Application Note

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Separation of a Critical Pair of N-Glycans Using a Quality by Design (QbD) Approach

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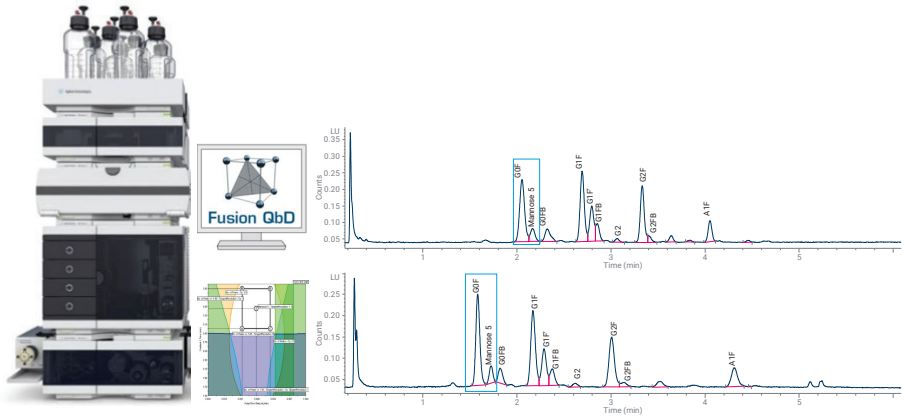

Abstract

N-glycosylation is one of the most complex post-translational modifications. It influences the structural characteristics of the Fc region of antibodies, potentially modulating effector function and pharmacokinetics.[1] This application note focuses on developing a method for the separation of a critical pair of glycans in a shorter run time using an Agilent 1290 Infinity II LC system with Fusion QbD Method Development software. To assess the separation of the critical pair G0F and Man5, the Agilent 2-AB labeled human IgG N-glycan standard spiked with the high mannose-type N-glycan 2-AB labeled oligomannose 5 (Man5) standard (formerly ProZyme) was used as the model glycan sample for the study. All the analysis was performed on an Agilent 1290 Infinity II Method Development system along with Fusion QbD software from S-Matrix. The 1290 Infinity II Method Development system with specialized column and solvent selection valves provides the perfect hardware for performing analytical method development. In this study, Fusion QbD software's multivariate design and analysis capabilities, which are aligned with a QbD approach are combined with Agilent OpenLab CDS experiment automation capabilities. The concept of design space in the Fusion QbD software provided a detailed understanding of the critical method parameter (CMP) effects on the various critical method attributes (CMAs) included in the study, and how their variation affects the analytical results.

Introduction

Glycosylation is one of the most commonly occurring post-translational modification. As per literature studies, mannose additions in the glycan profile are significant. This application note, as a proof of concept, demonstrates the separation of a critical pair of glycans, G0F and Man5, using an automated method development approach. The samples used in this study were Agilent 2-AB labeled human IgG N- glycan standard spiked with 2-AB labeled Man5 (formerly ProZyme).

Experimental

LC instrumentation

All experiments were carried out on Agilent 1290 Infinity II LC. Table 1 lists the LC modules used for the analysis.

Table 1. LC configuration used for the analysis.

LC Modules	Model Number
Agilent 1290 Infinity II Flexible Pump	G7104A
Agilent 1290 Infinity II Multisampler	G7167B
Agilent 1290 Infinity II Multicolumn Thermostat	G7116B
Agilent 1260 Infinity II FLD	G7121B
Agilent 1290 Infinity external valve drive with 12-position 13-port Solvent Selection valve.	G1170A

Software

- Agilent OpenLab CDS Chemstation: Rev.C.01.07 [27]
- Fusion QbD software from S-Matrix: Version 9.7.1 Build 5319.7.1

Materials and methods

Chemicals: Acetonitrile, ammonium formate, formic acid, and so forth were purchased from Sigma-Aldrich.

Samples

- Agilent 2-AB-labeled human IgG N-glycan standard library (p/n 5190-6996, 200 pmol)
- 2-AB Man5 standard (p/n GKSB-103, 100 pmol)

Sample preparation

A combination of 70:30 acetonitrile:water was used as the diluent for sample preparation. Agilent 2-AB-labeled IgG N-glycan standard and 2-AB-labeled Man5 standard were reconstituted with 200 and 100 µL of the diluent respectively. The glycan standard spiked with a 5 µL of Man5 was used as the sample for the entire study.

For released N-glycan sample analysis from biotherapeutic glycoproteins, a kit-based approach is available from Agilent (AdvanceBio Gly-X 2-AB Express kit (formerly ProZyme)) for a faster sample preparation. More details are mentioned in reference 2.

Results and discussion

Quality by Design (QbD) workflow

A quality by design approach to method development provides a multivariate approach to designing methods using different critical method parameters. This approach results in the generation of a robust design space, which meets with the assigned response goals. The QbD workflow involves two steps, screening and method optimization. Figure 1 shows a schematic representation of the QbD workflow.

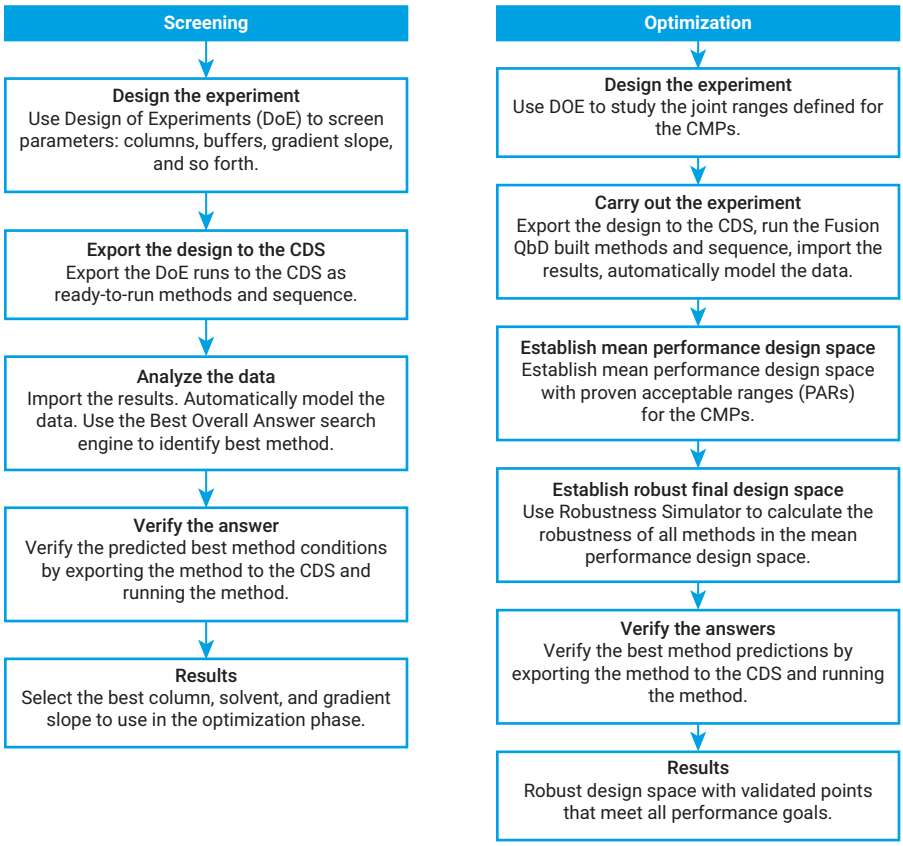

Figure 1. A schematic representation of the QbD workflow with Fusion QbD software.

Screening

Screening is the first step in the analytical method development. To achieve a good separation between analytes, the appropriate stationary phase and mobile phases are required. Therefore, in the screening phase, the main objective, or the Analytical Target Profile (ATP), is to identify the best column (stationary phase) and the solvent conditions (mobile phase) to attain a good chromatographic profile. The starting conditions for method development in this study were taken from a previously published Agilent application note.[4] Tables 2 and 3 summarize the CMPs and CMAs assigned for this screening study. The predicted DoE was then imported into Agilent OpenLab ChemStation software, and runs were performed using a 1290 Infinity II UHPLC.

Table 2. The CMPs for the screening.

Variables	Types/Ranges	Constants	Level Settings
Buffer Concentration	50 to 100 mM ammonium formate	Pump Flow Rate	1 mL
Column Type	**Column A:** Agilent AdvanceBio Glycan Mapping Column 2.1 × 100 mm, 1.8 µm **Column B** **Column C** **Column D**	pH	4.5
Gradient Slope	**Initial hold:** (Variable) 0.1 to 1 minute (% buffer concentration range 10 to 25%) Slope change from 30 to 55% buffer **Final hold:** (Variable) 0.5 to 1 minute (% of strong solvent buffer: 60%)	Detection	**Fluorescence:** excitation 260, emission 430
		Injection Volume	2 µL
		Equilibration Time	10 minutes
		Solvents	**Aqueous:** ammonium formate buffer **Organic:** 100% acetonitrile
		Gradient Time	10 minutes

Table 3. Response goals or critical method attributes that are set for the screening study.

Response Goals	Target	Relative Rank
Number of Peaks	Target	1
Number of Peaks ≥ 1.5 Tangent Resolution	Maximize	1

Figure 2 shows an overlay of the chromatograms obtained after the screening. The reprocessed chromatographic results were then exported back to the Fusion software, for best answer predictions. Table 4 shows the predicted best conditions. Column A (Agilent AdvanceBio Glycan Mapping column, 2.1 × 100 mm, 1.8 µm) and a buffer concentration of 50 mM ammonium formate were found to give the best result following the screening experiments (Figure 3).

Method optimization

The second phase used the best conditions from the screening study. Here the method was further optimized or fine-tuned to achieve the best separation between the chosen critical pair of peaks (G0F and Man5). The Man5 peak eluted as a shoulder peak of G0F. To achieve better separation between G0F and Man5, parameters that were expected to strongly influence the separation were studied. Parameters such as pump flow rate, oven temperature, pH, gradient time, and methanol % in acetonitrile (Solvent B) as a modifier, and so forth, were taken as CMPs for the optimization studies. Modification of acetonitrile with methanol % was used as a variable to study the effect of methanol addition in HILIC separation.[5] Tables 5 and 6 show the CMPs and the response goals assigned for the optimization study respectively. Table 7 shows the best answer predicted by the software from the optimization experiments. After the optimization studies, the effect of CMPs on the separation of the critical pair was very evident. A significant improvement in the separation of the critical pair of peaks was observed following method optimization.

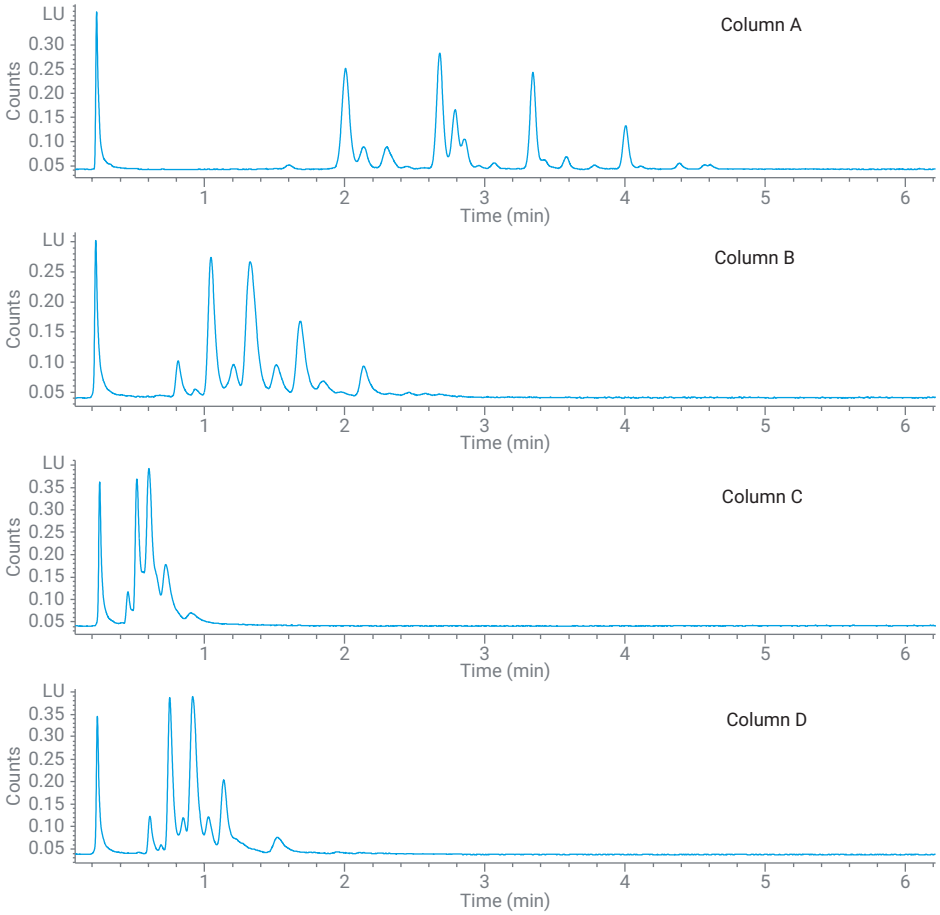

Figure 2. Chromatograms produced from screening different columns. It is evident from the results that Column A, the Agilent AdvanceBio Glycan Mapping column 2.1 × 100 mm, 1.8 µm, gave a good separation compared to the other columns.

Table 4. The best answer predicted by the software for the screening study.

Variable	Level Settings
Best Column	Column A: Agilent AdvanceBio Glycan Mapping column
Buffer Concentration	50 mM ammonium formate
Initial Hold Time	0.1 minute
Final Hold Time	0.6 minute
Initial Strong Solvent (Buffer) %	25%
Gradient Slope Change	30% buffer

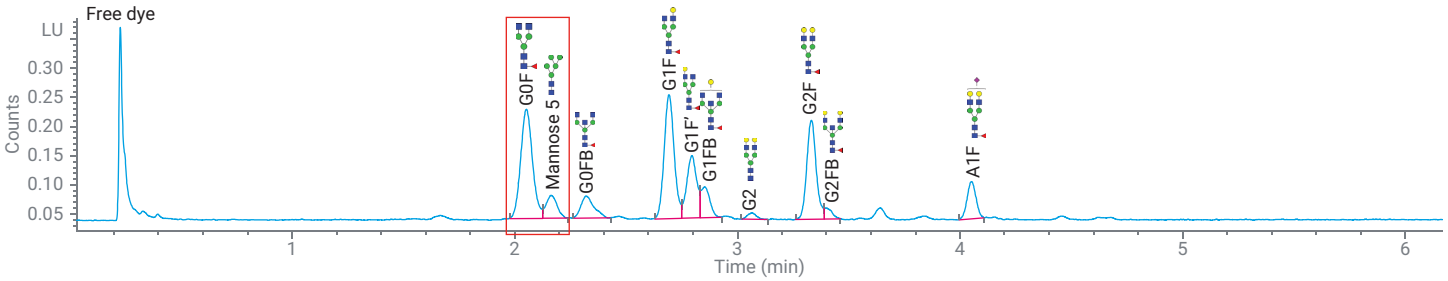

Figure 3. The chromatogram produced using the best conditions predicted by the software. Peak annotations were given according to the Agilent 2-AB-labeled glycan standard (formerly ProZyme).

Table 5. The CMPs for the optimization study.

Critical Method Parameters			
Variables	**Ranges**	**Constants**	**Level Settings**
Pump Flow Rate	0.8 to 1 mL	Column	Agilent AdvanceBio Glycan Mapping column, 2.1 × 100 mm, 1.8 µm
Oven Temperature	40 to 55 °C	Buffer Concentration	50 mM Ammonium formate
pH	3.5 to 5.5	Initial Hold	0.1 minute
Methanol % in Solvent B (Acetonitrile)	0 to 5%	Final Hold	0.6 minute
Gradient 1 (Time) Solvent Range 25% to 30% of Buffer	1.5 to 4 minutes	Injection Volume	2 µL
		Gradient 2 (Time)	1.0 minute 60%

Table 6. Response goals set for the optimization.

Response Goals	Target	Relative Rank
Number of Peaks	Target	1
Mannose 5 Peak Tangent Resolution	Maximize	1
Number of Peaks ≥ 1.5 Tangent Resolution	Maximize	0.9

Table 7. The best answers obtained from the optimization study.

Variable	Level Setting
Pump Flow Rate	0.856
Gradient 1 Time	3.3
Methanol	2.8%
Oven Temperature	47.5
pH	3.50

Design space

Design space is the primary region predicted by the Fusion QbD software, which defines the CMAs in terms of CMPs. Predicted results from the Fusion software after the optimization studies were used to create the design space. Figure 4 shows the Trellis graph with the design space (unshaded region). The shaded region in the Trellis graph shows the unfavorable conditions and the white unshaded region shows the favorable region or the region that defines the given response goals in terms of the analyzed CMPs. To establish a robust design space, quantify the robustness of methods in the mean performance space. This is done using the Fusion software's Robustness Simulator feature to characterize the independent and combined effects of the method parameters on method variability. The black rectangle box inside the unshaded white region demarcates the Proven Acceptable Region (PAR) or the robust space with five points (method conditions) A, B, C, D, and the center point T (final optimized method). Figure 5 shows the single graph view of the design space.

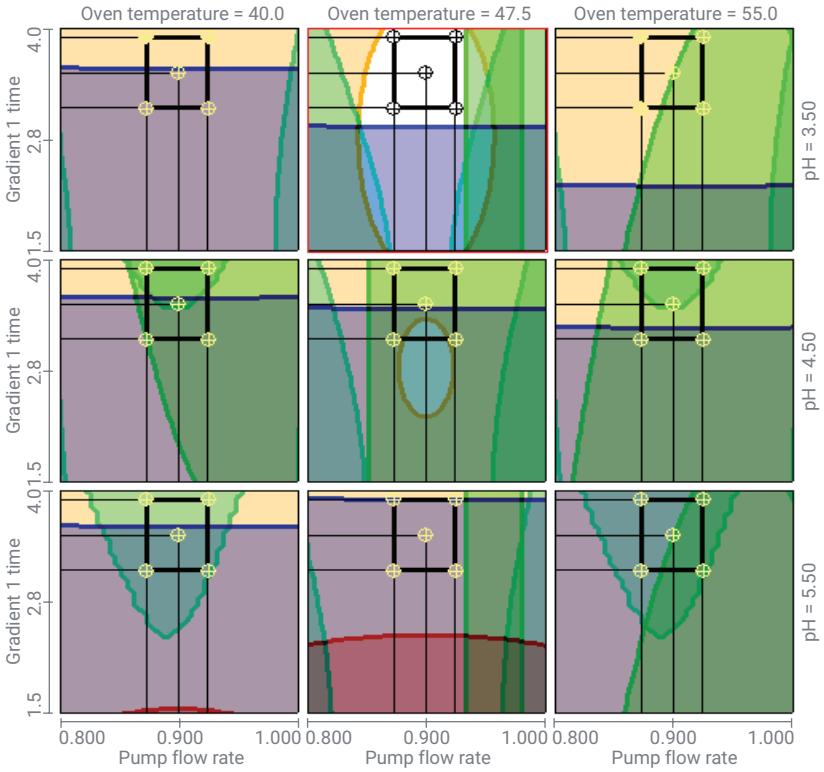

Figure 4. Trellis graph with the design space along with the PAR region. The black box inside the figure is the PAR (Proven Acceptable Region) with five points or method conditions A, B, C, D, and the center point T.

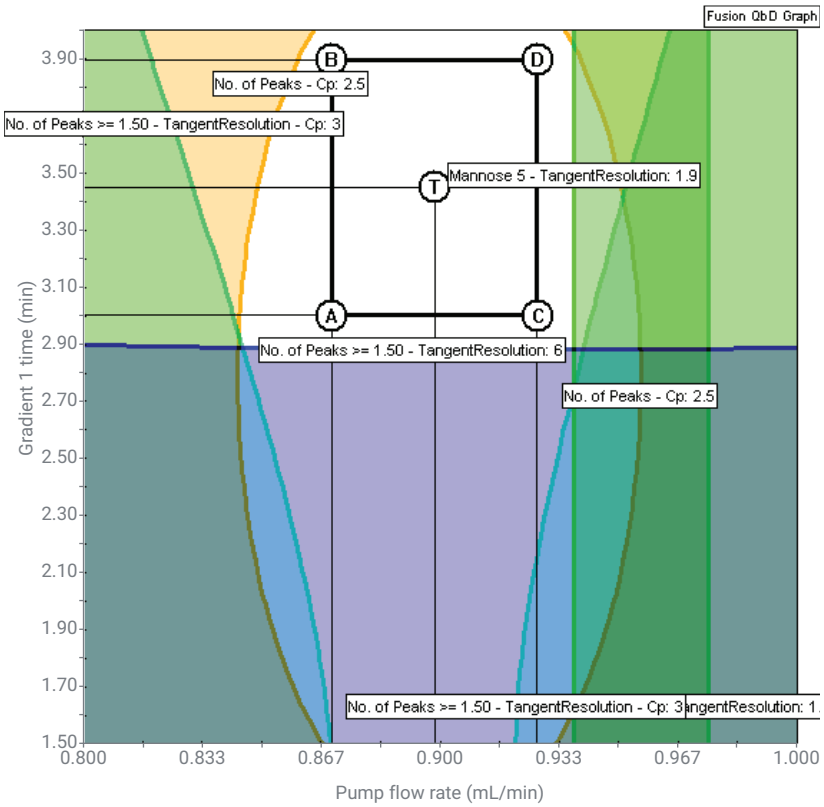

Figure 5. Single graph showing the enlarged design space with the PAR regions.

Point prediction

The point prediction feature in the Fusion QbD software gives the details of the software predicted method conditions. The five points in the PAR region are the predicted robust method conditions by the software from the optimization trials performed (Table 8). These points, or method conditions, were then validated using a 1290 Infinity II LC. Figure 6 shows an overlay of the chromatograms from the validation experiments. The highlighted part in the chromatogram with a rectangle shows the critical pair of peaks, which have good baseline separation. Table 9 shows the response goals and predicted versus experimental values obtained after the validation of software predicted method conditions.

Method robustness was further tested or validated by performing replicate injections of the sample using the final optimized result (condition from the center point T). Ten replicate injections were performed, and the six best results were taken for the RSD calculation. All peaks were separated within a 7.5 minute run time. Figure 7 shows the overlay of the chromatograms obtained after the replicate injections. The RSD results obtained from the replicate trials were tabulated in Table 10. The RSD % of all the peaks were found ≤2 demonstrating the method robustness.

After the validation, the chromatograms obtained were compared with chromatograms produced from the screening study. A dramatic difference was observed between the results. Figure 8 shows the comparison results. A good baseline separation between the critical pair of peaks was observed after the development. Other than the chosen critical pair, the separation was improved for the adjacent peaks such as G1F' and G1FB, G2F and G2FB, and so forth.

Table 8. The five conditions predicted by the software for validation.

Point	Run ID	Pump Flow Rate	Gradient 1 Time	Methanol	Oven Temperature	pH
A	Default_Graph_A	0.876	3	2.8	47.5	3.5
B	Default_Graph_B	0.876	3.9	2.8	47.5	3.5
C	Default_Graph_C	0.928	3	2.8	47.5	3.5
D	Default_Graph_D	0.928	3.9	2.8	47.5	3.5
T	Default_Graph_T	0.902	3.5	2.8	47.5	3.5

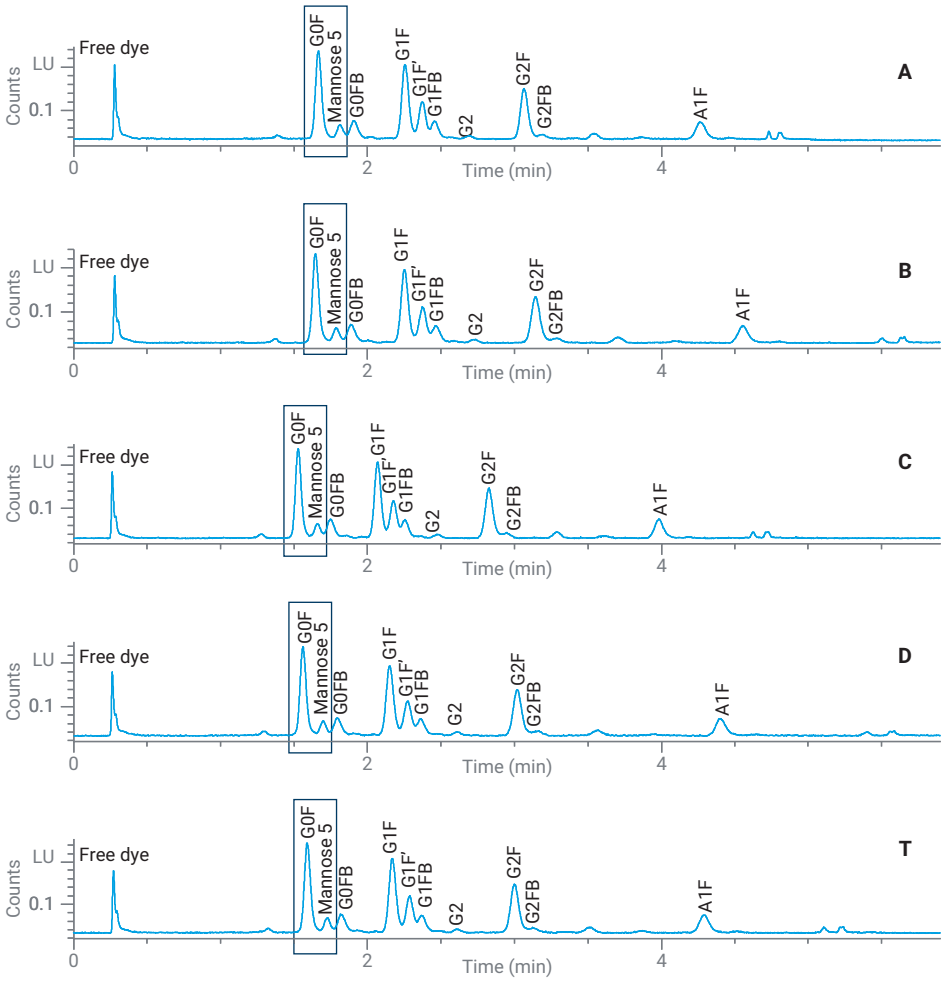

Figure 6. Chromatograms of the validated conditions from point predictions. The highlighted peak pair shows the baseline separated G0F from Man5 after the optimization study.

Table 9. Predicted and experimental results obtained for Response variables after the point prediction validation studies.

Response Variable Name (A)	Predicted Response Value	-2 Sigma Confidence Limit	+2 Sigma Confidence Limit	Experimental
Number of Peaks	10	9	10	10
Mannose 5 (Tangent Resolution)	1.93	1.84	2.45	2.25
Response Variable Name (B)	**Predicted Response Value**	**-2 Sigma Confidence Limit**	**+2 Sigma Confidence Limit**	**Experimental**
Number of Peaks	10	9	10	10
Mannose 5 (Tangent Resolution)	1.93	1.84	2.23	2.17
Response Variable Name (C)	**Predicted Response Value**	**-2 Sigma Confidence Limit**	**+2 Sigma Confidence Limit**	**Experimental**
Number of Peaks	10	9	10	10
Mannose 5 (Tangent Resolution)	1.90	1.81	1.99	1.97
Response Variable Name (D)	**Predicted Response Value**	**-2 Sigma Confidence Limit**	**+2 Sigma Confidence Limit**	**Experimental**
Number of Peaks	10	9	10	10
Mannose 5 (Tangent Resolution)	1.90	1.81	1.99	1.98
Response Variable Name (T)	**Predicted Response Value**	**-2 Sigma Confidence Limit**	**+2 Sigma Confidence Limit**	**Experimental**
Number of Peaks	10	9	10	10
Mannose 5 (Tangent Resolution)	1.91	1.82	2.12	2.04

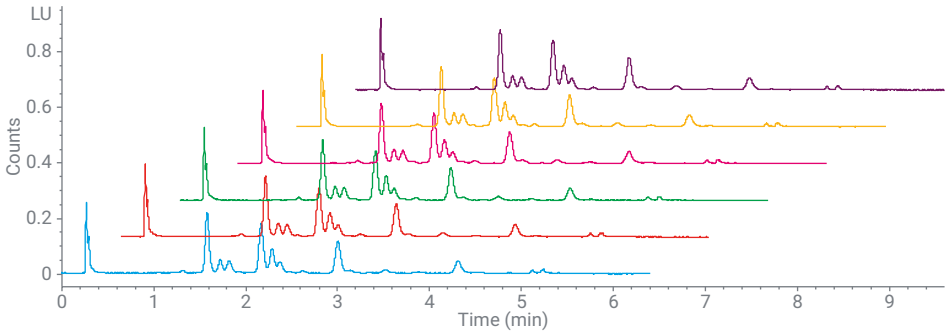

Figure 7. Replicate trials of the optimized method.

Table 10. RSD % calculated for all the glycan peaks.

	RSD % of Glycan Peaks		
Peaks	**Glycans**	**RT RSD**	**Area % RSD**
1	G0F	0.32	0.68
2	Man5	0.49	0.45
3	G0FB	0.23	0.82
4	G1F	0.39	0.26
5	G1F'	0.31	1.29
6	G1FB	0.39	1.87
7	G2	0.64	1.25
8	G2F	0.68	0.44
9	G2FB	0.56	1.62
10	A1F	0.36	1.67

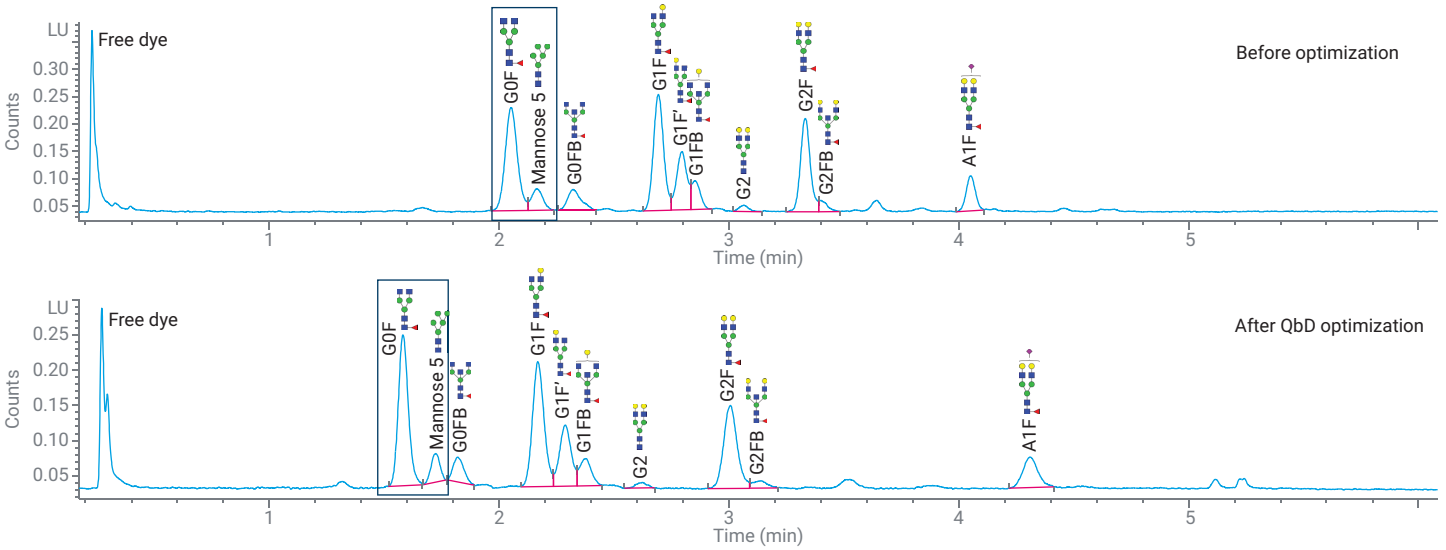

Figure 8: A comparison of chromatograms obtained from the screening as well as from the optimized results. A dramatic change in the resolution of the critical pair of peaks was observed after the optimization. The resolution of the Man5 peak before optimization was 1.3 and after it was 2.04.

Conclusion

This application note demonstrates systemic method development for the separation of glycans using an Agilent 1290 Infinity II LC with Fusion QbD Method Development software from S-Matrix. As a proof of concept, we have tried to separate a critical pair of glycans, G0F and Man5. After the analysis, a good baseline separation between the critical pair of peaks was achieved. A comparison of the chromatograms obtained with and without optimization showed a dramatic change in the separation of the glycan peaks. Other than the critical pair of peaks, the optimized results showed improved separation between the other adjacent peak sets (G1F' and G1FB, G2F and G2FB) as compared to the screening study. A combination of Agilent hardware and Agilent OpenLab CDS ChemStation software along with the Fusion QbD from S-Matrix with its automated experiment execution technology resulted in a robust method for the separation of glycans. This method was achieved in less than two weeks, including instrument run time, analyst time for instrument setup, and chromatogram processing. Agilent hardware with Fusion QbD software provided good separation between the critical glycan pairs in dramatically less time than manual method development.

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