Peptide Mapping: A Quality by Design (QbD) Approach

Application Note

Bio-Pharmaceutical

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Abstract

Peptide mapping is a widely used technique for the characterization of proteins. This Application Note demonstrates a robust LC method and design space for peptide mapping using a Quality by Design (QbD) approach. A therapeutic monoclonal antibody (mAb) was employed as the model protein in the current peptide mapping study. Fusion QbD software's multivariate design and analysis capabilities, which are aligned with a QbD approach, in combination with its OpenLAB CDS experiment automation capabilities, provided a detailed understanding of the critical method parameter (CMP) effects on the various critical method attributes (CMAs) included in the study. This knowledge was translated directly into a robust final peptide mapping method with maximum number of peaks and good separation in far less method development time.

Agilent Technologies

Introduction

Peptide mapping is the most commonly used identity test for proteins[1]. Peptide mapping is protein fingerprinting; it involves several processes including digestion, separation of peptides, and data analysis, which should result in comprehensive understanding about the protein being analyzed[1]. Reversed-phase liquid chromatography (RPLC) with UV detection is the most commonly used method for peptide mapping in quality control (QC). One of the major challenges in traditional LC method development for peptide mapping is the time consumed in screening the various chromatographic parameters using a one-factor-at-a-time (OFAT) approach.

The goal of analytical Quality by Design (QbD) is to achieve quality in measurement, leading to consistent quality of a drug product. Quality principles in the ICH guidelines can be implemented in the design of analytical method development work to meet that goal[2,3]. Many of the traditional LC method development limitations can be overcome with the QbD approach. For example, design of experiment (DOE) methods can reduce the large number of runs normally needed to support the OFAT approach. DOE can provide increased understanding of method performance and variability from a limited number of experiments selected to specifically characterize independent and multivariate interactions between method parameters[4].

This Application Note describes the application of the analytical QbD approach to peptide mapping method development using an Agilent 1260 Infinity Bio-inert LC with Fusion QbD method development software for a tryptic digested monoclonal antibody (mAb). The Fusion QbD software delivers a totally automated DOE-based experimental approach that evolves systematic method development from start to end.

Materials and Methods

LC instrumentation

All chromatographic analyses were performed on an Agilent 1260 Infinity Bio-inert Quaternary LC system consisting of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316C, option 19)
- Agilent 1260 Infinity DAD VL with a 10-mm bio-inert standard flow cell (G1315D, option 28)

Software

- Agilent OpenLAB CDS ChemStation Edition, Version C.01.05.
- Fusion QbD Software Platform, version 9.7.0 Build 494 (S-Matrix Corporation).

Reagents and chemicals

The mAb protein was a proprietary therapeutic molecule. Tris buffer, urea, iodoacetamide (IAA), dithiotreitol (DTT), trifluoroaceticacid (TFA), and formic acid were obtained from Sigma-Aldrich. Trypsin is from Agilent Technologies. Acetonitrile, methanol, and isopropanol (IPA) were obtained from Labscan.

Tryptic digestion

The mAb (2 mg) was treated with DTT and IAA for reduction and alkylation. After the reduction and alkylation steps, the pH of the solution was adjusted to pH 7–8, and trypsin digestion (20:1, protein to protease w/w) was performed overnight, incubating at 37 °C. The incubated sample was then quenched with 0.05 % TFA. The samples were either immediately analyzed or stored at –20 °C until use.

Results and Discussion

QbD Workflow

In this study, the QbD workflow consists of two phases: 1) screening and 2) method optimization. Figure 1 presents a schematic representation of the QbD workflow for each phase. In each phase, given a defined set of variable inputs, the Fusion QbD software generates a statistical DOE experiment design. It then exports the design to the Agilent OpenLAB ChemStation Software as ready-to-run sequences and methods, which were run on an Agilent 1260 Infinity Bio-inert Quaternary LC system. The software then imports and models the data from the experiment chromatograms, and generates an automated report identifying the best conditions, in terms of meeting the defined performance goals for the critical method attributes (CMAs)[5]. Table 1 summarizes the CMA (response goals) for the current study.

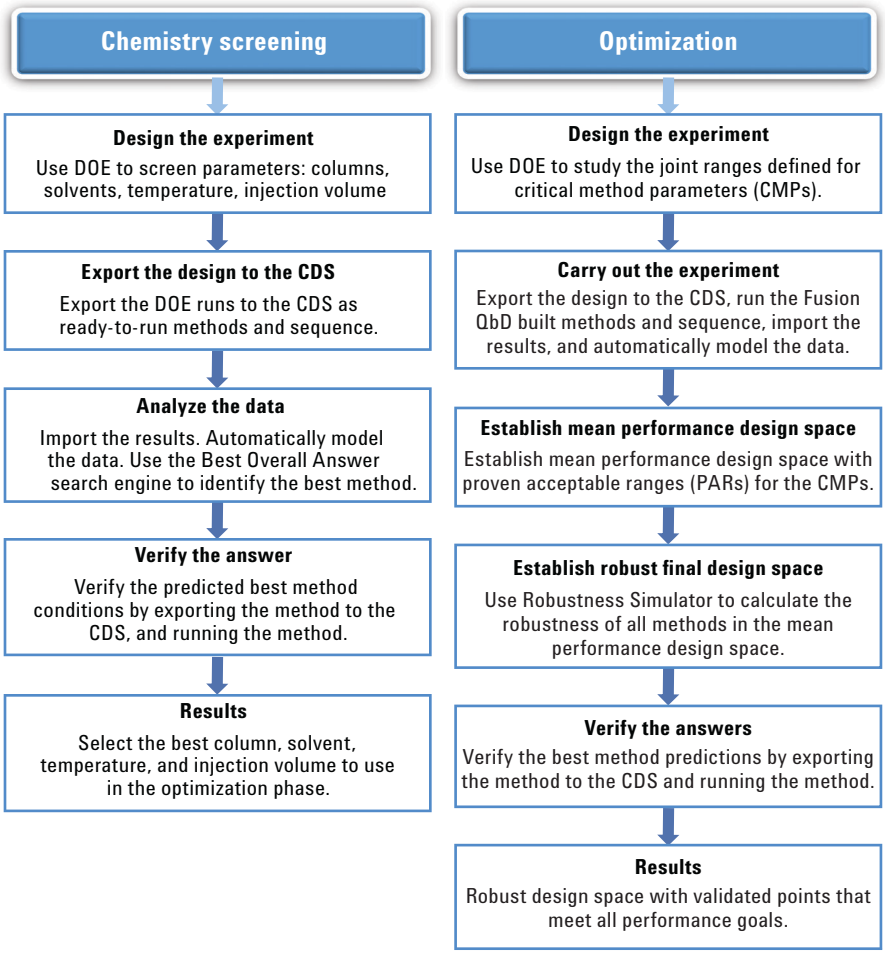


Figure 1. Schematic representation of the QbD workflow with Fusion QbD.

Table 1. Critical method attributes (CMAs) for QbD peptide mapping analysis.

Response goals (critical method attributes)	Target	Relative rank
No. of peaks	Maximize	1
No. of peaks ≥ 1.50 (tangent resolution)	Maximize	1
No. of peaks ≥ 2.00 (tangent resolution)	Maximize	1

Screening

This first phase involved screening column and solvent type combinations using a generic gradient[6]. The experiment setup required specifying critical method parameters (CMPs) as either variables or constants. The parameters specified as variables in the screening study included different columns (Column A: AdvanceBio Peptide Mapping column, Column B, and Column C), different strong solvents (acetonitrile, methanol, IPA + acetonitrile + water), injection volume (1–5 µL), and column temperature (40–60 °C). Gradient profile, flow rate (1 mL/min), and additive concentration (0.08 % TFA) were kept constant. In this phase, the goal was to identify the conditions that provide better peptide separation in terms of the number of separated peaks. Figure 2 shows example chromatograms obtained from various phase 1 DOE experiment runs. Table 2 summarizes the outcome of the screening phase results.

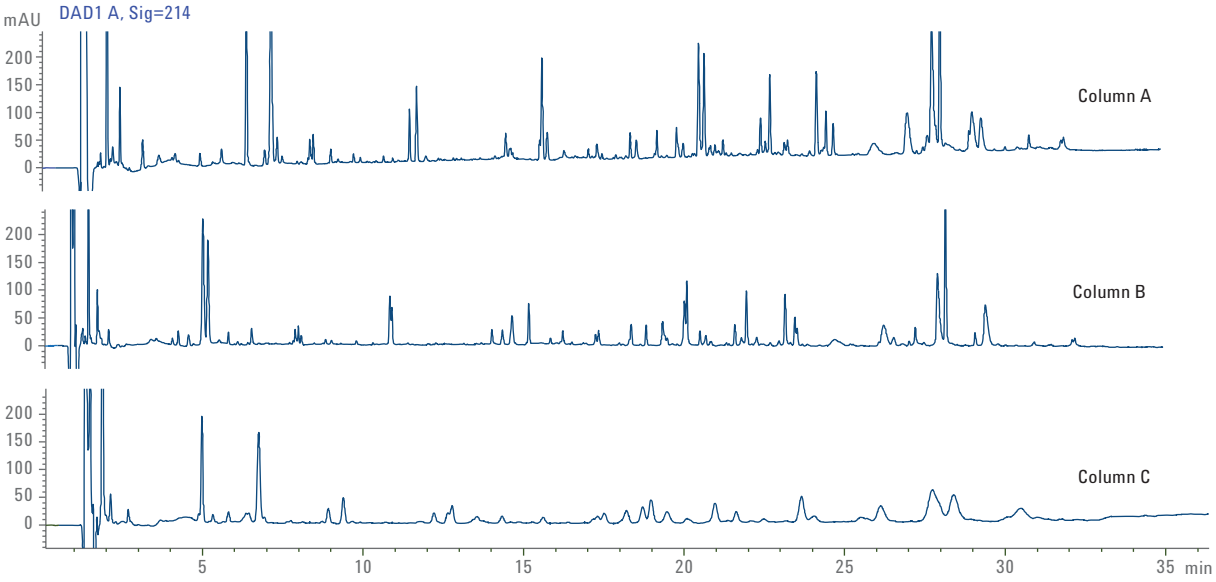


Figure 2. Chromatographic profiles of tryptic digested mAb under three different column conditions. Column A: Agilent AdvanceBio Peptide Mapping column (4.6 × 150 mm, 2.7 µm); Column B: Agilent Poroshell 120 SB (4.6 × 100 mm, 2.7 µm); Column C: Agilent ZORBAX SB (4.6 × 150 mm, 5 µm).

Table 2. Screening phase output predicted by the Fusion QbD software.

Best overall answer	
Variable	Level setting
Column	AdvanceBio Peptide Mapping column 4.6 × 150 mm, 2.7 µm
Strong solvent	Acetonitrile
Oven temperature	53 °C
Injection volume	5 µL

The Advance Biopeptide column (Column A) and acetonitrile (Solvent A) mobile phase were found to be the best conditions to obtain the maximum number of peaks with tangent resolution >1.5 and 2. Figure 3 presents an example of the response surface graphs used to identify the optimum injection volume and oven temperature.

Method optimization

The second phase used the best column, strong solvent, and injection volume identified in the screening study. In this phase, the method was further optimized by studying pump flow rate, gradient slope, and additive concentration – parameters expected to strongly influence the method's mean (average) performance and the method's robustness. This phase also included the higher end of the column (oven) temperature range, which was shown to be advantageous in the screening study. Including column temperature enables characterizing the effect of temperature variation on method robustness. Tables 3 and 4 respectively summarize the critical method variables included in the study, and the resulting optimized method. Figure 4 presents a response graph showing the combined (interactive) effects of pump flow rate and strong solvent % on the maximum number of separated peaks – one of the CMAs in this study.

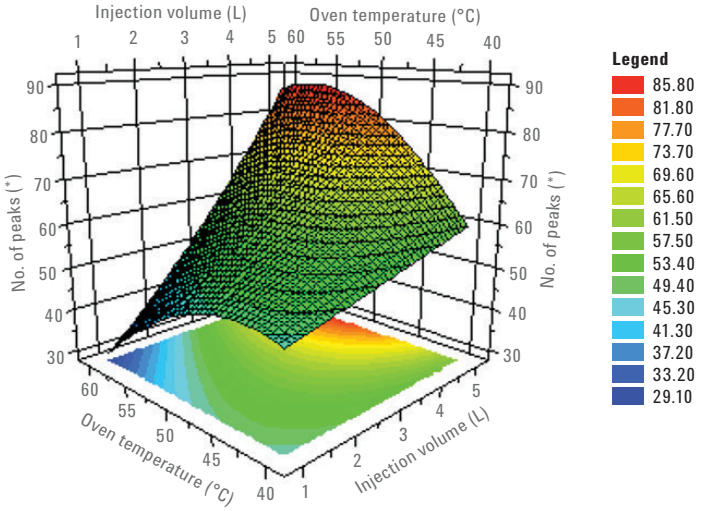


Figure 3. Fusion QbD software response graph showing the impact of an interaction between injection volume and oven temperature. The graph shows the combined (interactive) effect of increasing both injection volume and oven temperature on the maximum number of separated peaks.

Table 3. The constants and variables tested during optimization phase.

Experimental details		
Constants		
Gradient	Equilibration	10.0 minutes 2 %B
	Initial hold	0.01 minutes, 2 %B
	Final hold	3 minutes at 95 %B
	Re-equilibration	1 minute at 2 %B
Injection volume	5 µL	
Wavelength	214 nm ± 4 nm	
Variables		
Pump flow rate	0.5, 1.25, 2.0 mL/min	
Final % of strong solvent	10–30 %	
Intermediate hold	40, 50, and 60 minutes	
Oven temperature	50, 55, and 60 °C	
Additive concentration (TFA)	0, 0.04, and 0.08 %	

Table 4. Optimization phase output predicted by the Fusion QbD software.

Best overall answer	
Variable	Level setting
Pump flow rate	0.5 µL
Intermediate hold time	60 minutes
Final % strong solvent	18 %
Oven temperature	50 °C
Additive concentration	0.07

Design space

Design Space is a primary region, predicted by the Fusion QbD software, that defines the CMAs in terms of the CMPs[7]. To establish a robust final design space, it is important to quantify the robustness of all possible methods in the mean performance design space. This is done using the software's Robustness Simulator feature to characterize the independent and combined effects of the method parameters on method variability. Figure 5 shows a Trellis graph illustrating the impact of the CMPs on the CMAs (response goals). These graphs facilitate understanding the combined effects of the study variables on the separation of the peptide mixture.

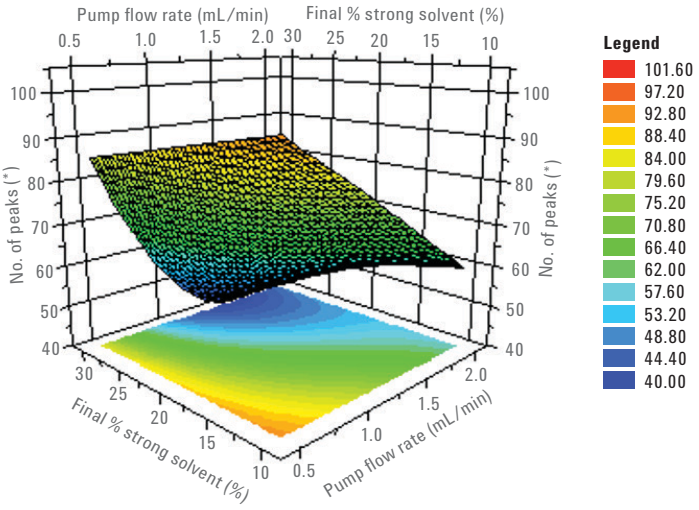


Figure 4. Fusion QbD software response graph showing the combined (interactive) effects of pump flow rate and strong solvent % on the maximum number of separated peaks. The graph shows that the best result occurs by combining low pump flow rate with high final % strong solvent.

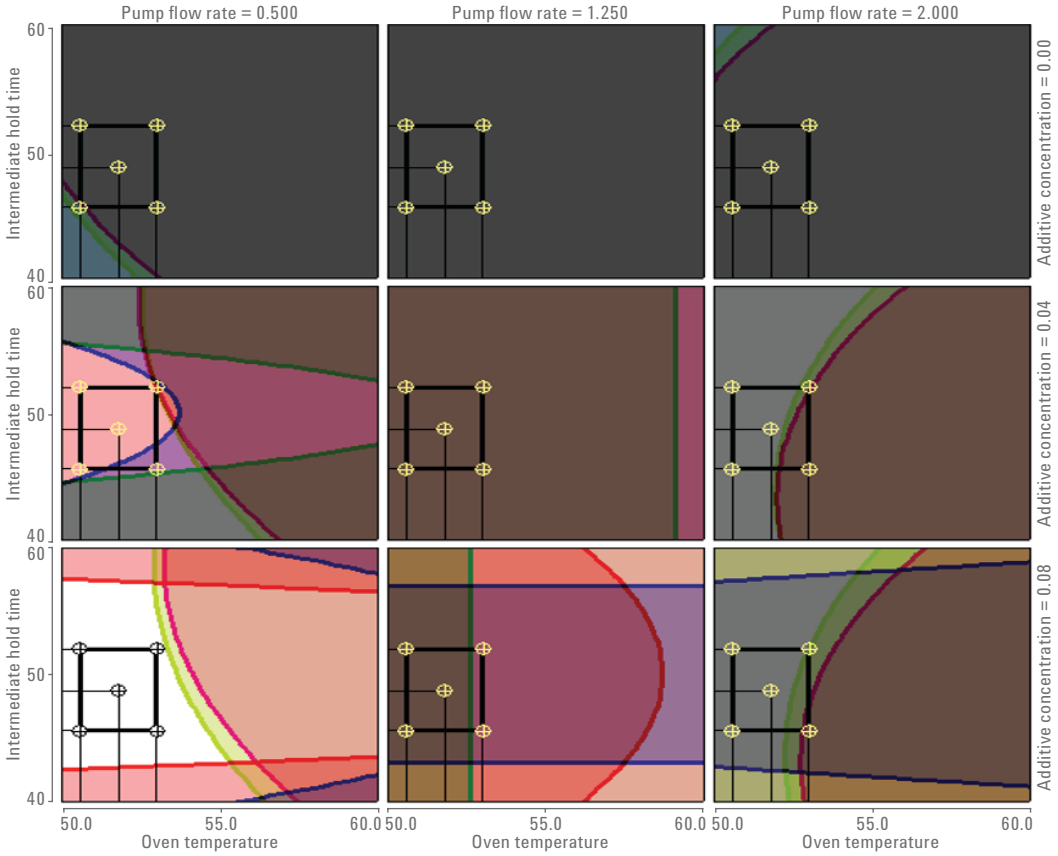


Figure 5. Trellis Graphs show the design space (unshaded region), proven acceptable ranges (the PAR rectangle), and final method settings (center dot in rectangle) for oven temperature and intermediate hold time achievable at low pump flow rate and high additive concentration.

In Figure 5, the unshaded (white) region in the bottom left graph of the trellis is the robust design space – the region containing methods that meet required mean performance and robustness. In this graph, the rectangle demarcates the joint proven acceptable ranges (PARs) for oven temperature, intermediate hold time, and the final method settings (the center point of the rectangle). The Fusion QbD software automatically selects the center point and the four border points of the PAR rectangle for point prediction verification, and automatically includes the pump flow rate and additive concentration associated with the graph. Figure 6 is an expanded view of the selected 2D contour graph containing the robust design space and PARs for the combined method performance requirements (CMAs) of the greatest number of separated peaks and the highest number being baseline resolved and well resolved (Tangent Resolution ≥1.5 and ≥2.0).

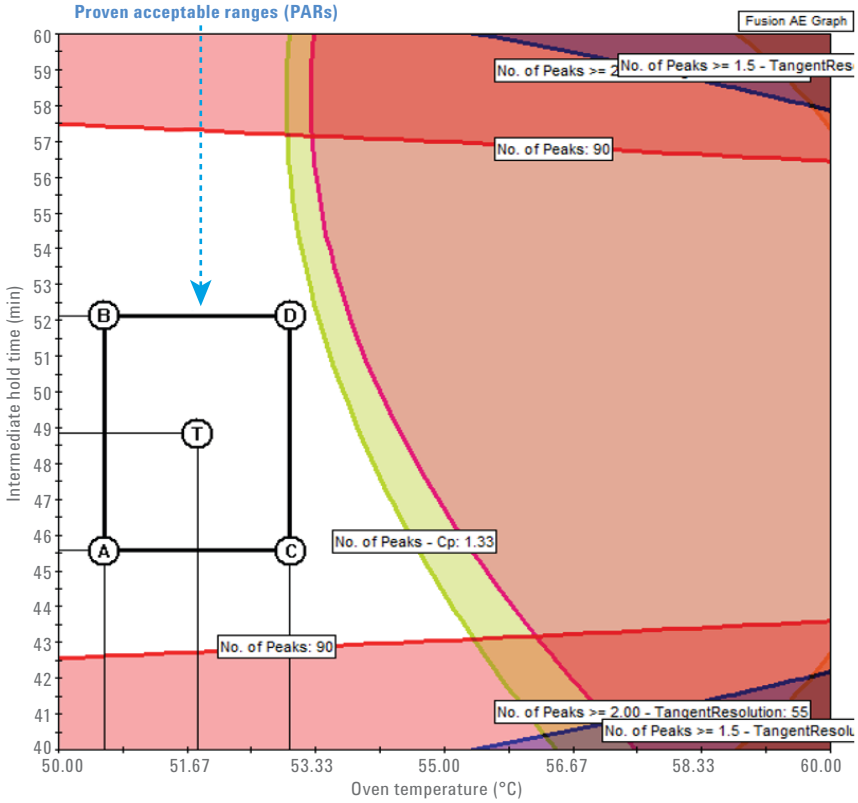
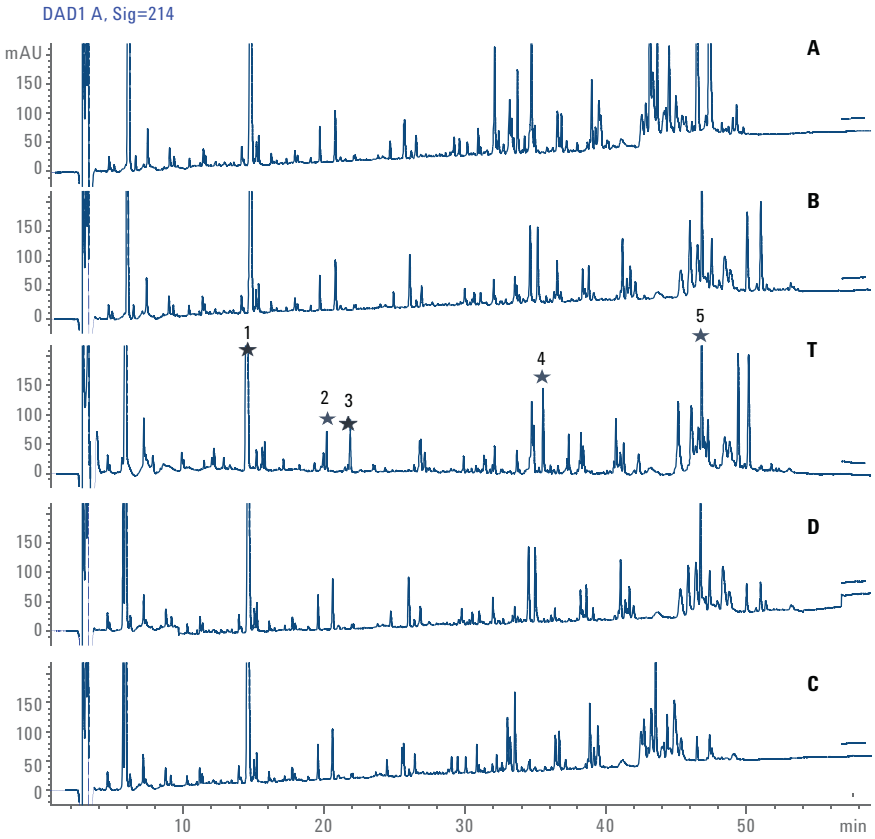


Figure 6. Fusion QbD software design space graph with PAR rectangle showing proven acceptable ranges for oven temperature and intermediate hold time.

Method verification

The final step involved verifying that the optimized method's predicted performance met all response goals. To do this, the Point Predictions feature in Fusion QbD was used to automatically export the center point and the four border point verification methods from the PAR rectangle to OpenLAB for automated execution. These points are identified as A, B, C, D, and T in the 2D graph presented in Figure 6. The experimentally verified chromatographic runs at these five different conditions are presented in Figure 7, along with the results for the CMAs.



	Response variables	Predicted	Experimental	±2 Sigma confidence limit
A	No. of peaks	93	98	80–105
	No. of peaks ≥1.5 (Tangent resolution)	75	78	66–84
	No. of peaks ≥2.0 (Tangent resolution)	60	69	51–69
B	No. of peaks	94	88	81–107
	No. of peaks ≥1.5 (Tangent resolution)	76	74	66–86
	No. of peaks ≥2.0 (Tangent resolution)	61	62	51–70
T	No. of peaks	94	86	81–108
	No. of peaks ≥1.5 (Tangent resolution)	78	72	68–87
	No. of peaks ≥2.0 (Tangent resolution)	60	58	51–69
D	No. of peaks	94	88	80–107
	No. of peaks ≥1.5 (Tangent resolution)	79	70	69–88
	No. of peaks ≥2.0 (Tangent resolution)	60	57	50–69
C	No. of peaks	93	86	80–105
	No. of peaks ≥1.5 (Tangent resolution)	78	72	68–87
	No. of peaks ≥2.0 (Tangent resolution)	59	55	50–68

Figure 7. The chromatographic runs of the center (T) and four (A, B, C, and D) robust operating range points along with the response goal values. *selected peaks for %RSD calculations (Table 5).

All the values obtained after the point prediction validation studies were within the predicted range (±2 Sigma confidence limit). The method's reproducibility was also evaluated by replicate injections, and the %RSD calculations presented in Table 5 show the precision of the method.

Figure 8 shows the comparison of the chromatogram obtained from the initial screening study, and the chromatogram obtained from the final QbD optimized method. A comparison of these chromatograms shows the dramatic improvements in both the number of separated peptide peaks and the number of well resolved peptide peaks obtained from method optimization using the Fusion QbD software and the QbD-aligned approach.

Table 5. Precision of the final LC method. Upper limits of acceptable %RSD: Retention time = 0.1 %, Area = 2.0 % and Area% = 5 %.

%RSD values for selected peaks			
Peaks	Retention time	Area	Area%
1	0.05	1.15	0.40
2	0.03	0.63	1.06
3	0.03	0.60	0.66
4	0.02	0.97	4.40
5	0.02	1.80	2.10

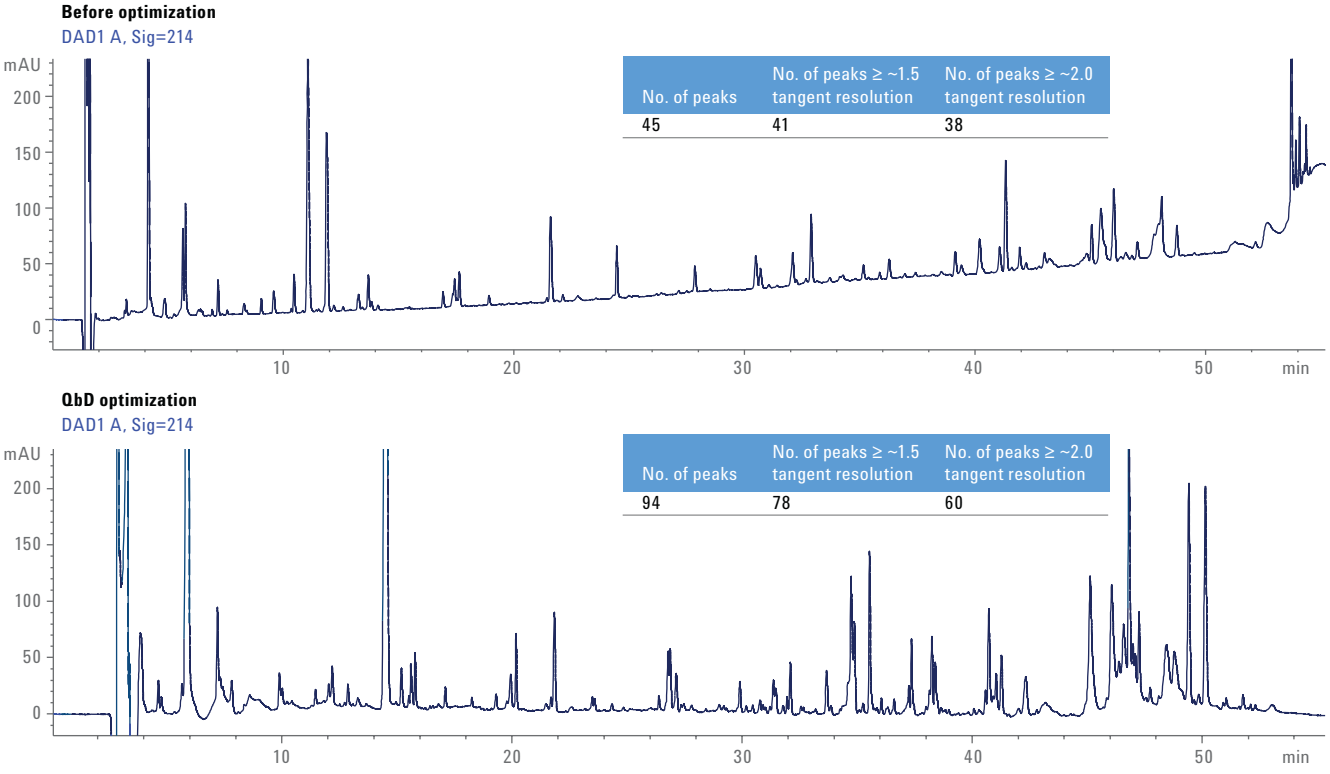


Figure 8. Comparison of chromatograms obtained before and after QbD optimization, along with the response goal values.

Conclusion

This Application Note demonstrates systematic LC method development for peptide mapping using QbD principles on an Agilent 1260 Infinity Bio-inert Quaternary LC system. Fusion QbD method development software with OpenLAB ChemStation facilitates the automation of LC method development by evaluating multiple parameters such as column, solvent, oven temperature, injection volume, additive concentration, and gradient slope. The Fusion QbD software's multivariate experiment design and analysis capabilities, combined with its automated experiment execution technology, enabled a robust final method for mAb peptide mapping to be obtained in less than two weeks total development time, including instrument run time and analyst time for instrument setup and chromatogram processing. Therefore, the automated QbD method development approach using Fusion QbD software has provided a far better performing and more robust method in dramatically less time compared to manual method development.

References

1. Recombinant Protein Characterization, *Agilent Primer*, publication number 5990-8561EN, August 08, **2011**.
2. Vogt, *et al.* Development of Quality-By-Design Analytical Methods, *J. Pharma. Sci. Vol*. **March 2011**, *100*, No. 3, p.797-812.
3. G. Reid, *et al.* Analytical Quality by Design (AQbD) in pharmaceutical Development. *American Pharmaceutical Review* **August 2013**, 144191.
4. https://smatrix.com/pdf/Fusion%20AE%20%20LC%20Method%20Development%20Brochure.pdf
5. L. Jones, *et al.* Rapid Peptide Mapping Method with High Resolution Using a sub 2-µm Column, *Agilent Technologies*, publication number 5990-4712EN.
6. J. Martosella, A. Zhu, N. Tang, Fast and Efficient Peptide Mapping of a Monoclonal Antibody (mAb): UHPLC Performance with Superficially Porous Particles, *Agilent Technologies*, publication number 5991-3585EN.
7. S. Lateef, A. K. Vinayak, Automated QbD based Method Development and Validation of Oxidative Degraded Atorvastatin, *Agilent Technologies*, publication number 5991-4944EN.

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