% TFA in 3/7 acetonitrile/water (v/v) , (P/N acetonitrile TS-51101)
Gradient	Isocratic
Flow Rate	0.2 mL/min
Temperature	25 °C
Injection Volume	1 µL
Detection	214 nm Data Collection Rate: 10 Hz Response Time 0.4 s
Flow Cell	10 mm LightPipe

Experimental Conditions - MS	
Source	HESI-II
Sheath Gas Pressure	35 psi
Auxiliary Gas Flow	10 arbitrary units
Capillary Temperature	250 °C
S-lens RF Voltage	60
Source Voltage	3.5 kV
Full MS Parameters	
Full MS Mass Range	1500–5000 / 400–3000 *m/z*
Resolution Settings	15.000 / 240.000
Target Value	3e6
Max Injection Time	200 ms
Microscans	10 / 1
SID	10–100 eV

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2 SR3 was used for data acquisition, and the data analysis was performed using Thermo Scientific™ ProteinDeconvolution™ software version 3.0.

Results and Discussion

The mixture of four standard proteins (cytochrome c, myoglobin, ribonuclease A, and transferrin) was used to evaluate the setup. The separation and corresponding mass spectrum for each protein is shown in Figure 1. The measured mass after deconvolution has a mass deviation to the calculated theoretical mass of 0.75–1.23 ppm and shows the outstanding mass accuracy of the Q Exactive mass spectrometer.

The concentration of the additive TFA is a critical parameter in this method and has to be optimized for protein separations; it varies between 0.01 and 0.2%. The quaternary pump of the Vanquish Flex system enables a straightforward mobile phase optimization using one channel containing an adequate concentration of TFA. All common therapeutic mAb products are dissolved in formulation buffers with MS-incompatible components like polysorbate 80 and salts to stabilize the protein. The established SEC-MS setup is able to separate the small buffer components from the protein, can be used as a desalting step prior to mass detection and provides an alternative to IP-RPC separations. (Figure 2A and 2C).

The isocratic separation allows fast separations, due to the absence of an equilibration step and the low carryover of SEC separations. This method can also be used to separate the intact mAb from the detached chains for product related impurity characterizations, due to a retention time difference of 0.7 minutes from the intact mAb to the next eluting peak of the heavy chain. The disulfide bridges of the mAb were reduced with TCEP to separate light (23 kDa) and heavy chain (50 kDa). The two chains were baseline separated and the measured protein spectra are shown in Figure 2B and 2D.

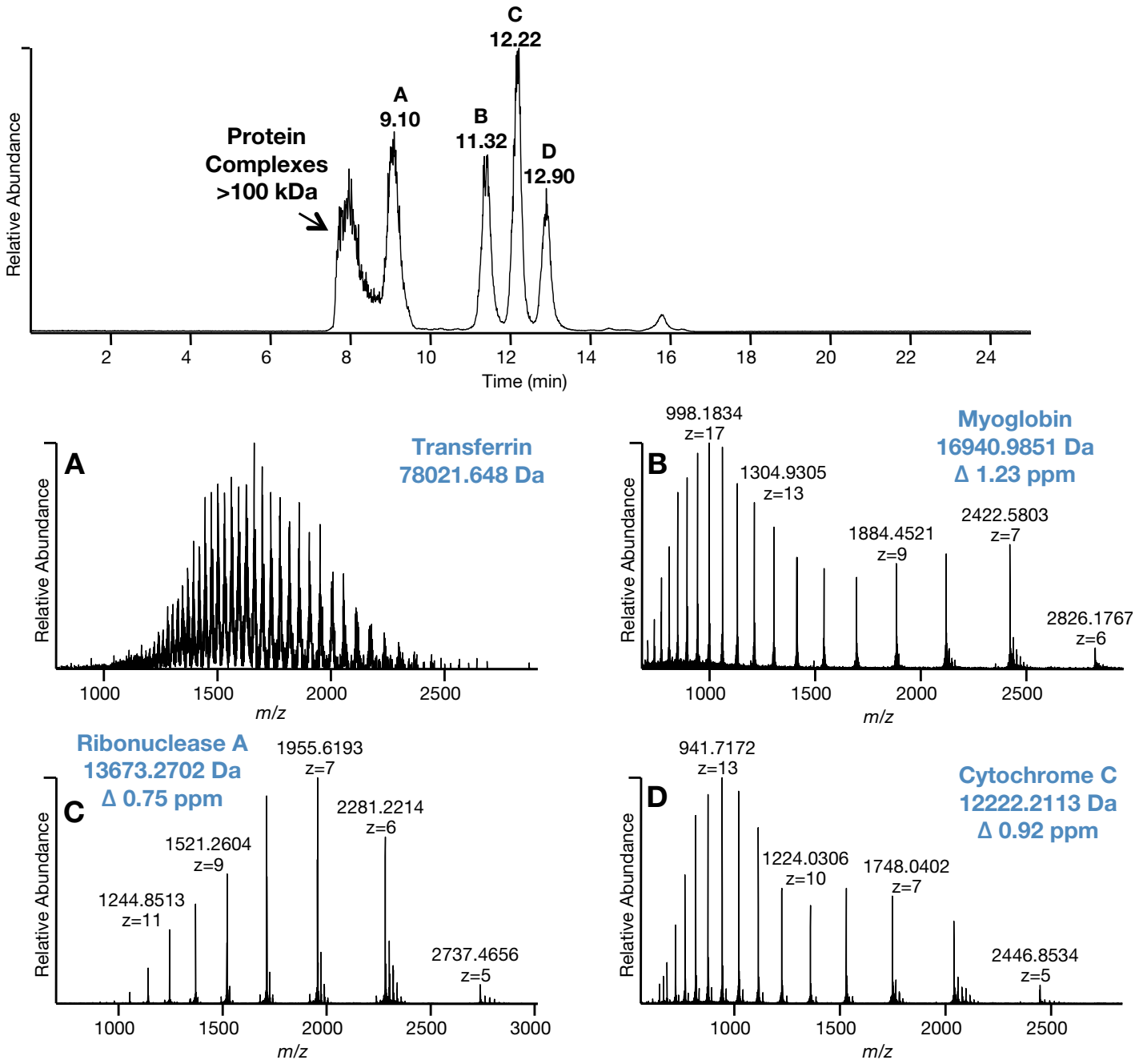

Figure 1. Separation of a mixture of the four standard proteins and the measured mass spectra for cytochrome c (1D), myoglobin (1B), ribonuclease A, (1C) and transferrin (1A). The protein complexes at RT = 8 min can be minimized with increased TFA concentrations (~ 0.1%), with the drawback of decreased signal intensities of the protein spectra.

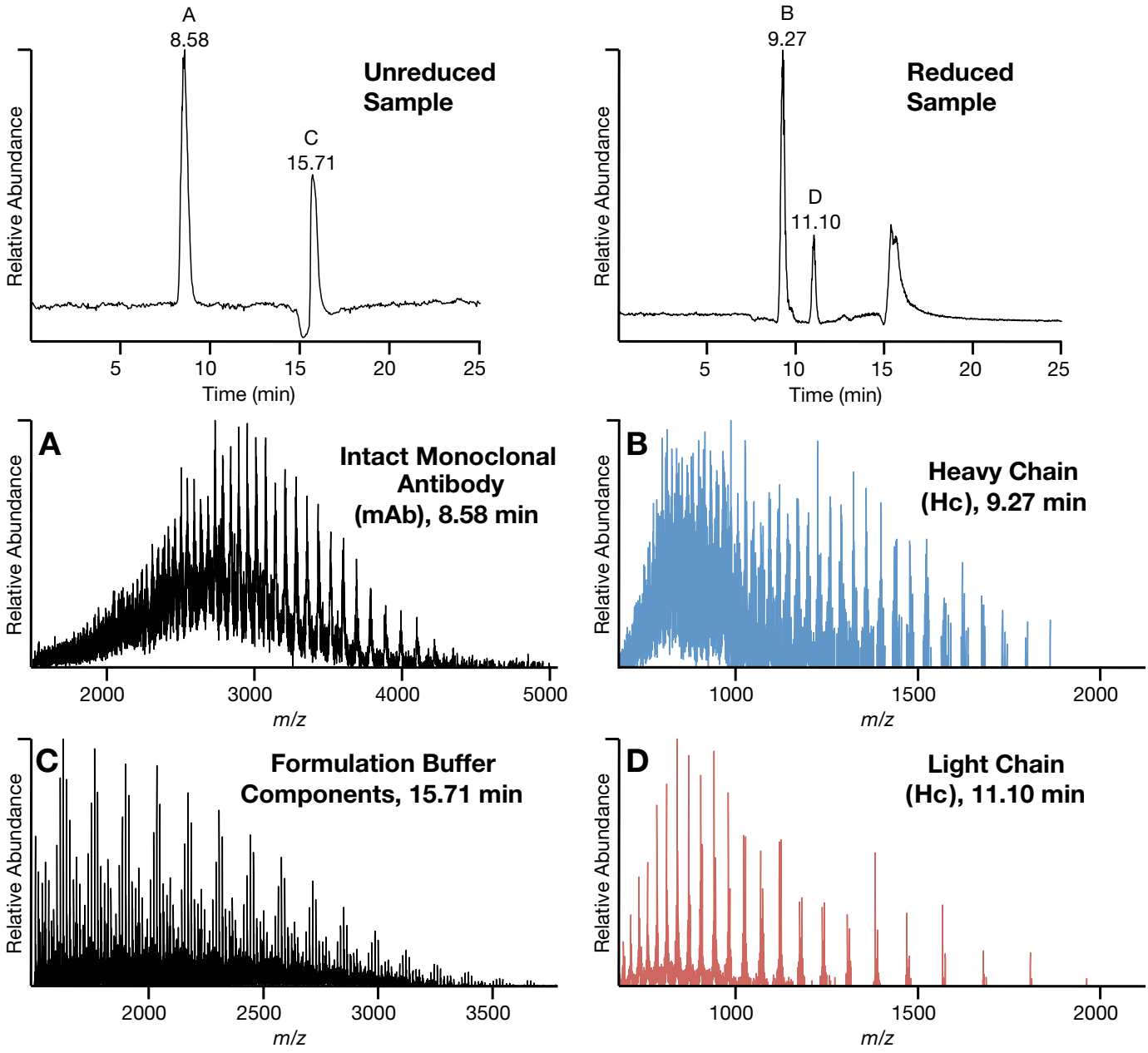

Figure 2. Separation of the unreduced and reduced monoclonal antibody rituximab.

Conclusion

The use of TFA and FA as additives for volatile SEC buffers enables the possibility for MS detection and delivers sufficient separation efficacy to characterize biopharmaceuticals. The biocompatible flow path of the Vanquish Flex system and the solvent flexibility of the quaternary pump delivers the ideal system for SEC-MS analysis and even establishes the possibility of method scouting.

References

1. Thermo Scientific Application Note 591: LC/MS Analysis of the Monoclonal Antibody Rituximab Using the Q Exactive Benchtop Orbitrap Mass Spectrometer. 2013.
2. Arakawa, T., et al., The critical role of mobile phase composition in size exclusion chromatography of protein pharmaceuticals. *J Pharm Sci*, **2010**, *99*(4), 1674-92.

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