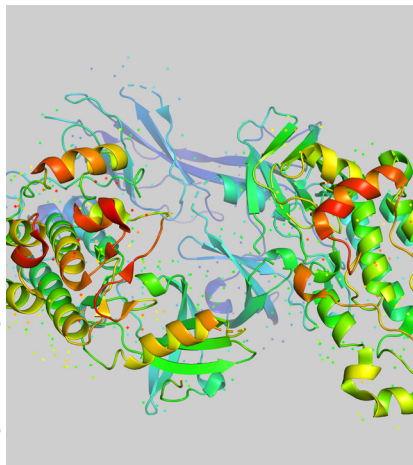


Development of a Size-Exclusion Chromatography Method to Characterize a Multimeric PEG–Protein Conjugate

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Conjugation of poly(ethylene glycol) (PEG) to therapeutic molecules is a widely used technique to increase in vivo half-life of therapeutics. A multimeric PEG–protein conjugate, which contains eight antigen-binding fragments (Fabs) conjugated to an 8-arm PEG core, was developed as a therapeutic candidate for age-related macular degeneration (AMD). The increased molecular size of the conjugate, compared to the Fab alone, produces a significant longer half-life, and requires less frequent intravitreal (ITV) injections, which can greatly benefit the patient. Due to the major impact that molecular size has on in vitreous half-life, it is crucial to have an analytical method to monitor the size attribute of the conjugate. Here we report a simple and robust size-exclusion chromatography (SEC) method that was developed for the conjugate. A wide range of size variants of the conjugate, ranging from 50 kDa to >1000 kDa, can be resolved and quantitated. The method was evaluated for precision, accuracy, linearity, and robustness, and was deemed suitable for routine use in product quality control.

Poly(ethylene glycol) (PEG) is a water-soluble, nontoxic, non-antigenic, biocompatible polymer that has long been utilized in the fields of biotechnology and pharmacology (1,2). PEGylation of peptides, proteins, and other therapeutic entities can improve their pharmacological properties by increasing water solubility (3), reducing immunogenicity (4), and increasing half-life (5,6). These advantages have made PEGylation one of the most attractive and prevalent techniques in drug delivery. More than 10 PEGylated drugs have been approved by the FDA since the 1990s, and new PEGylated agents continue to enter and expand clinical pipelines (7).

In PEGylation, linear PEGs are the simplest and most commonly used conjugate agents. Most of the approved PEGylated drugs are conjugates with one or multiple linear PEG chains, such as PEGylated bovine adenosine deaminase (Adagen), PEGylated L-asparaginase (Oncaspar), and PEGylated

α -interferon (PEG-INTRON) (8). On the other hand, novel PEG geometries with varied branching, chain length, and polydispersity have recently been utilized more in drug PEGylation, providing improved pharmacological properties compared to linear PEGs. One example is multi-armed PEG. With a star-like structure carrying multiple conjugation sites, it is shown to increase the active ingredient ratio, while simultaneously offering advantages of increased half-life and reduced immunogenicity (9).

A novel multi-armed PEG-protein conjugate, which contains eight antigen-binding fragments (Fabs) conjugated to an 8-arm PEG core, was developed as a drug candidate for age-related macular degeneration (AMD) (10). With an increased hydrodynamic radius (R_{H1}), the multimeric PEG–Fab conjugate produces a significant longer vitreal half-life than Fab alone. The large Fab:PEG ratio also enables more protein delivery compared to the con-

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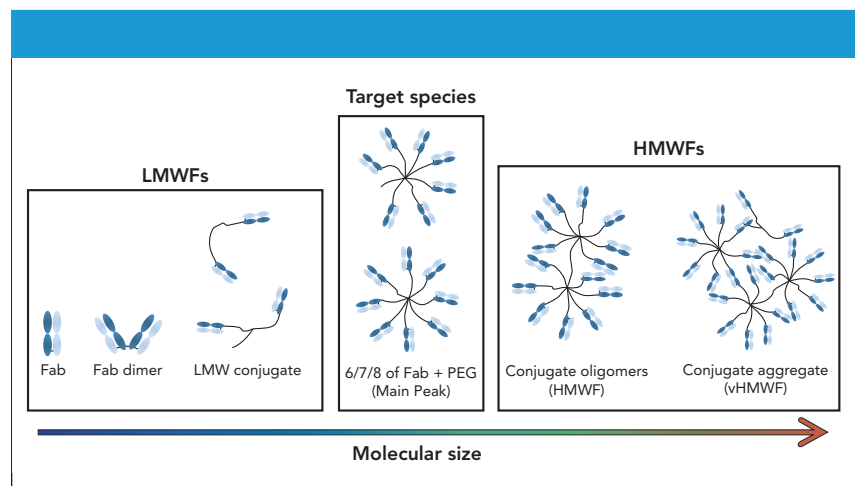


Figure 1: Schematic of the expected size variants in the PEG–Fab conjugate sample.

jugate with low Fab:PEG ratio. These improved properties make the multi-meric PEG–Fab conjugate a promising ocular therapeutic with increased exposure and less dosing frequency, which can greatly benefit the patients.

As part of drug development, reliable qualitative and quantitative methods are required for product characterization and quality control. For the PEG–Fab conjugate, it is crucial to have an analytical method to monitor the size attribute, which is proven to be a key determinant of vitreal half-life (6). Due to the large molecular size of the PEG–Fab conjugate target form, a product specific size-exclusion chromatography (SEC) with optimal resolution for all the product-related size variants was developed. A qualification study was further conducted to assess the method for specificity, precision, accuracy, linearity, and robustness.

Experimental

Reagents and Samples

Sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, L-arginine monohydrochloride, sodium azide, and sodium sulfate were purchased from Sigma-Aldrich. Isopropyl alcohol was purchased from EMD Millipore. Hydrochloric acid standard solution (5.996 mol/L) and sodium hydroxide solution (5.0 mol/L) were purchased from Fluka. Water was obtained with a Milli-Q Purification system from Millipore.

Samples

PEG–protein conjugate used in this study consists of eight Fabs conjugated to an 8-arm 40 kDa PEG core. The Fab protein was expressed in *E. coli* and it contains an unpaired hinge cysteine on

the C-terminal of the heavy chain. The PEG molecule used in the conjugation is an 8-arm PEG with a maleimide group on the end of each arm. The PEG–Fab conjugate was formed via the covalent bondage between the maleimide of the PEG and the C-termini cysteine of the Fab.

The stock solution of the PEG–Fab conjugate had a Fab concentration of 50 mg/mL and was diluted to the target concentration, 5 mg/mL, with 5 mM histidine hydrochloride, pH 5.5 buffer before the injection.

Instrumentation and Chromatographic Conditions

All measurements were performed using a Waters Alliance HPLC with multiple wavelength detector (MWD) or diode array detector (DAD). A Wyatt μ DAWN multi-angle light scattering (MALS) detector and a Wyatt Optilab UT-rEX differential refractive

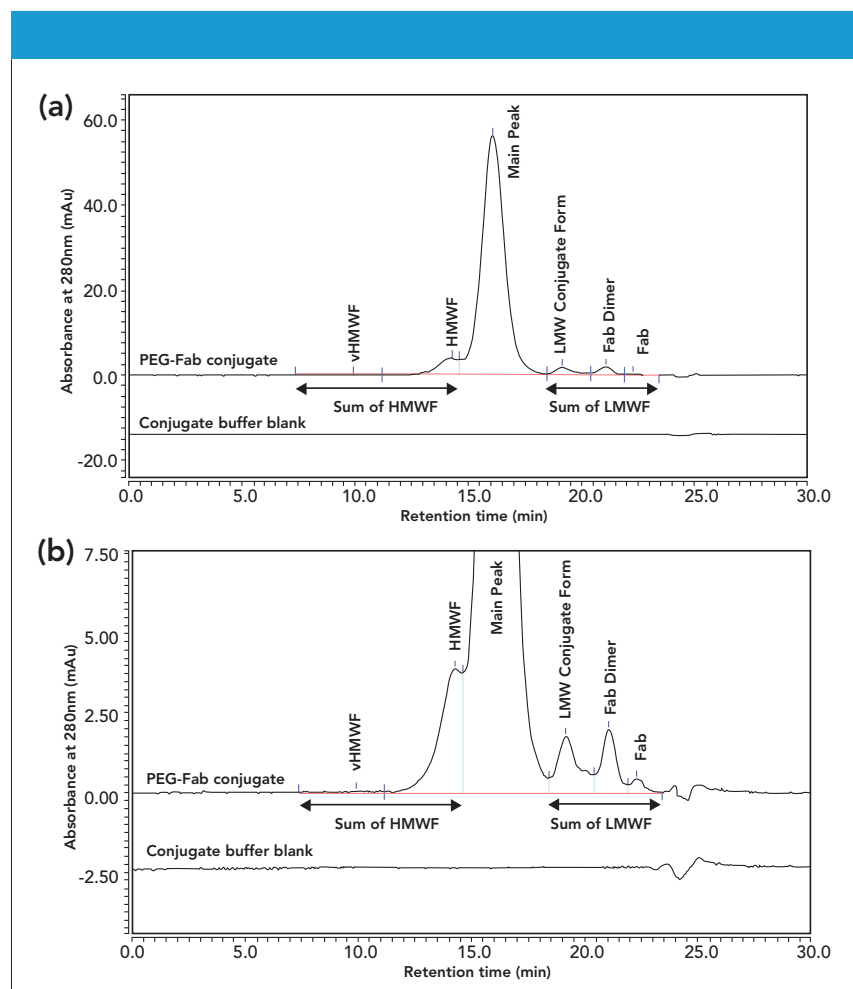


Figure 2: (a) Full-scale, and (b) enlarged representative SEC chromatogram of the PEG–Fab conjugate using a Tosoh TSKgel G4000SWXL column.

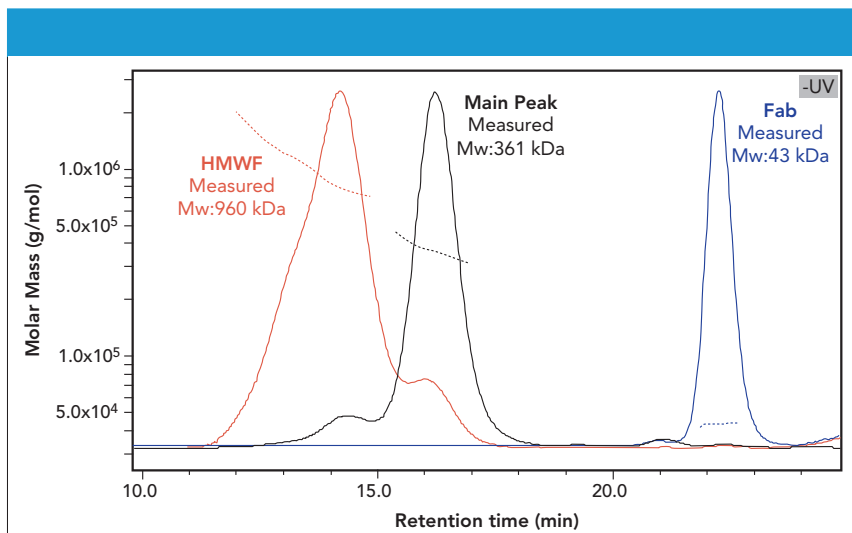


Figure 3: SEC-MALS for enriched HMWF sample, PEG–Fab conjugate and Fab, with measured molecular weight across ultraviolet (UV) trace demonstrates the effective separation of the size variants.

index (dRI) detector were coupled to the HPLC sequentially to measure the molecular weight of the eluting peaks.

Tosoh TSKgel G4000SWXL column (7.8 mm x 300 mm, 8- μ m) was purchased from Tosoh Bioscience LLC. All separations were in isocratic mode at a flow rate of 0.5 mL/min and with a ultraviolet (UV) detection at 280 nm. The optimized mobile phase consisted of 100 mM sodium phosphate, 300 mM arginine, pH 6.2 with 10% IPA. Injection volume was 10 μ L with a Fab concentration of 5.0 mg/mL.

Results and Discussion

Method Development

Column Screening

Consisting of 8 Fabs of 47 kDa and a PEG core of 40 kDa, the theoretical molecular weight (MW) of the PEG–Fab conjugate is 416 kDa. Due to conjugate aggregation, heterogeneity of PEG core, incomplete conjugation, and dimerization of Fab, many size variants can form. As shown in Figure 1, conjugate with 6, 7, or 8 Fabs are the target species. Potential low molecular weight forms (LMWFs) include free Fab, Fab dimer, and LMW conjugate that consists of a low number (2 or 3) of Fab and LMW PEG. Conjugate oligomer and aggregate can form as high molecular weight form (HMWF) and very high molecular weight form (vHMWF). Effective monitoring of these variants requires a SEC method capable of

resolving both LMWFs and HMWFs with a range of MW from ~50 kDa to over 1000 kDa.

After screening a few SEC columns with the desired mass range (data not shown), Tosoh TSKgel G4000SWXL column (7.8 mm x 300 mm, 8- μ m) showed the best resolution of HMWFs, main peak and various LMWFs and was selected for further optimization. A representative chromatogram of the PEG–Fab conjugate obtained with the Tosoh TSKgel G4000SWXL column is shown in Figure 2. To confirm the identity of the SEC peaks, enriched HMWF and Fab samples were run together with the conjugate sample by SEC-MALS (Figure 3). Average molecular weight of each peak was determined and

showed agreement with the theoretical values, confirming the effective separation of size variants by the Tosoh TSKgel G4000SWXL column.

Mobile Phase Screening

The SEC mobile phase used during the initial column screening experiment was 20 mM sodium phosphate, 300 mM sodium chloride, pH 6.2. However, during an extended method evaluation, the repeatability of the results for the same sample on different columns and during different runs was not acceptable. More specifically, in three testing runs on two columns of different lots, the relative peak area of the sum of HMWF region has a %RSD of 29.6% and the relative peak area of the sum of LMWF has a %RSD of 40.0%. Inconsistent SEC profiles can often appear as a result of nonspecific interactions between the sample and the column resin. Sodium chloride is commonly used to reduce the electrostatic interaction between the sample and resin surface, but it cannot suppress the hydrophobic interaction. On

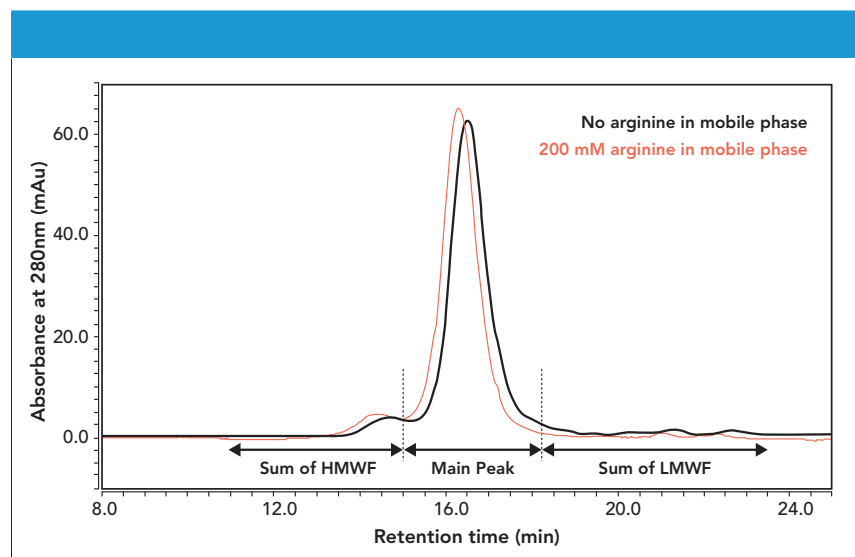


Figure 4: Comparison of chromatograms with and without 200 mM arginine in the mobile phase (20 mM sodium phosphate, 300 mM NaCl, pH 6.2).

the other hand, arginine is typically added to the mobile phase to suppress the hydrophobic interaction between sample and resin without aggregating or dissociating proteins (11). To assess the effect of arginine, the PEG–Fab conjugate sample was analyzed by SEC with and without 200 mM arginine in the mobile phase (Figure 4). With the presence of arginine, all peaks eluted earlier and the total peak area increased, proving the nonspecific interactions were reduced between the sample and the column resin.

After screening different arginine concentrations in the mobile phase, 300 mM arginine was selected because it resulted in the lowest peak retention time and the largest peak area, demonstrating minimal sample-resin interactions. In addition, 10% (*v/v*) IPA was added to the mobile phase as a microbial inhibitor after bacterial growth observed in the mobile phase during storage at ambient temperature. This extended the mobile phase storage time to 2 weeks without significant impact on the SEC separation. The optimized mobile phase was 100 mM sodium phosphate, 300 mM arginine, pH 6.2 with 10% IPA.

Optimization of Other SEC Parameters

In all modes of chromatography, high sample load tends to lead to higher sensitivity but generally reduces the resolution. For this method, injection

volume was optimized to 10 μ L with a Fab concentration of 5.0 mg/mL as a compromise between sensitivity and resolution.

The impact of column temperature on method performance was also tested by changing the column temperature from 24 $^{\circ}$ C to 30 $^{\circ}$ C. Higher column temperature resulted in lower column pressure and sharper peaks compared to the lower temperature. A column temperature of 28 $^{\circ}$ C was selected to achieve better performance while accommodating the column's recommended maximum temperature of 30 $^{\circ}$ C.

Method Qualification

After the SEC conditions were optimized, a method qualification was conducted to assess the specificity, stability indicating property, precision, accuracy, and linearity. Method robustness was also evaluated through a design of experiment (DOE) study.

Specificity and Stability Indicating Property

To assess the specificity and stability indicating property, the conjugate buffer blank, PEG–Fab conjugate (t_0), and the thermally stressed conjugate sample (4 weeks, 40 $^{\circ}$ C) were run with the optimized conditions. As shown in Figure 5, there was no significant interference from the matrix components for the quantification of the size variants, demonstrating a suitable specificity of the method. In comparison with the t_0 sample, the stressed sample showed an increase in relative peak area for both HMWF and LMWF regions. The increased HMWF is likely due to the formation of aggregate, and the increased LMWF can result from the deconjugation of Fab under the elevated temperature. These results demonstrated that the SEC method is stability indicating.

Precision, Accuracy and Linearity

To assess the precision of the method, 18 injections of the conjugate sample were made using two HPLC instruments and two columns from different

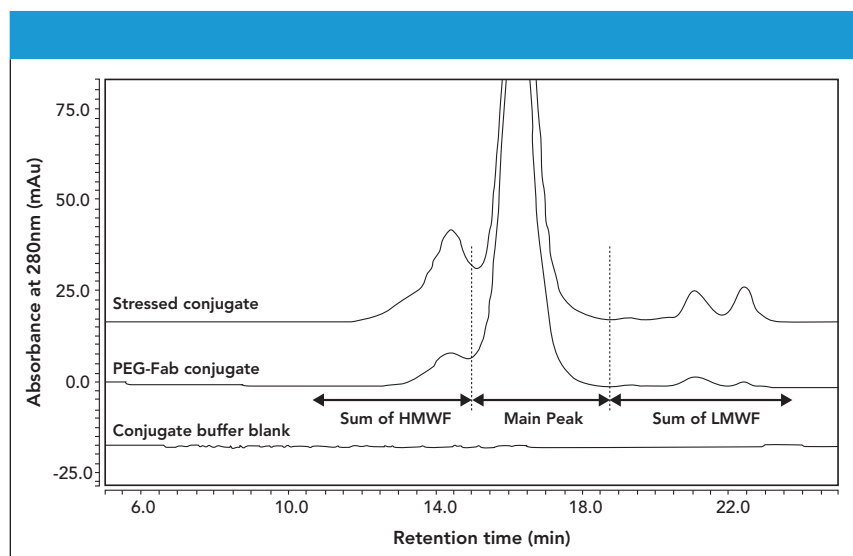


Figure 5: SEC chromatograms of the conjugate buffer blank, PEG–Fab conjugate (t_0), and the stressed conjugate sample (4 weeks at 40 $^{\circ}$ C).

Table I: Test conditions for each parameter in the DOE study

Parameter	Test Conditions:		
	Low	Center	High
Column temperature	26	(28)	30°C
Mobile phase [arginine]	270	(300)	330 mM
Mobile phase pH	6.1	(6.2)	6.3

lots. The %RSD of the relative peak area of the sum of HMWF, the main peak and the sum of LMWF is 1.2%, 0.1%, and 2.5%, respectively, demonstrates suitable precision.

For the linearity and accuracy assessment, five samples were prepared and injected at the concentrations of 2.5 mg/mL, 3.75 mg/mL, 5.0 mg/mL, 6.25 mg/mL and 7.5 mg/mL, corresponding to a range of 50% to 150% of the target protein loading (50 µg). The peak area of each region in the linearity samples was plotted against the theoretical protein loading and linear regression analysis was performed. The Pearson correlation coefficient (r) of the sum of HMWF, the main peak, and the sum of LMWF was 1.00 for each, and the percent recovery ranged from 100% to

114% at all loading levels. These results demonstrated a suitable linearity and accuracy of the method in the assessed range of 25 µg to 75 µg of protein loading.

Robustness by DOE

A DOE study was conducted to assess the method robustness by multivariate data analysis. Three parameters were identified as the potential significant sources of variation: column temperature, arginine concentration in the mobile phase, and the pH of the mobile phase. These parameters were evaluated in a full-factorial study design with eight test conditions and four replicates of the center point (Table I, Figure 6). Across all test conditions, the impact of each factor on the method performance

was minimal. The resulted chromatograms overlaid well, and no significant changes in retention time and resolution between peaks of interest could be observed. The relative peak areas were further analyzed with a main effect plot (Figure 7). The parameters with the most significant impact on the quantitation were the level of arginine for the sum of HMWF and the column temperature for both the main peak and the sum of LMWF. However, the observed differences in the relative peak area were very close to the standard deviation of the center points; therefore, the SEC method was deemed robust for these tested parameters and no changes were made to the final SEC method.

Conclusion

In this study, we describe the development and optimization of a SEC method for monitoring of the size variants of a protein-polymer conjugate. Specific challenges related to the multimeric format of the conjugate, such as undesired secondary hydrophobic interactions with the column resin, were minimized with

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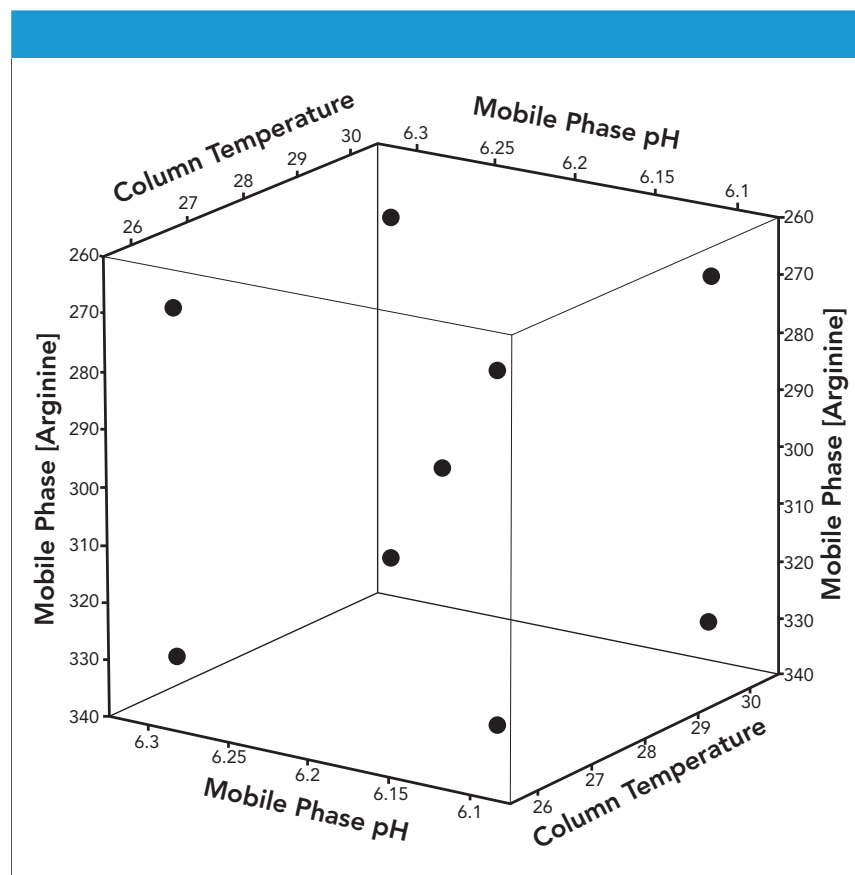


Figure 6: Cube plot showing the experimental conditions tested in the DOE study.

the final optimized method. The optimized method was further tested for specificity, precision, linearity, accuracy, stability indicating properties,

and robustness. This study demonstrated that the SEC method is suitable for monitoring the size variants of the PEG–Fab conjugate and appropriate

to support the manufacturing of clinical batches.

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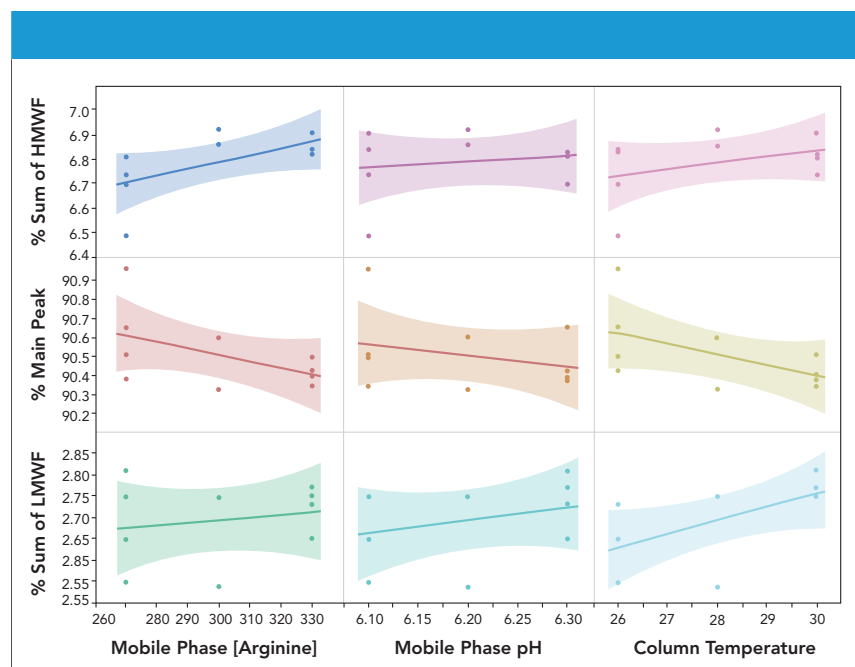


Figure 7: Main effect plot of the relative peak area of each peak region from the three-variable DOE study.