Introduction
During various stages in the development of biopharmaceuticals, purification and analytical characterization of the product are required. In the early phase of recombinant antibody process optimization, a large number of samples must be screened for titer, aggregation, and charge variants. Two variations of major importance occurring in monoclonal antibody (MAb) biopharmaceuticals are the presence of variants of the target molecule and their aggregation. These product-related substances can have different efficacies than the main product and may cause serious side effects, such as antidrug-antibody formation. Protein aggregates are mostly the consequence of suboptimal production, purification, or handling conditions (e.g., temperature, pH).

In the purification of antibodies, a protein-affinity separation is generally the first step. Affinity chromatography on protein A or G columns typically yields a purity of more than 95% in a single step. Although the purification on affinity columns yields information on the titer of the product, it is not selective with respect to related substances. To verify the purification efficiency, sample purity, or antibody quality, a technique such as ion-exchange chromatography (IEC) or size exclusion chromatography (SEC) is required. The SEC technique provides the necessary selectivity to identify agglomerates and size-based variations of the main component. Ion-exchange stationary phases provide good selectivity for separation of charge variants of the protein biopharmaceutical. The variations may be very subtle or small, and finding the optimal chromatographic conditions requires optimization.

The work shown here discusses the development of an automated and unattended solution for purification and separation (by IEC and SEC) of monoclonal antibodies using a single HPLC system (Figure 1A). In this two-dimensional (2D) process, multiple protein separation steps are performed automatically. The autosampler of the HPLC system was configured to perform the injection, fraction collection, and reinjection of the collected fractions. The intelligent program capabilities of the Thermo Scientific Dionex Chromeleon™ Chromatography Data System (CDS) software take care of all of the automation.
Instrumentation and 2D-LC Workflow

HPLC experiments were carried out using a Dionex UltiMate 3000 Titanium System equipped with:
- SRD-3600 Membrane Degasser
- DGP-3600BM × 2 Dual-Gradient Pump System
- TCC-3000SD Thermostatted Column Compartment with valve actuators and biocompatible pods
- WPS-3000T(B)FC Analytical Dual-Valve Well Plate Sampler (Figure 1B)
- VWD-3400RS UV Detector equipped with a 2.5 µL flow cell

The Chromeleon CDS extended fractionation software driver is added in the server configuration to control the advanced features of the Dionex WPS-3000T(B)FC. This software extension is capable of managing time- and peak-based fractionation, including a wide range of settable parameters to adapt the fractionation to the specific needs of the analysis. Fractionation can be operated in continuous or pooled mode, as used in enrichment experiments. Further, this extension enables Chromeleon CDS postacquisition steps, which allows fractions to be reprocessed by another chromatographic method.

The WPS-3000T(B)FC

This dual-valve well plate autosampler is capable of fractionating into various types of vials and well plates. It can operate at elevated flow rates by using the second valve as a diverter valve. The dual-needle design with a metal prepuncture needle allows fractionation in both capped and uncapped vials and well plates, as well as Eppendorf tubes. Both puncture depth and sample needle protrusion depth can be programmed for each sample container format. In addition, the WPS-3000T(B)FC also offers sample handling and derivatization capabilities (e.g., in-well digestion, pH adjustment, dilution). It has a thermostatted sample compartment (4–45 °C) and easy-to-adapt fluidics to suit the application needs, including full-loop injection volume ranges from 1 to 500 µL. It also accommodates multiple well plate types simultaneously. Finally, the module’s excellent repeatability for injection and fractionation provides a robust platform for any analysis.

The workflow and LC conditions for automated offline 2D-LC includes the following:
- Injection of 50–250 µL of an unpurified monoclonal/polyclonal antibody sample
- A first-dimension (1D) 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 peak detection followed by fraction collection into a well plate in the autosampler
  - The protein A is reconditioned for the next analysis
- The total analysis time: approximately 3 min
- A second-dimension (2D) separation (one of the following):
  - Strong cation-exchange separation, applying a linear NaCl salt gradient (20 mM MES buffer, pH 5.6)
  - SEC separation (200 mM sodium phosphate, 250 mM NaCl, pH 6.8)

Prior to the 2D separation, add a neutralization buffer (Tris, 0.1 M) to each fraction to raise its pH, using the derivatization capabilities of the autosampler.
LC Conditions

Protein A Affinity Separation

Used Applied Biosystems Poros® protein A column (20 µm, 4.6 mm i.d. × 50 mm, 0.8 mL) for 1D affinity-based separations with UV detection at 214 and 280 nm. Apply between 50 and 250 µL of sample, making use of the large-volume injection kit and a user-defined program (UDP) to fill the sample loop.

Affinity LC Conditions
Mobile Phase A: 10 mM Sodium phosphate, 150 mM NaCl, pH 7.0
Mobile Phase B: 50 mM Glycine-HCl, 150 mM NaCl, pH 2.8
1D Gradient: Wash and equilibration step for 0.75 min at 100% A, followed by a 1 min elution step at 100% B
Flow Rate: 2.0 mL/min
Temperature: 25 °C

Ion-Exchange Separation of Protein Variants

Used Thermo Scientific MAbPac™ SCX-10 column (4 mm i.d. × 250 mm) for the 2D weak cation-exchange LC separation applying a linear salt gradient of MES buffer/ sodium-chloride solution, detection at 214 and 280 nm.

Mobile Phase A: 20 mM MES buffer, pH 5.6 + 60 mM NaCl
Mobile Phase B: 20 mM MES buffer, pH 5.5 + 300 mM NaCl
2D Gradient: From 15 to 45% B in 30 min; 6 min wash step at 100% B; and 10 min equilibration time at 15% B
Flow Rate: 1.0 mL/min
Temperature: 30 °C

Size-Exclusion Separation of Protein Aggregates

Used Thermo Scientific MAbPac SEC-1 column for the aggregation analysis (4 mm i.d. × 300 mm). The mobile phase is 200 mM sodium phosphate, 250 mM NaCl, pH 6.8.

Results

Typically, the antibody is manually injected onto the affinity column. Here, the autosampler was used to introduce the sample to this column. After an equilibration step with mobile phase A, the antibody was eluted from the Protein A column with mobile phase B and fractionated into a 96-deep well plate using UV-based peak triggers (Figure 3). These triggers can be optimized easily to either collect or discard the breakthrough peak and to collect the antibody peak with a trigger based on retention time and signal properties. A minimum fractionation/decision delay time of 5 s should be considered for peak-based fractionation.

Fractions can be visually and numerically reported in the Chromeleon CDS software. The fraction volume can be extracted and transferred automatically to the 2D sequences to enable numerous derivatizations. A neutralizing buffer of 15% of the fraction volume was added to increase the fractions’ pH, using the derivatization capabilities of the autosampler (Figure 4). When a fraction is found that meets the requirements (e.g., titer/peak area), the Chromeleon CDS software can be used to initiate a postacquisition step for the 2D separation(s). Examples of 2D separations are: a strong cation-exchange-based separation yielding selectivity towards small charge differences in the antibody sample (Figure 4A, IEC of a monoclonal IgG1 fraction); or an SEC to separate the antibody from its dimers or aggregates (Figure 4B, SEC of a MAb). The ion-exchange chromatogram clearly shows the heavy chain C-terminal lysine heterogeneity variants of the MAb described by the three largest peaks. A series of basic and acidic variants are also clearly visible. A more detailed look into monitoring MAb heterogeneity and stability by IEC can be found in AN 127 and AN 1281,2 and the MAbPac column data sheet.3

In the applied workflow all 1D separations are carried out, followed by the automatically generated 2D separation sequence. The sample throughput can be further increased by accelerating the flow rate for the affinity and the SEC separations and shortening the different steps required in the affinity separation. The IEC separation also may be accelerated by modifying the gradient.
Acceleration of Analysis

Depending on the requirements for the separation, several options are available. Utilizing short columns allows high-throughput separations. Emerging techniques such as pH-gradient-based IEC are good alternatives to accelerate the screening process.\(^4,5\) Examples of fast IEC as well as SEC are depicted in Figures 5A and 5B.
Conclusions

The dual-valve WPS-3000T(B)FC autosampler enables high-flow fraction collection and re-injection of fractions as used in 2D workflows. Such workflows include automated offline 2D column coupling (e.g., affinity with IEC and SEC; RP with RP utilizing different pH levels; SPE with RPLC); as well as IEC with fractionation, trapping, and desalting prior to mass spectrometric detection. It is clear that this module can serve as a platform for coupling various chromatographic techniques for biopharmaceuticals and proteins analysis.

The Chromeleon software package allows this high degree of automation with a wide range of flexibility to accommodate the most demanding applications. Sample handling and derivatization, purification, and HPLC analysis can be programmed both easily and in depth and gives the instrument unique capabilities in the field of sample purification, standard HPLC, and QC.

References