Application Note 1154

Utilizing the Native Fluorescence of Monoclonal Antibodies for the Sensitive Detection of Charge Variants

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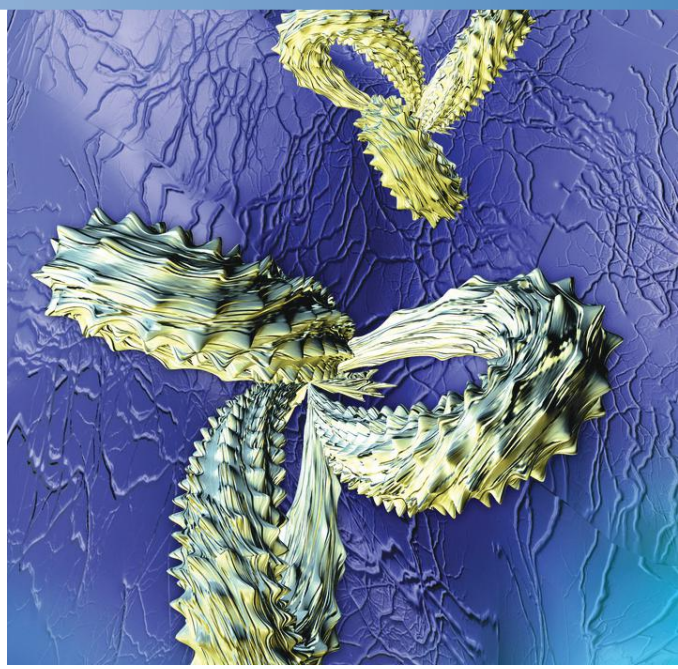
Key Words
Vanquish Flex, Biocompatible UHPLC, MabPac SCX, Biotherapeutics Characterization, Biopharma

Goal
Evaluate the use of native fluorescence detection compared to UV absorption for measurement of mAb charge variants



Introduction

Monoclonal antibodies (mAbs) are widely used in targeted therapies, and their clinical use is constantly increasing. Due to their size and molecular complexity, mAbs require a variety of analytical techniques to be fully characterized. One analytical approach, ion exchange chromatography (IEC) with UV detection, is used to provide information about charge variants and their relative amounts. In some cases, for instance after a size-exclusion chromatography (SEC) purification step, the sample to be separated by IEC may contain high concentrations of salt (e.g., ≤ 300 mM NaCl). High salt concentrations provide the strong ionic eluent necessary in IEC. Good chromatography practice, however, recommends injecting samples dissolved in solvents of equal or lower elution strength than the mobile phase. Failure to do so can cause additional band broadening resulting from peak distortion during the injection. One way to mitigate the limitation is to simply dilute the sample prior to the injection and thus reduce the elution power of the sample solvent. However, this approach may result in insufficient sensitivity when using UV detection. An alternative detection technology is fluorescence detection (FLD), which utilizes the native fluorescence of proteins (e.g., immunoglobulin) primarily resulting from the presence of tryptophan, tyrosine, and phenylalanine. FLD is often regarded as more sensitive than UV absorption. In addition, some studies have evaluated the opportunity of LC-FLD for the analysis of mAbs and proteins.[1-2]

In this work, the impact of a salt-rich matrix on the quality of an IEC separation was evaluated and detection by FLD and UV absorbance compared. For the separation of the charge variants, a linear pH gradient with the Thermo Scientific™ MAbPac™ SCX-10 RS column was used. For detection, the Thermo Scientific™ Vanquish™ Variable Wavelength Detector and the Thermo Scientific™ Vanquish™ Fluorescence Detector were used. The method was optimized for high throughput rather than for highest resolution. The high data collection rate capabilities of the FLD (up to 200 Hz) and the low gradient delay volume of the Thermo Scientific™ Vanquish™ Flex Binary System are ideal for high-throughput applications.

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Experimental

Sample Preparation

Infliximab powder (100 mg) was dissolved in 10 mL deionized water according to the manufacturer's manual to give a final concentration of 10 mg/mL.

The solution was frozen in 250 µL aliquots at -20 °C until used. Prior to use, the samples were thawed and diluted with 250 µL of water (low salt concentration sample) or 600 mM NaCl (300 mM NaCl sample matrix) to obtain a final concentration of 5 mg/mL. Further dilutions of the salt-rich 5 mg/mL stock solution were prepared with deionized water.

Instrumentation

- Thermo Scientific Vanquish Flex UHPLC system consisting of:
  - System Base (P/N VF-S01-A)
  - Binary Pump F (P/N VF-P10-A-01)
  - Split Sampler FT (P/N VF-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Active Pre-heater (6732.0110)
  - Variable Wavelength Detector (P/N VF-D40-A)
  - Variable Wavelength Detector Flow Cell: Semi-micro flow cell 2.5 µL, biocompatible (P/N 6077.0300)
  - Fluorescence Detector F (P/N VF-D50-A)
  - Fluorescence Detector Flow Cell: Micro flow cell 2 µL, biocompatible (6079.4330)

Experimental Conditions

Column	MAbPac SCX-10 RS, 5 µm, 2.1 x 50 mm (P/N 082675)
Buffers	Thermo Scientific™ CX-1 pH Gradient Buffer A (pH 5.6) 125 mL (P/N 083273) Thermo Scientific CX-1 pH Gradient Buffer B (pH 10.2) 125 mL (P/N 083275)
Mobile Phase	A: CX-1 pH Gradient Buffer A (pH 5.6) diluted 10× in deionized water B: CX-1 pH Gradient Buffer B (pH 10.2) diluted 10× in deionized water
Gradient	For standard application: 0–10 min: 0–100% B, 10–10.1 min: 100–0% B, 10.1–15 min: 0% B For high-throughput application: 0–0.8 min: 21–28% B, 0.8 min: 21% B, 0.8–2 min 21% B
Flow Rate	For standard application: 0.45 mL/min For high-throughput application: 1.2 mL/min
Temperature	30 °C Still air mode of the Column Thermostat
Injection Volume	10 µL
Detection VWD	280 nm Data Collection Rate: 20 Hz
Detection FLD	Excitation: 280 nm, Emission: 348 nm Data Collection Rate: 20 Hz and 50 Hz for high-throughput application Lamp Mode: High Power Sensitivity: 4

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2, was used for data analysis.

Results and Discussion

In Figure 1 the effect of the salt content in the sample matrix is illustrated. The low salt sample shows well-separated variants and sharp peaks. The chromatogram related to the high salt concentration matrix showed much broader peaks, with loss of resolution between variants. Moreover, the peaks eluted earlier when high salt concentration sample matrix was injected. Since all conditions were identical except the sample matrix composition, the experiments clearly indicate that the high salt concentration in the matrix negatively affects the IEC resolving power.

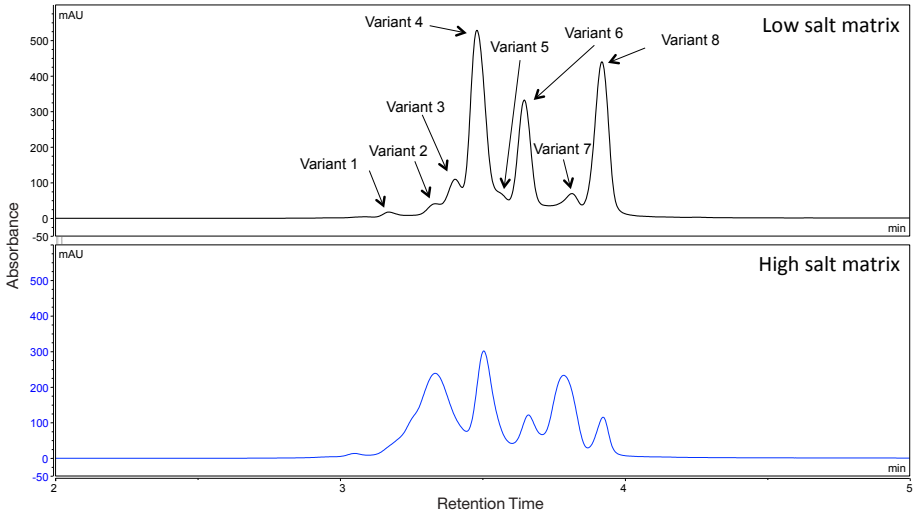

Figure 1. Separation of charge variants using a pH gradient of the low salt and high salt samples. Top trace shows a well separated chromatogram of a sample with low amount of salt, while the bottom trace shows separation distortion due to high salt levels. The samples were analyzed by LC-VWD.

The negative matrix effects on the chromatography can be avoided by diluting the sample with deionized water. Figure 2 shows the chromatograms of samples at several degrees of dilution. A 10-fold dilution of the original sample already significantly improves the separation. The chromatographic pattern of the sample related to the 30 mM NaCl matrix was very similar to the chromatogram of the low salt matrix sample of Figure 1. However, sample dilution always results in a loss of sensitivity, which may be problematic if very low abundant variants of an antibody need to be quantified. To address this issue, the use of fluorescence detection was evaluated. In Figure 3, the same sample (0.01 µg/µL, 6 mM NaCl) was analyzed with a UV detector (top trace) and FLD (bottom trace). The comparison clearly demonstrates that the FLD is significantly more sensitive than the UV detector. Table 1 presents the signal-to-noise ratios achieved for both detection techniques. On average, the FLD is more than five times more sensitive than the UV detector. Since most of the fluorescent amino acids are part of the conserved regions, this approach is suitable for the vast majority of mAbs.[3]

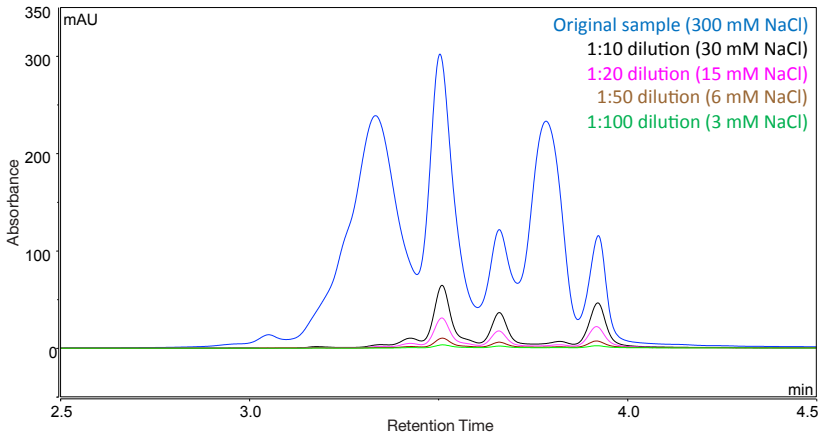

Figure 2. Dilution series of a high-salt-containing mAb sample to demonstrate the effect of salt concentration on ion-exchange separation. The samples were analyzed by LC-VWD.

Table 1. Signal-to-noise ratio for the detection with UV and fluorescence (sample: 0.05 µg/µL). The variants not listed in the table were not detected by UV at the concentration of 0.05 µg/µL.

Substance	S/N UV	S/N FLD	Gain Factor
Variant 3	10	51	5.0
Variant 4	146	839	5.8
Variant 6	81	450	5.6
Variant 7	7	30	4.2
Variant 8	105	569	5.4

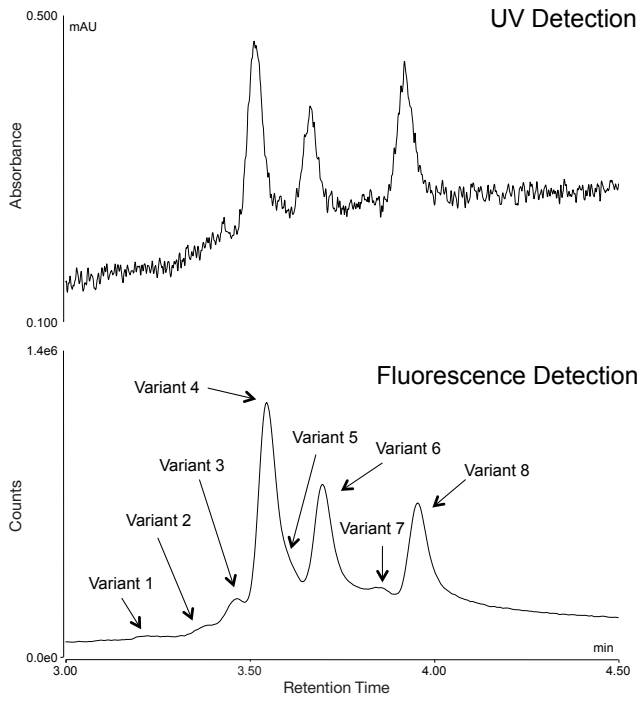

Figure 3. Comparison of a UV chromatogram (top trace) and a fluorescence chromatogram (bottom trace), showing improved S/N of the fluorescence approach (sample: 0.01 µg/µL).

For the routine measurement of charge variants, short analysis times are preferred to meet high-throughput demands. The new Vanquish Flex Binary system is able to run exceptionally fast gradients and is ideally suited to meet this challenge. This is being demonstrated in Figure 4, which shows the fast separation of infliximab. The chromatographic pattern was conserved between the moderate flow rate of the 10 min gradient and the high flow rate of the rapid sub-one-minute gradient. Impressively, the resolution between the different variants was not negatively affected by the use of a high flow rate gradient.

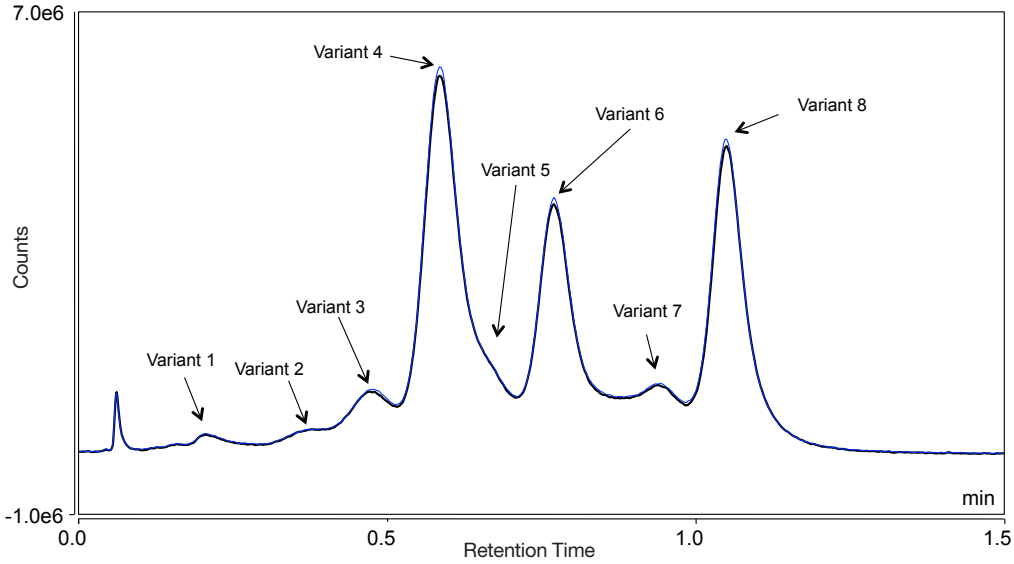

Figure 4. Separation and fluorescence detection of the monoclonal antibody with a 48-second gradient and a total run time, including equilibration, of 2 minutes (overlay of two runs).

Conclusion

The presence of salt in samples for IEC has a negative effect on the separation, but this cannot always be avoided given the combination of methods used for mAb analysis. This application note demonstrates the effects of simply diluting the sample prior to IEC and ways to overcome the reduced sensitivity. Signal-to-noise gains achieved with fluorescence detection were typically 5× greater than UV absorption at 280 nm, allowing the detection of minor components. The combination of the new Vanquish Flex Binary system and well-established, high-pressure rated, small-particle-size ion exchange columns easily enables charge variants separation. Furthermore, the Vanquish system, with its default sample capacity up to 1500 samples and up to nearly 9000 samples with the Thermo Scientific™ Vanquish™ Charger module, readily supports high sample throughput.

References

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