White Paper

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Rapid Development and Validation of HPLC Methods Using QbD Software

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Overview

This white paper is a resource for liquid chromatography (LC) users for method development or validation using Agilent OpenLab Chromatography Data Systems (CDS) or OpenLab ChemStation software. It is the responsibility of the user and their organization to ensure that the functionalities of the S-Matrix and Agilent software are used appropriately and function correctly. This white paper is intended as a resource for general guidelines and should be understood as a generic example of using the S-Matrix Fusion QbD software (Version 9.7.1) in conjunction with the Agilent LC software.

Introduction

There is a constant need to develop and validate high-performance liquid chromatography (HPLC) methods to meet the FDA's expectation for protein analysis and quality control of antibodies. Using the systematic approach of quality by design (QbD) experiments ensures a more efficient and robust process for method development and approval. However, QbD setup can be time-consuming. Fusion QbD is a sophisticated software from S-Matrix that pairs seamlessly with Agilent OpenLab CDS or OpenLab ChemStation software to automate QbD method execution and analysis for HPLC methods. The QbD software accelerates the entire process from method development and robustness to validation by automating the design of experiment (DoE) setup, quantifying the relationship between method parameters, and optimizing the HPLC method based on USP-recommended chromatographic outputs. In this white paper, the Fusion QbD software was used alongside OpenLab ChemStation software to fully develop and validate a size exclusion chromatography (SEC) HPLC method. The analytical target profile (ATP) for the SEC method was achieved with performance specifications for each output based on the critical quality attributes (CQAs). The optimized method gave consistent quantitation and improved resolution, peak symmetry, and peak capacity. The QbD software supplemented the OpenLab ChemStation software analysis, and the method development and validation was three times faster than the conventional development and validation approach.

Method development outline

An SEC method was developed to quantify antibody purity through size distribution. During method development, the method parameters were optimized based on the chromatographic outputs to develop the most resolved, robust, and efficient SEC method. The method parameters include injection amount, mobile phase pH, mobile phase concentration, flow rate, and column temperature. The chromatographic outputs include: peak-to-valley ratio, peak symmetry, number of peaks, and theoretical plate number.

Once the method parameters were finalized, the robustness was tested by slightly fluctuating each method parameter to ensure that adequate chromatographic outputs were attained within the robustness window. Lastly, the method was validated by confirming accuracy, intermediate precision, repeatability, specificity, lower limit of quantitation (LLOQ), linearity, range, and stability.

Traditional method development strategy

The initial development of the SEC method began with mobile phase pH using the traditional method development strategy of one factor at a time (OFAT). A set of runs was dedicated to testing the pH range, and it was found that the pH range from 6.8 to 7.2 gave high peak resolution and symmetry. The method parameter for mobile phase pH was set to 7.0. Dedicating an entire set of runs to determine just one parameter was time-consuming and did not address the interactions on other parameters. After establishing the pH using an OFAT method, a better development strategy was engaged, and for the rest of the method development QbD strategies were used.

QbD method development strategy

The QbD method development began with exploring the flow rate, salt concentration, and column temperature in a QbD environment.

To better optimize the parameters in relationship to each other, a DoE was performed, testing combinations of extremes and center points for each parameter. A DoE was automatically designed using a QbD software from S-Matrix, a partner of Agilent, to automatically make a design matrix calculated to gain the maximum information from a single set of runs. The DoE type was an A- and G-optimal process applying a cubic model type with 32 runs, two center point repeats, two noncenter point repeats, and six lack-of-fit degrees of freedom points. The design matrix was exported to OpenLab ChemStation as ready-to-run sequence and method programs.

The sequence was run and the chromatograms were integrated using the OpenLab ChemStation software. The integrated chromatograms were labelled as high molecular weight species (HMWS) or main peak.

The analyzed data were exported from the OpenLab ChemStation software and imported back to the QbD software for final analysis. It was important to monitor these outputs because they support the separation goal of the ATP to achieve the peak purity required for repeatable and accurate quantitation. In accordance with USP <621> recommendations, the following chromatographic outputs (Figure 2) were used to optimize resolution and efficiency: peak-to-valley ratio, peak symmetry, number of peaks, and theoretical plate number.

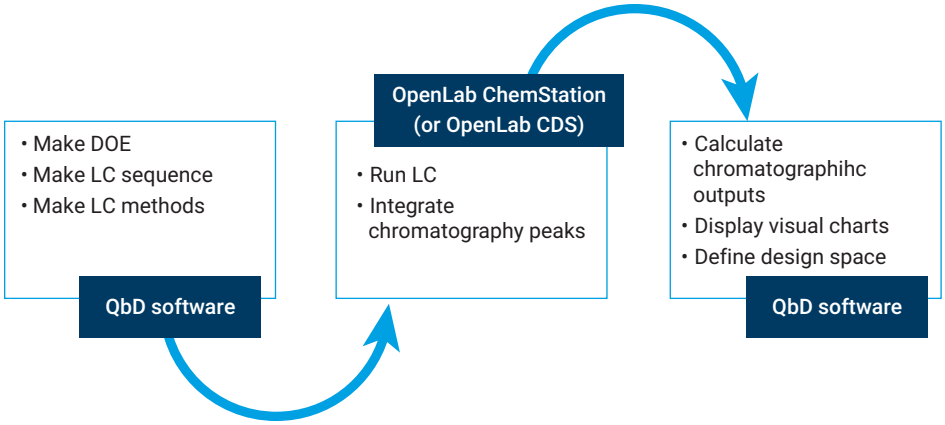

Figure 1. A flow chart showing which steps the QbD software uses and when the Agilent software is used for the method setup, development, and analysis.

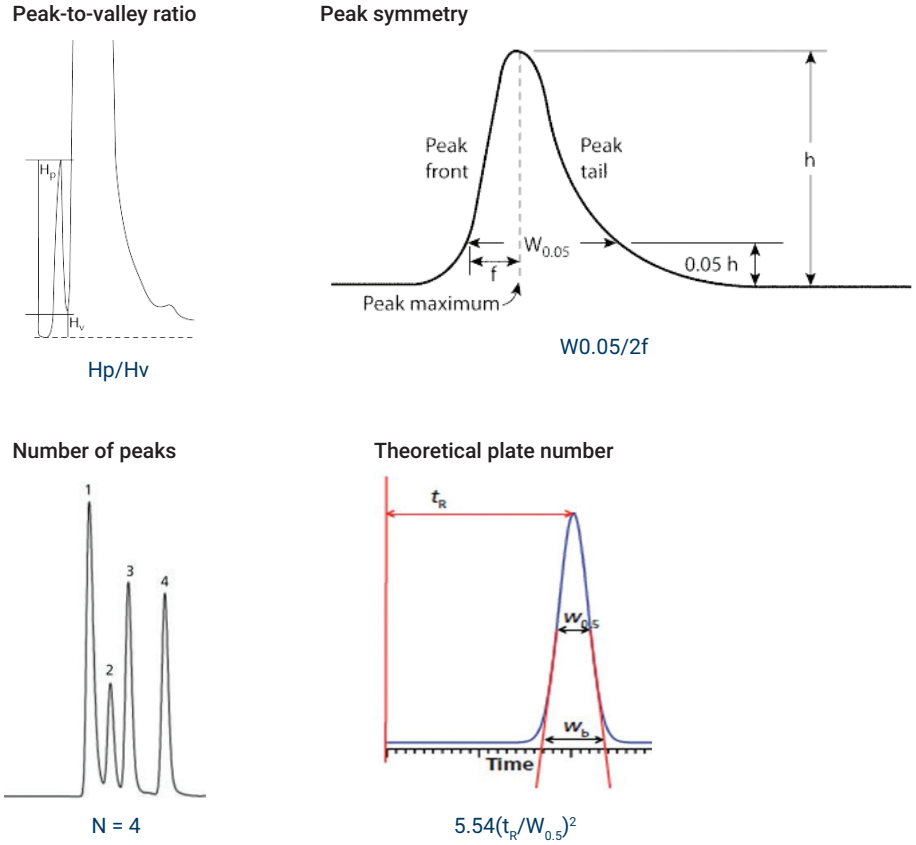

Figure 2. Chromatographic outputs used to optimize the SEC method.

QbD results

The Pareto chart and the effects plot are two of many visualization tools generated by the QbD software, and are extremely useful in guiding method development.

A Pareto chart displays which method parameters have the biggest impact on the chromatographic outputs. An example of a Pareto chart used for this method development is shown in Figure 3. It shows that temperature followed by concentration had the largest effect on the peak symmetry of the HPLC SEC method. The Pareto chart also shows that the linear, curvilinear, and the two-way interaction of temperature and concentration account for approximately 85% of the overall variation of the peak symmetry results. Using the QbD software, the Pareto charts can be displayed for any output to quickly visualize the effect of method parameters on resolution and efficiency. Development can then continue with an emphasis on the parameters that impact the method the most. In this case, the temperature and buffer concentrations were the focus of future development to improve peak symmetry.

An effect plot visually displays the interactions of a method parameter at various levels (Figure 4). The steeper the slope of the line, the more the parameter affects the chromatographic output. If the line is not sloped and is horizontal, it has no effect. For example, the upper left chart of Figure 4 shows that the oven temperature did not affect the number of peaks at 200 mM, as shown by the horizontal red line. However, with a 100 mM buffer, as the column temperature increased, so did the number of peaks, as seen with the upward-sloped blue line. Having these effect plots automatically displayed by the QbD software greatly increased the understanding of the method at a quick glance.

In the final step in the method development, a design space was made. The design space is a range where the method gives acceptable

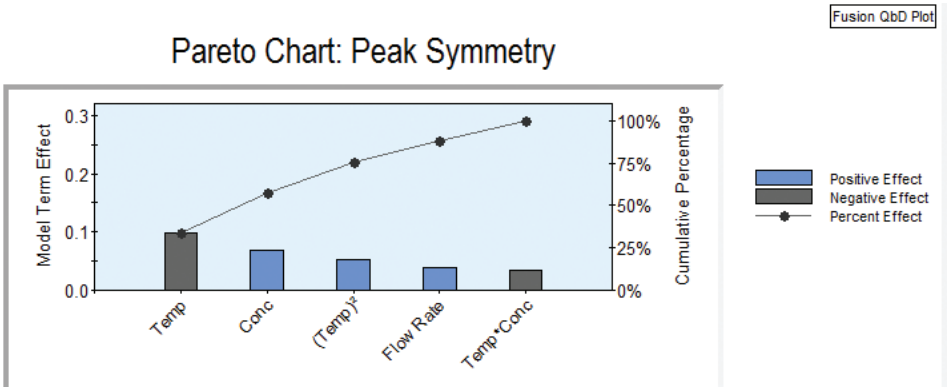

Figure 3. A Pareto chart from the SEC study, which highlights the relative impact of several method parameters on peak symmetry.

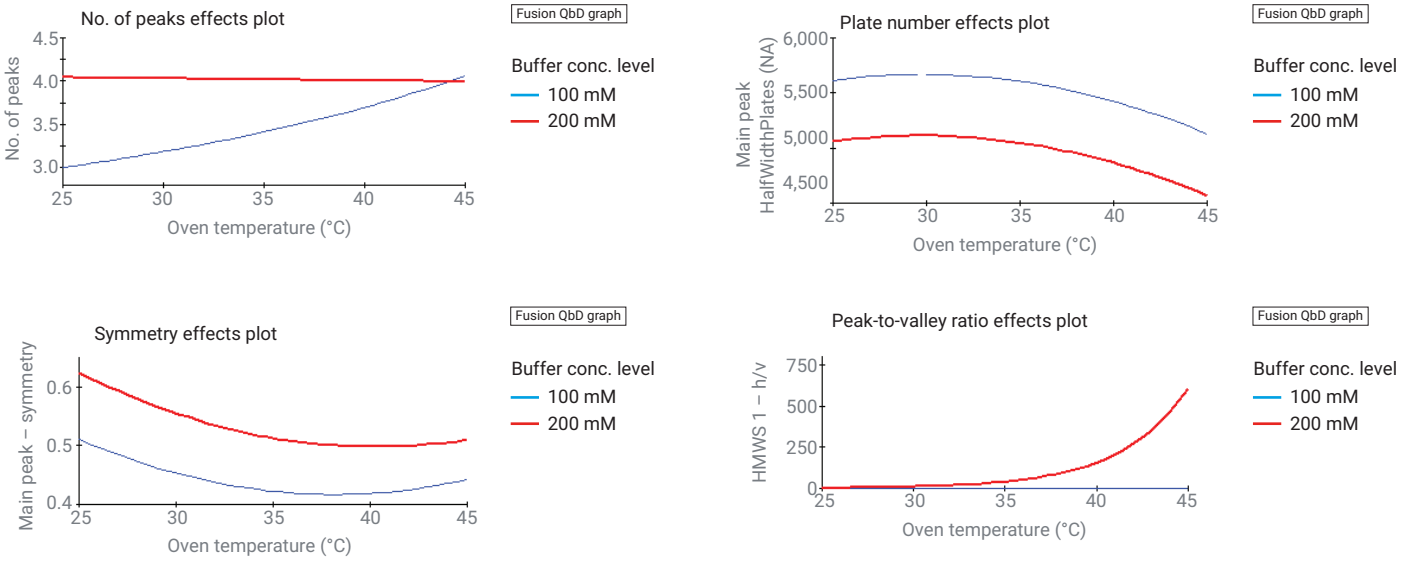

Figure 4. Effect plots from the SEC study used to understand the effects of a combination of chromatographic outputs.

chromatographic peak resolution and efficiency. Limits were set for each parameter to ensure quality chromatographic outputs. Ideally, the method should be set at the center point of the design space region to ensure a robust method. However, there may be other restraints such as manufacturer's recommendations, pressure restrictions, or shelf-life concerns that could change the method regardless of the development results.

Figure 5 is a chart generated by the QbD software using the results from the SEC development to find the design space. The white regions on the graphs are the design space; the shaded region delineates where the quality of the SEC chromatogram is no longer acceptable based on the resolution, and efficiency as measured by the chromatographic outputs.

The shaded green regions show where the resolution between peaks was poor based on the peak-height-to-valley ratio. The blue regions show where the column efficiency was low based on the theoretical plate numbers. The orange region shows where the number of peaks were outside the targeted range. The pink region shows where the peak shape was poor based on the peak symmetry. The gray region shows where the resolution was poor based on main peak width. That leaves the white region as the design space. The black box in Figure 5 visualizes the design space where the parameters can be independently varied without compromising any of the method performance requirements, placing it within the proven acceptable range (PAR). The PAR can then be used to help determine method robustness.

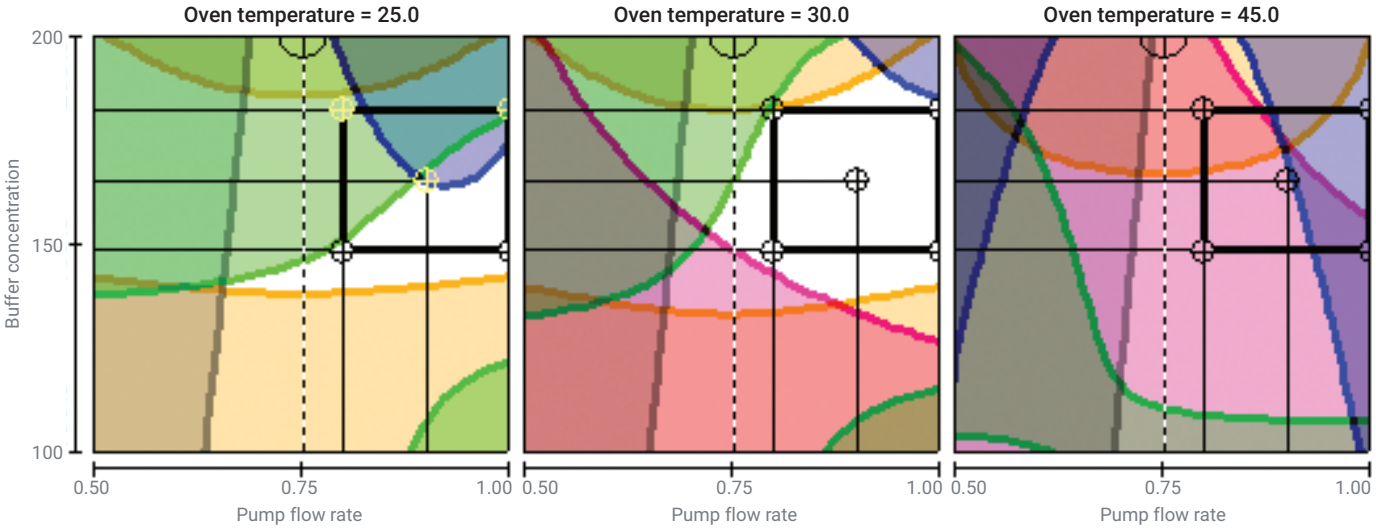

Figure 5. Three charts visually displaying the design space at various buffer concentration and flow rates at a column temperature of 25 °C (left), 30 °C (middle), and 45 °C (right).

Method development final conditions

Based on resolution, column efficiency, peak shape, and quantitation consistency, the final SEC method conditions for flow rate, salt concentration, and column temperature were 1.0 mL/min, 165 mM sodium phosphate, and 30 °C, respectively. The final optimized SEC chromatogram is displayed in Figure 6. In the expanded view, the HMWS, which are each 0.5% of the protein peak area, are well separated and easily integrated. The next step of robustness testing was assessed with confidence since the parameters were already known to be within the proven acceptable range based on the method development.

Results

The PARs from method development were used as limits for the robustness testing. The passing criteria assigned for each output is based on the method development of maintaining the CQA. For this SEC method, the CQA is the percent peak area of HMWS, and the acceptable range of each output was assigned accordingly. The percent relative standard deviation (% RSD) of the HMWS was important to capture in the robustness to show that there was little variation in the CQA reported. It is important to note that passing criteria are specific for each method, and for new methods, the passing criteria needs to be re-assessed for that specific method during the development based on the CQA.

Having the chromatographic outputs calculated by the QbD software was essential for quickly proving robustness. The HMWS accounted for 0.5% of the sample, and even at this small amount, the % RSD of the HMWS was tight, confirming a very robust SEC method (Table 1). Additionally, having a peak-to-valley ratio over 1 showed that the shoulder peak of the HMWS is routinely pulled out and reliably quantified.

The accuracy, precision, specificity, and linearity of the SEC method were validated using Fusion QbD, and all passed the acceptance criteria with ease. Different operators, on different days, using freshly prepared buffers had less than 0.1% RSD in quantitation of the SEC method. These results show that the method development was satisfactorily optimized.

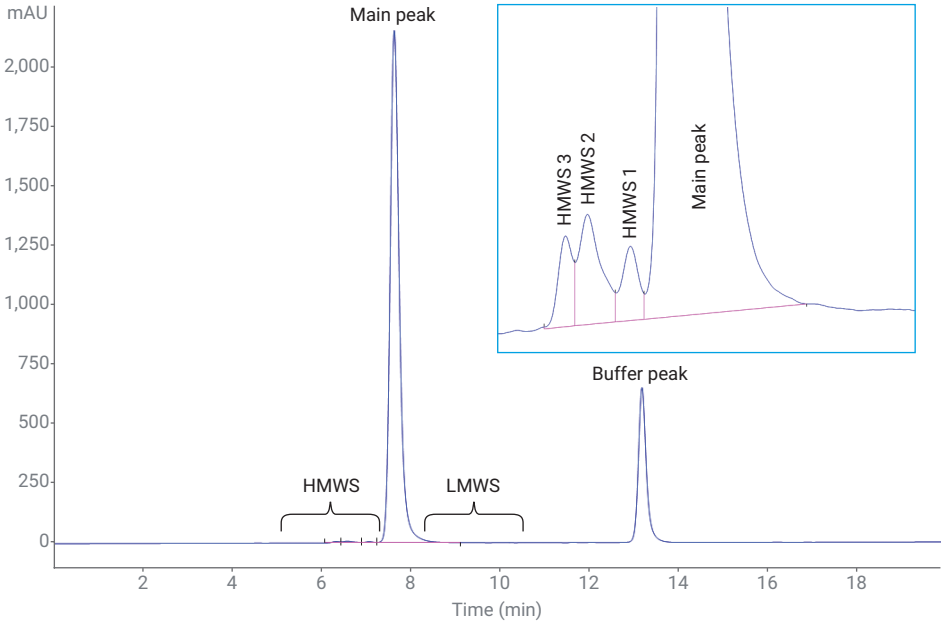

Figure 6. An SEC chromatogram of the final optimized method using nominal conditions. The full view of the chromatogram is displayed, with the protein peaks eluting between 6 to 9 minutes, and the buffer peak eluting at 13 minutes. An expanded view of the protein peaks is embedded in the upper right corner.

Table 1. Parameters applied within robustness testing.

Robustness Parameter	Ranges Tested	Number of Peaks (= 4)	Peak-to-Valley Ratio (>1)	% RSD of Peak Area (<15%)	Robustness Conclusion
pH	7.0 ±0.2	4	>2	1.9%	Robust
Salt Concentration	165 ±17 mM	4	>5	2.4%	Robust
Column Lots	3 lots	4	>1	6.6%	Robust
Temperature	30 ±3 °C	4	>2	2.8%	Robust
Flow Rate	1.0 ±0.1 mL/min	4	>2	2.2%	Robust

Conclusion

Fusion QbD software was easy to use and integrated effortlessly with OpenLab ChemStation on an Agilent HPLC. The automatic setup of DoE methods and sequences, along with the automatic analysis of the integrated chromatograms made the development, robustness, characterization, validation, and data analysis over three times faster (Figure 7). More importantly, the ability to compare so many different variations of parameters made the development cover a larger array of combinations, and in return gave a more robust method.

The flexibility of OpenLab ChemStation software's integration parameters, along with the automated integration options, allow the HPLC method development to work seamlessly with Fusion QbD to provide high-quality, robust methods in a very short timeline.

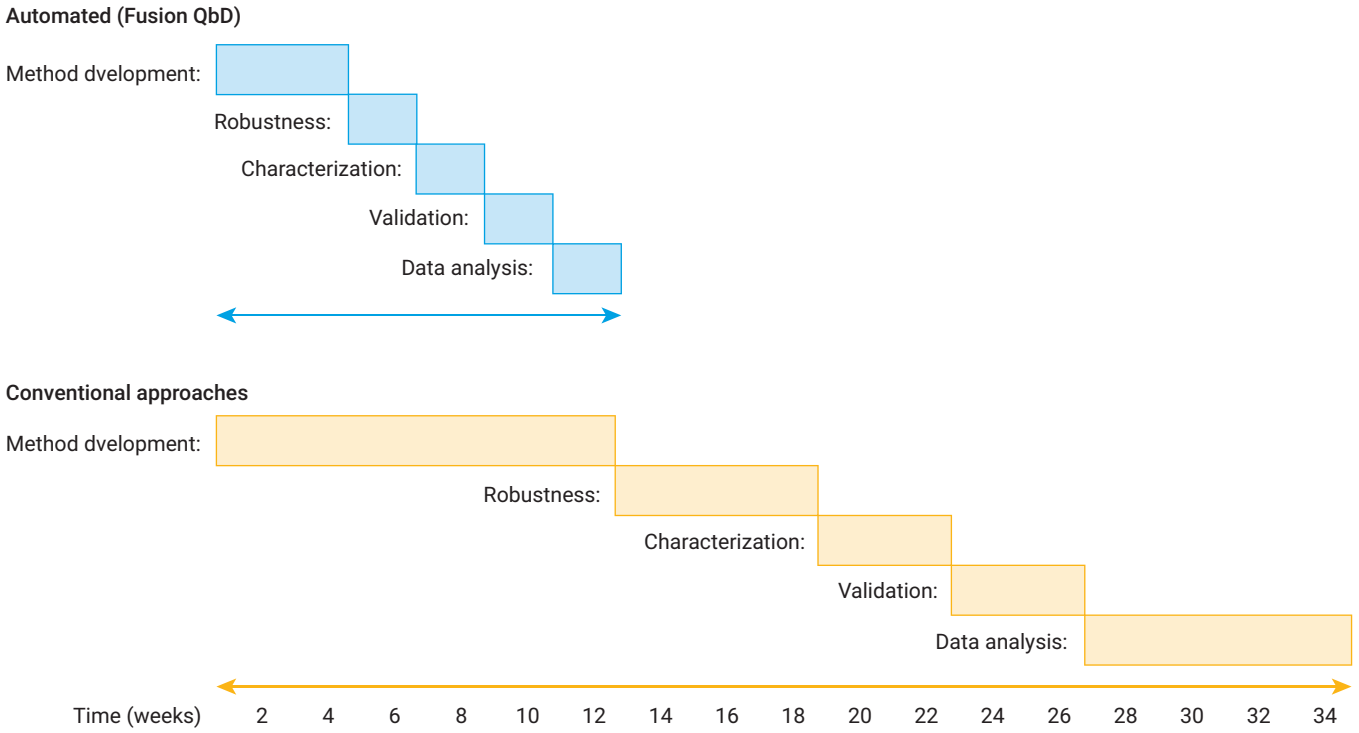

Figure 7. Time comparison of creating an HPLC method using the automated Fusion QbD software and conventional approaches.

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