

A Complete Solution for Accelerating and Revalidating HPLC Methods—Start Working with Your New Methods in Less than One Week

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INTRODUCTION

A straightforward approach to speeding up an existing LC method involves using a shorter column to reduce run times, and a smaller particle size to counteract the drop in resolution. For example, many methods are typically run on a 15 cm x 4.6 mm column with 5- μ m particle size material. These methods can generally be run on a 7.5 cm x 3 mm column with 3- μ m particle size material, and using an adaptation of the original instrument method (e.g., new gradient profile). It is possible to determine the new method parameters by applying the mathematics behind the van Deemter theory, theoretical peak volumes, and the gradient volume concept. Once the user has selected a new fast LC column with a stationary phase that provides the appropriate selectivity, method details such as flow rate, injection volume, and gradient program of the optimized method are automatically predicted by a software package that incorporates the mathematical formula.

However, successful method development is only the first step of accelerating a method. The next step is complete method revalidation required in many laboratories. This revalidation is generally a barrier to developing a new method, as the time required to revalidate can be longer than the time savings gained by reducing the analysis time.

In this presentation we show the systematic software assisted speed-up of an impurity profiling method in pharmaceutical quality control. The new method provides analysis time and solvent savings of up to 80%. The instrument used automates significant parts of the validation effort, and the software package used automatically calculates all validation results and prepares the final validation report in only a matter of minutes. The new solution allows laboratories to speed-up existing methods and revalidate them in less than one week.

ULTIMATE® 3000 RAPID SEPARATION LC

A complete solution for optimized LC methods includes appropriate instrumentation to achieve the required performance on a dedicated separation column design. In addition, software tools assist the development and implementation of the optimized LC method. The goal of optimization is to achieve the required chromatographic resolution in as short a time as possible, without compromising the quality of the generated data. A validation of the new method according to the ICH guidelines can be considered an appropriate tool for assessing the

quality of the new method. A chromatography data system can be of help to coordinate and partially automate the related tests and provide the complete templates for a seamless documentation of the validation results.

The UltiMate 3000 achieves the maximum resolution in minimal time. This optimized binary gradient based system (see Figure 1) features:

- A pressure range up to 500 bar, and a column temperature range up to 85 °C
- A pressure-independent gradient delay volume of < 100 μ L (< 60 μ L with sample loop bypass)
- Superior separation efficiency due to an extra column volume of < 13 μ L (including eluent temperature preconditioner)
- A fast and accurate well plate autosampler with injection cycle times < 15 s
- A high speed variable wavelength UV-detector with a data collection rate up to 100 Hz



Figure 1. The UltiMate 3000 Rapid Separation LC system delivers superior resolution in minimal time.

Acclaim® Rapid Separation LC Columns

The Acclaim Rapid Separation LC (Acclaim RS) columns feature:

- Ultrapure 120-Å silica-based 3-µm particle stationary phase
- Choice of a standard C18, a sulfonamide polar embedded C16 (Polar Advantage), and a carbonamide polar embedded C18 (Polar Advantage II) bonding
- Enhanced sensitivity, optimal radial temperature balance, and solvent savings with 3-mm internal diameter column hardware
- Superior separation acceleration using a column length of 33 cm or 75 cm

Dionex Method Speed-Up Calculator

The Dionex method speed-up calculator facilitates the transfer of methods to new column formats that differ in length, internal diameter, and particle size of the stationary phase. For further details, refer to the Figure 2.

INSTRUMENTS AND EXPERIMENTAL CONDITIONS

The Original HPLC Method

The method and the samples were provided from a customer and represent a standard example for impurity profiling in pharmaceutical quality control.

Original Instrumental Configuration

- UltiMate 3000 Binary Analytical HPLC System (600 µL total gradient delay volume) consisting of the following modules (and 0.25 µm ID connection tubing):
 - HPG-3200A binary analytical pump
 - WPS-3000SL analytical autosampler
 - TCC-3000 thermostatted column compartment
 - VWD-3400 four-channel variable wavelength detector with analytical flow cell (11 µL, 10 mm)
- Eluent preconditioner (11 µL), in-line filter with 0.5 µm SST frit
- Chromeleon® 6.80 Chromatography Management Software

Original Chromatographic Conditions

- Column: Dionex Acclaim 120 C18, 3 µm, 150 × 4.6 mm
- Eluent A: 5 mM octane-1-sulfonic acid sodium salt, 10 mM potassium dihydrogen phosphate, pH = 2.5; eluent B: methanol gradient: 0–60 min 5–40% B, 60–61 min 40–5% B, 61–66 min 5% B
- Column temperature: 55 °C, flow: 1.2 mL/min, injection volume: 100 µL
- Detection wavelengths: 205 nm, data collection rate: 2.0 Hz, time constant: 1.2 s

Computer Assisted Method Acceleration

To find the appropriate method parameters for separations on the Acclaim RS LC columns, the Dionex method speed-up calculator was used. The original column dimensions and particle size, flow rate, injection volume, and gradient table were entered, followed by the targeted column dimensions and particle size for the optimized method. We set the boost factor initially to 1 for no increase in the linear velocity relative to the original method (particle diameter remained unchanged). The calculated flow rate, injection volume, and gradient profile were used as a first speed-up approach involving the Acclaim RS column (C18) and an optimal setup with the UltiMate 3000 Rapid Separation LC system. The values of backpressure and resolution of the critical peak pair (e.g., compound 4 and 5) were predicted from the observed values of the original method. Figure 2 shows the method speed-up calculator sheet with the respective initial values and results. Finally, the boost factor was increased to 3 to achieve an additional threefold acceleration of the separation.

Speed-Optimized HPLC Methods

Optimized Instrumental Configuration for Rapid Separations

- UltiMate 3000 Rapid Separation LC System with analytical autosampler (245 µL total gradient delay volume) consisting of the following modules that differ from the original configuration and 0.13 µm ID connection tubing (see Figure 2 for a photo of the system):
 - HPG-3200M binary analytical pump with 50-µL static mixer
 - VWD-3400 four-channel variable wavelength detector with semi-microflow cell (2.5 µL, 7 mm)

All other conditions as in the original configuration (see above)

Optimized Chromatographic Conditions (Boost Factor 1)

- Column: Dionex Acclaim RS 120 C18, 3 µm, 75 × 3.0 mm
- Gradient (Boost Factor 1): 0–30.0 min 5–40% B, 30.0–30.5 min 40–5% B, 30.5–33.0 min 5% B
- Flow: 0.510 mL/min, injection Volume: 30.1 µL
- Data collection rate: 4.0 Hz, time constant: 0.36 s

All other conditions as in the original method (see above)

Optimized Chromatographic Conditions (Boost Factor 3)

- Gradient (Boost Factor 3): 0–10.00 min 5–40% B, 10.00–10.17 min 40–5% B, 10.17–11.00 min 5% B
- Flow: 1.531 mL/min

All other conditions as in the original or boost factor 1 configuration (see above)

Auto Dilution

For method calibration and revalidation, seven standard solutions were sampled automatically by the WPS-3000SL. A 200% stock solution containing 42 µg/L of compound 6 and 1.28 µg/L of compound 4 was prepared manually and subsequently diluted to obtain standard solutions at 10%, 25%, 50%, 75%, 100%, 125%, and 150% levels. The diluent was first dispensed into separate HPLC vials with 250-µL glass inserts by the WPS-3000SL. Subsequently, the 200% stock solution was added and mixed with the diluent in each vial. Mixing was enhanced by fast movement back and forth of the sampler carousel. The total volume of each standard was 140 µL. To minimize dispersion or carry-over, air was drawn into the needle before drawing the diluent. After preparation of the standard solutions an outside needle wash (300 µL) and sample loop wash (1 mL) was performed.

METHOD SPEED-UP RECOMMENDATIONS											
Column Used					Planned Column						
Length (mm)	150 mm				Length (mm)	75 mm					
Diameter (mm)	4.6 mm				Diameter (mm)	3.0 mm					
Particle Size (µm)	3.0 µm				Particle Size (µm)	3.0 µm					
Peak Details (Critical Pair)											
Actual Rs	2.86				Predicted Rs Change Factor	0.71					
					Predicted Rs	2.02 Baseline resolution achieved					
Used Instrument Settings					Recommended Instrument Settings						
Flow	1.200 ml/min				Flow	0.510 ml/min					
Injection Volume	100 µL				Injection Volume	30.1 µL					
Max Pressure	137 bar				Est. Max Pressure	69 bar					
No of Samples	20				No of Samples	20					
Boost Factor:	1.0										
Gradient Table					Gradient Table						
Step	Time (min)	%A	%B	%C	%D	Step	Time (min)	%A	%B	%C	%D
1	0.000	95.0	5.0			1	0.000	95.0	5.0		
2	60.000	60.0	40.0			2	30.000	60.0	40.0		
3	61.000	95.0	5.0			3	30.500	95.0	5.0		
4	66.000	95.0	5.0			4	33.000	95.0	5.0		
5						5					
6						6					
7						7					
8						8					
9						9					
10						10					
End Time	66.000				End Time	33.000					
TOTALS					TOTALS					SAVINGS	
Eluent Usage	1584.00 ml				Eluent Usage	336.86 ml =				79%	
Time	1320 min				Time	660 min =					
	22.00 hr					11.00 hr =				50%	
Sample Usage	2000.00 µL				Sample Usage	601.51 µL =				70%	

Figure 2. The Dionex method speed-up calculator sheet shows the transfer from the original method to a 75-mm column format without a further method boost (boost factor = 1).

RESULTS AND DISCUSSION

Faster Separation on an Optimized Column Design

The main disadvantage of the original method is the relatively long run time of 66 min. As the method involves a large gradient volume on a relatively long column, there is a high potential to transfer it to a shorter column whilst maintaining the respective gradient volume. This would speed up the separation and still provide adequate peak resolution. The new column format is 75 × 3.0 mm, packed with the identical stationary phase. The method speed-up calculator automatically adapted the flow rate, injection volume and gradient table to the new column format. A prediction of the peak resolution with the speed optimized method was provided, too. Figure 3 shows the separation on the new column format performed with the same linear velocity as the original method. We could reduce the run time to 33 min while maintaining good resolution of the critical pair (Rs=2.19, relative to a predicted value of only 2.02).

Further Acceleration with the Boost Factor

With the new 33 min method, the linear velocity was only about half the theoretical value according to van Deemter. Since gradient methods usually give a large potential to achieve appropriate selectivity at higher flow rates with constant gradient volume, there was a room for further optimization. We used the available boost tool of the method speed-up calculator at a boost factor of three to increase linear velocity of the mobile phase three-fold. The gradient table was adapted automatically. This step reduced the total run time to 11 minutes, as shown in Figure 4. This run time compares to 66 min of the original method. The resolution of the critical peak pair is now 1.88—still appropriate for pharmaceutical QC laboratories.

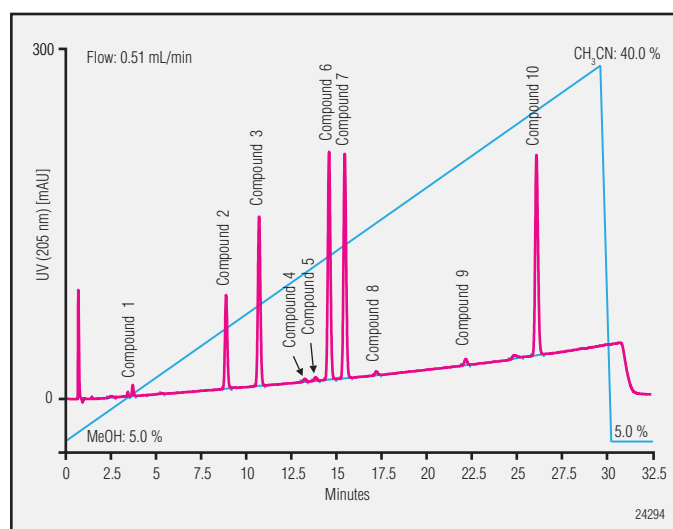


Figure 3. Speed-up of impurity profiling method (compare to Figure 4) run on a 3.0 × 75 mm column format. For further details, please refer to experimental section.

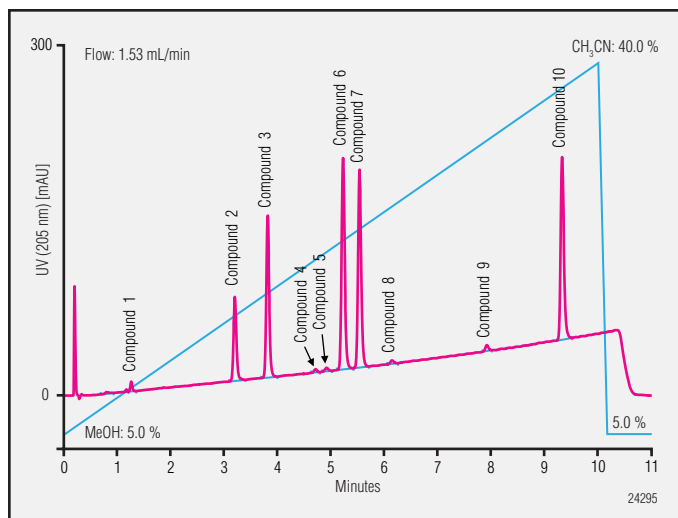


Figure 4. Boost of the fast impurity profiling method (compared to Figure 3) using a three-fold flow rate increase and adapted gradient table. For further details, please refer to the experimental section.

Table 1: Most Important Parameters of the Three Different Methods			
	Original Method	Fast Method (Boost Factor 1)	Boosted Method (Boost Factor 3)
Resolution (Compound 4/5)	2.86	2.19 (2.02)1	1.88 (n.a.)2
Resolution (Compound 5/6)	3.89	2.69 (2.75)	2.86 (n.a.)
Resolution (Compound 6/7)	4.28	3.20 (3.03)	2.85 (n.a.)
Pressure maximum during gradient (bar)	137	92 (69)	290 (206)
Run Time (min)	66	33	11

1. Predicted values from the method speed-up calculator in brackets
2. Resolution prediction disabled at boost factors > 1

Figure 5 shows a comparison to scale of the three different methods. The comparison clearly depicts the significant acceleration of the method. Table 1 shows the resolution figures of each critical peak pair for all three methods. The overall run time and the generated column pressure is also listed in this table. The shorter column and the use of 3- μm stationary phases allows the resulting back pressure of the “boosted method” to remain at a very moderate level of 225 bar. The predicted resolution figures closely matched the actual resolution at boost factor 1. The more pronounced differences between the predicted and measured pressure are due to pressure generated in the instrument outside the column (e.g., the calculator does not factor that the connection tubing id is reduced from 0.25 mm to 0.13 mm).

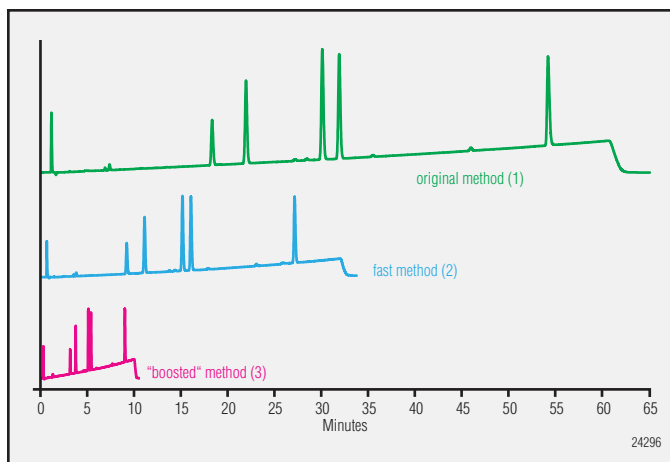


Figure 5. Comparison to scale of the three different methods. Note that the optical path length of the detector flow cell differs from method 1 (10 mm) to method 2 (7 mm).

Computer-Assisted Validation of the Optimized Method

For the validation of the method, we tested linearity, accuracy, and repeatability. Additional tests such as specificity, LOD/LOQ, intermediate precision, and robustness were scheduled for later. We compared the linearity of the method using the manually prepared solutions and the auto-diluted solutions. The accuracy was checked only with the auto-diluted solutions.

With the auto-diluted solutions, we obtained a correlation coefficient of 0.9997, with a slope of 1.431 and an intercept of -0.6811 (see Figure 6). The origin of the plot was outside the 95% confidence limits of the intercept, showing that the negative intercept value was not acceptable (see Figure 7). Data from a plot using the manually diluted standards did not show this effect, suggesting that the cause of the low intercept is a nonoptimization of the automated dilution. However, the excellent correlation coefficient shows that the auto dilution process is a workable one and that an optimized method is likely to provide an acceptable intercept value.

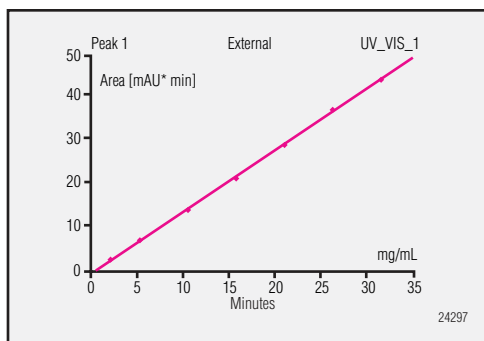


Figure 6. Linearity plot with five different concentration levels, and three injections per level.

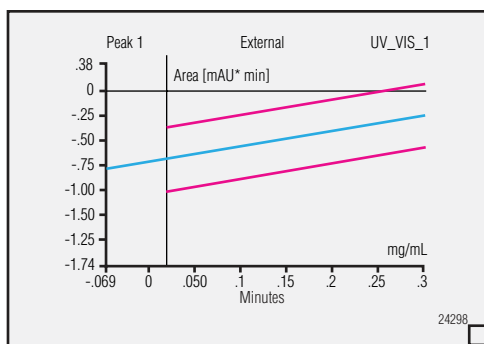


Figure 7. Ninety-five percent confidence limits enclosed the origin of the plot.

The accuracy was tested by reinjecting the auto-diluted standards and calculating the recovery against the linearity plot. In this experiment, we performed three injections (for repeatability assessment) at three different concentration levels: 15.75, 21.0, and 26.25 mg/mL (75–125% of the nominal concentration). At each level we saw recoveries of greater than 95% and %RSD values at each concentration level of less than 0.2%. For a similar experiment performed with manually diluted samples, all recoveries were in the range of 99–100%. It is likely that an optimized auto-dilution process that yields a more acceptable intercept will also improve recovery figures.

Table 2. Accuracy and Repeatability Data					
Range level %	Expected amount (mg/mL) Peak 1 UV-Vis 1	Calculated amount (mg/mL) Peak 1 UV-Vis 1	Recovery % Peak 1 UV-Vis 1	Average	RSD %
75.0000	15.7500	15.2823	97.0302		
75.0000	15.7500	15.2527	96.8429		
75.0000	15.7500	15.2578	96.8750	96.9160	0.1034
100.0000	21.0000	20.8602	99.3341		
100.0000	21.0000	20.8036	99.0649		
100.0000	21.0000	20.8561	99.3150	99.2380	0.1514
125.0000	26.2500	26.7110	101.7562		
125.0000	26.2500	26.7067	101.7399		
125.0000	26.2500	26.7353	101.8487	101.7816	0.0576

CONCLUSIONS

1. A speed-up from 66 min run time to 11 min was achieved with an acceptable decrease in critical peak pair resolution from 2.86 to 1.88.
2. The new method could be created and optimized within one day
3. The data generated with manually diluted solutions shows that the faster method can be validated according to the ICH guidelines (further improvement of the auto-dilution process required if it is to be used for automated validation)
4. The complete validation and report generation can be accomplished within three days

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LPN 1957-01 07/07
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