Automated MAb Workflow: from Harvest Cell Culture to Intact Mass Analysis of Variants

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Overview

Purpose: Demonstrate an automated monoclonal antibody (MAb) analysis two-dimensional (2D) workflow and intact mass detection.

Methods: Automated analysis is achieved with the Thermo Scientific Dionex UltiMate 3000 x2 Dual Titanium Biocompatible Analytical LC System using Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software. The intact mass information is acquired on the Thermo Scientific Q Exactive mass spectrometer.

Results: This workflow enables the completion of affinity purification, size-exclusion analysis, and charge variant analysis in less than one hour. The intact mass analysis characterizes the structural difference of the MAb variants.

Introduction

During development of recombinant MAbs, a large of number of harvest cell culture (HCC) samples must be screened for IgG titer, aggregations, and charge variants. Affinity chromatography is often used first to purify MAbs, with typical yields of more than 95%. Size-exclusion chromatography (SEC) is used to identify and quantify MAb aggregations. Finally, ion-exchange chromatography (IEC) characterizes charge variants. For the final biopharmaceutical product approval and subsequent manufacturing processes, a comprehensive characterization of MAb purity, aggregate forms, and charge variants is required by the regulatory agencies.

In the present study, we automate a 2D high-performance liquid chromatography (HPLC) workflow using an integrated HPLC system. This system consists of a dual-gradient pump, a UV/VIS detector, a column oven, and an autosampler capable of both sample injection and fraction collection. First, the HCC is injected onto the POROS® A Protein A Affinity column and IgG fractions are collected by the autosampler. Subsequently, the IgG fractions are injected separately onto Thermo Scientific MAbPac SEC-1 and MAbPac™ SCX-10 columns for further analysis. The MAbPac SCX-10, 3 µm column was recently introduced in 4 × 50 mm format for high-throughput MAb variant analysis. This column delivers high resolution separation with a shorter run time using either salt or pH gradients. Incorporating this column into the workflow, we completed affinity purification, SEC and charge variant analyses in less than one hour. Furthermore, the fractions collected off the MAbPac SCX-10 column were analyzed by mass spectrometry (MS), and intact mass information of the MAbs demonstrated the presence of lysine variants.

Methods

Harvest Cell Culture

MAb HCC was a gift from a local biotech company. The HCC was filtered through a 0.22 µm membrane prior to sample injection.

Columns

- MAbPac SCX-10, 3 µm, 4 × 50 mm (P/N 077907)
- MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
- MAbPac SEC-1, 4 × 300 mm (P/N 074696)
- POROS A Protein A Affinity 20 µm Column, PEEK™, 4.6 mm x 50 mm, 0.8 ml (P/N 1-5022-24)

Liquid Chromatography System

HPLC experiments were carried out using an UltiMate™ 3000 x2 Dual Titanium System equipped with SRD-3600 Integrated Solvent and Degasser Rack, DGP-3600BM x 2 Dual-Gradient Micro Pump, TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves, WPS-3000T(B)FC Analytical Dual-Valve Wellplate Sampler, VWD-3400RS Four Channel Variable Wavelength Detector equipped with a Micro Flow Cell, and PCM-3000 pH and Conductivity Monitor.

pH-Based Ion-Exchange Chromatography

In a scale-up purification, 1 mL of IgG was purified from the 3.8 mL HCC using Thermo Scientific Pierce Protein A Plus Agarose beads (P/N 22810). The protein concentration was determined at ~ 0.5 mg/mL. Approximately 100 µL of the purified IgG was injected onto a MAbPac SCX-10, 10 µm, 4 × 250 mm column and separated via pH gradient from pH 7.8 to pH 10.8. Mobile phase buffers contained 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine with pH values of either 6.8 (Buffer A) or 10.8 (Buffer B). The column was equilibrated at 40% B. Three min after sample injection, a linear gradient was run from 40% to 100% B in 30 min. Fractions were collected onto a 96-wellplate at a rate of 0.2 min per fraction from 17 to 27 min.

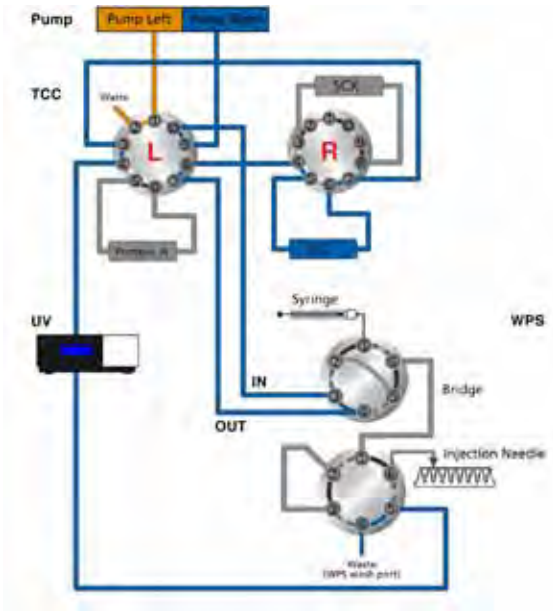
2D-LC Workflow

The workflow and LC conditions for automated off-line 2D-LC include the following:

- Injection of 50 µL of an unpurified HCC sample
- A first-dimension (^{1}D) affinity chromatography separation at a flow rate of 2.0 mL/min using the following steps:
  - A column wash/equilibration step of 0.75 min
  - An elution step of 1 min
  - Automated time-based fraction collection into a wellplate in the autosampler
  - Protein A column is regenerated by a 20% acetonitrile wash and reconditioned for the next analysis

  Total analysis time is approximately 3 min.
- A second-dimension (^{2}D) separation of the collected fraction includes one of the following:
  - SEC separation at a flow rate of 0.3 mL/min using an isocratic mobile phase
  - Strong cation-exchange separation at a flow rate of 0.6 mL/min using a salt gradient

FIGURE 1. Fluidic configuration of the automated off-line 2D-LC system using the wellplate bio-inert autosampler



LC-MS

HPLC: Thermo Scientific ProSwift RP-10R Monolithic Capillary Column (1.0 mm i.d. × 5 cm) was used for desalting. LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 50 ºC during analysis. Flow rate was 100 µL/min. After injection of MAb, a 5 min gradient from 10% B to 95% B was used to elute MAbs from the column.

MS: Using Q Exactive™ instruments, intact MAb was analyzed by ESI-MS for intact molecular mass. The spray voltage was 4 kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 ºC . S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500. The AGC target was set at 3E6 for full scan. Maximum IT was set at 200 ms.

Data Processing: Full MS spectra of intact MAbs were analyzed using Thermo Scientific Protein Deconvolution software 1.0 that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the MAb. The averaged spectra were subsequently deconvoluted using an input *m/z* range of 2000 to 4000 *m/z*, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and minimum of at least 8 consecutive charge states from the input *m/z* spectrum to produce a deconvoluted peak.

Results

In the first step of the chromatographic separation, HCC was injected onto the Protein A Affinity column. In order to collect sufficient amounts of IgG material for the ^{2}D analysis, 50 µL of HCC was injected. The IgG fraction was collected into a 96-wellplate using time-based triggers (Figure 2). The total collection time was 0.1 min. At 2 mL/min flow rate, the total volume collected was 200 µL. Chromeleon CDS software is capable of fraction collection using UV-based peak triggers, or both time and peak triggers together. In the configuration presented here, there was a 0.1 min delay time in fraction collection.

A transition sequence was used to switch the valves and direct the flow path to each ^{2}D analysis column. The ^{2}D analyses can be either SEC (Figure 3) or IEC (Figure 4). Collected fractions can be directly injected onto the ^{2}D column without further modifications. The injection volume for each ^{2}D was 25 µL.

The IEC analysis of the Protein-A purified fractions which used a linear salt gradient revealed many variants in the purified IgG fractions. A one-hour carboxypeptidase digestion (data not shown) eliminated several peaks and enhanced others, suggesting the presence of lysine variants. Use of the MAbPac SCX-10 3 µm column reduced the analysis time from ~60 to 20 min. The total analysis time for all three chromatographic steps was <60 min, which included the transition programs between different analyses. All these steps are automated, and therefore multiple HCC samples can be cycled through without user intervention.

Over the last few years, researchers have demonstrated that pH-gradient-based IEC is an effective method to separate acidic and basic proteins. In this study, we applied pH gradient to the separate MAb variants on a MAbPac SCX-10 column. As shown in Figure 5, separation of at least three variants was achieved. Major peaks 1, 2, and 3 eluted at 19.8, 20.8, and 22.1 min, respectively. Use of the PCM-3000 allowed real-time monitoring of the pH and conductivity of the eluent during all the analyses. The pH values for fractions containing Peaks 1, 2, and 3 were 8.5, 8.6, and 8.7, respectively. These fractions were analyzed on a Q Exactive mass spectrometer (Figure 6). On-line desalting using a reversed phase monolithic column was carried out prior to MS detection. The deconvoluted spectra (Figure 7) showed that the major component in Peak 1 has a 147992.703 *m/z*. Adjacent peaks at 148155.503 and 148315.903 *m/z* correspond to different glycoforms with 1 and 2 additional hexoses. The major component in Peak 2 has a 148210.650 *m/z*. The delta mass between Peak 1 and Peak 2 is 128 amu, corresponding to one lysine. Similarly, the delta mass between Peak 2 and Peak 3 (at *m/z* 148248.641) is also 128 amu. These data suggest that Peak 1 and Peak 2 correspond to lysine truncation variants of Peak 3.

FIGURE 2. Example of a ^{1}D affinity purification of IgG from HCC: the vertical yellow stripe indicates fractionation time.

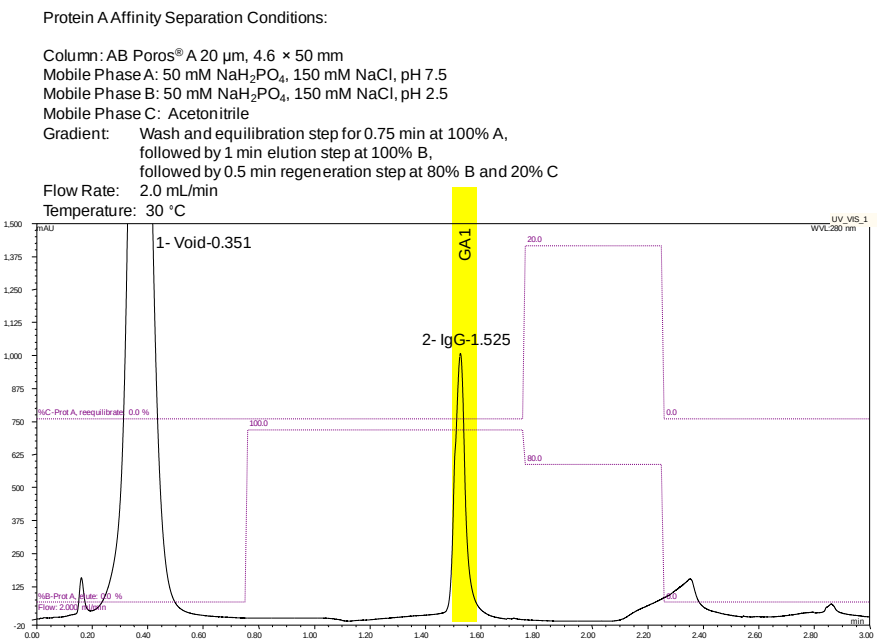


FIGURE 3. Example of an isocratic ²D SEC separation of a purified IgG fraction collected from the MAbPac SEC-1, 4 × 300 mm column

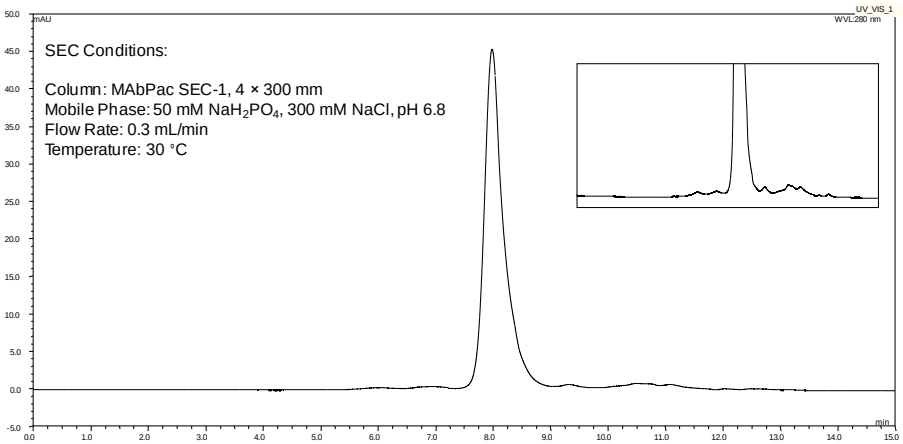


FIGURE 4. Example of a ²D SCX separation of a purified IgG fraction collected from the MAbPac SCX-10, 3 µm, 4 × 50 mm column

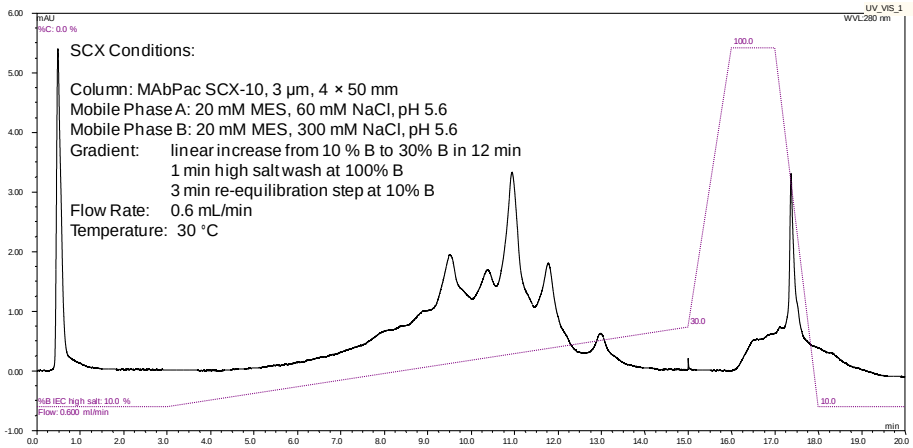


FIGURE 5. pH gradient separation of purifed IgG on a MAbPac SCX-10 column

pH-Gradient Separation Conditions:

Column: MAbPac SCX-10, 10 µm, 4 × 250 mm
Mobile Phase A: 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine, pH value 6.8
Mobile Phase B: 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine, pH value 10.8
Gradient: 3 min pre-equilibration at 40% B
followed by linear increase from 40 % B to 100% B in 30 min
followed by 7 min high pH wash at 100% B
followed by 15 min re-equilibration step at 40% B
Flow Rate: 1.0 mL/min
Temperature: 30 °C
Fraction Collection Rate: 0.2 min/well

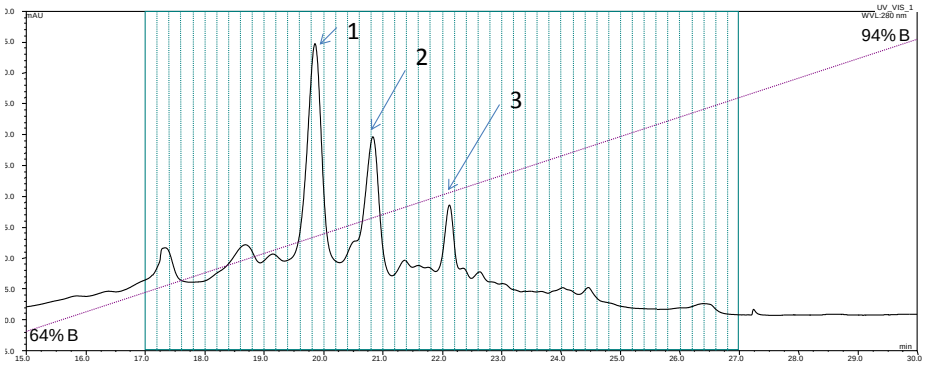


FIGURE 6. Full scan MS spectra

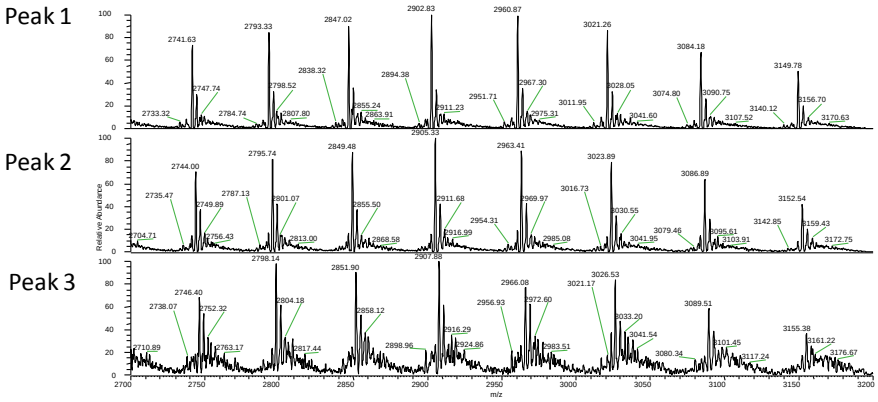
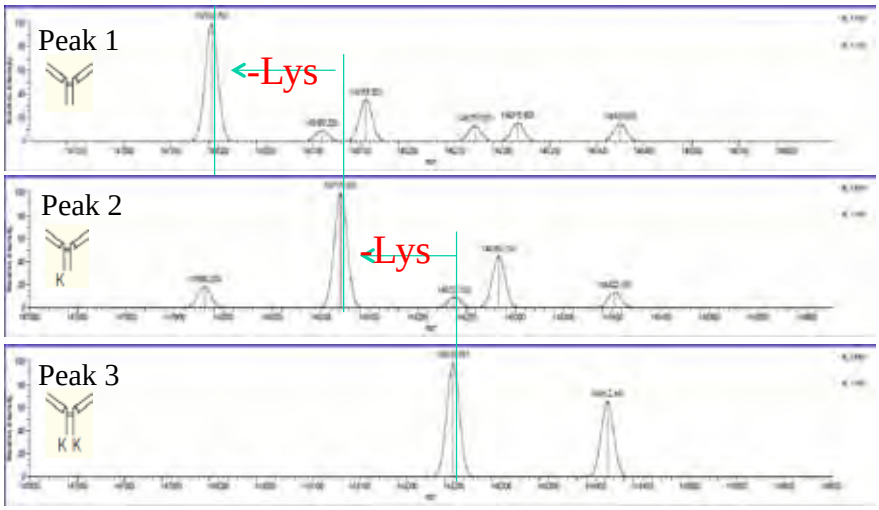


FIGURE 7. Deconvoluted MS spectra



Conclusion

- Using Protein-A Affinity, MAbPac SEC, and MAbPac SCX-10 columns, HCC was characterized by affinity purification, followed by SEC and charge variant analysis in less than one hour.
- The separation of the lysine variants demonstrated that the pH-based gradient method is an effective approach, orthogonal to salt gradient separation.
- The combination of off-line IEC separation and on-line LC MS detection provides an efficient way to obtain structural information of MAb variants.

References

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2. Rea, J., Moreno, T., Lou, Y., and Farnan, D. Validation of a pH Gradient-Based Ion-exchange Chromatography Method for High-Resolution Monoclonal Antibody Charge Variant Separations. *J. Pharm. Biomed. Anal*. **2011** *54* (2), 317–23.

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