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Technical Article

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Strategy for Improving HPLC

Retention Time Prediction Accuracy Using Chromatography Simulation Software

The introduction of Quality by Design, a systematic approach to product and process design and development, has shifted the paradigm in HPLC methods development from a retrospective approach to a prospective, systematic, risk-based approach in order to develop enhanced method understanding.[1]

The basis for Quality by Design of chromatographic methods can be broadly categorized into two areas: knowledge space and design space. The knowledge space encompasses all considerations made, all experiments conducted, and all knowledge gained in the development of a method (i.e., column screening or pH screening experiments). The knowledge space forms the basis for delineating a design space within which one can modify the chromatographic factors (i.e., gradient slope, temperature, and buffer concentration, all at a defined pH) without significantly impacting the final quality of the method and making sure all of the chromatographic figures of merit can still be met (resolution of critical pairs, tailing factor, selectivity, etc.).

There is an inherent need to understand the main effects of critical method factors and their mutual interactions on the critical method attributes (the response variables that are the quality characteristics of the method). Since there are many factors a scientist must consider when developing or validating a robust stability-indicating chromatographic method, statistical experimental design and analysis allow for an efficient and effective means of execution. This will lay the basis for defining the design space boundaries and a control strategy to ensure that quality is built into the method and further ensure the integrity of the results.

In most cases, reversed-phase chromatographic separation can be achieved with an appropriate column selection and evaluation of different variants of mobile phase parameters. The first stage of chromatographic method development is to identify the most suitable column, mobile phase pH, aqueous phase buffer, and organic solvent to separate the active pharmaceutical ingredient (API) and its impurities in a particular sample. After initial starting conditions have been identified, the next stage is to determine optimal and robust separation conditions. This task becomes more complicated as the number of operating variables increases, which leads to a larger number of experimental runs required. To simplify and accelerate the optimization process, computer simulation software packages have been employed.[2]

Liquid chromatography simulation software such as DryLab® (**Molnár Institute**, Berlin, Germany), LC-Simulator® (**Advanced Chemistry Development**, Toronto, Ontario, Canada), and ChromSword® (**Merck**, Darmstadt, Germany) have been shown to be effective tools in modulating gradient and column temperature during method development. These programs can use a small set of well-defined experimental data on a particular stationary phase at a defined variable such as pH to predict optimal separation based on changes in mobile phase composition and temperature.[3] DryLab software uses retention data from scouting runs for subsequent retention and resolution prediction via simulation.[3] ChromSword, another optimization software, takes a somewhat different approach, using structure fragments and dipole–dipole interactions to predict retention behavior.[4,5] Both of these methods work without any direct connection to the chromatographic apparatus. More sophisticated software utilizes artificial intelligence. An early example is the EluEx (**CompuDrug**, Budapest, Hungary), which can suggest initial experimental conditions based on chemical structures.[5]

Many authors reported successful use of chromatography simulation software for method development purposes. A polar neutral compound (ethinylestradiol, EE) and its related substances (6-alpha-hydroxy-EE; 6-beta-hydroxy-EE; 6-keto-EE; 9,11-didehydro-EE; and estradiol) were separated on a Pinnacle® C18 column (**Restek**, Bellefonte, PA) (50 × 2.1 mm, d_p = 1.9 µm). Two gradients with different slopes (7- and 21-min gradient time) were run at two different column temperatures (35 and 65 °C). The predicted retention times (RT) were in close agreement with the experimental times; the average retention time error was 1.6% (minimum 0.44, maximum 3.09).[6] Similar results were observed in the simultaneous optimization of gradient program and mobile phase pH for basic molecules separated on a Zorbax SB C18 column (**Agilent Technologies**, Santa Clara, CA) (50 × 2.1 mm, d_p = 1.8 µm) with methanol and buffer as mobile phase. Two initial gradients with different slopes (7- and 21-min gradient time) were run within a narrow pH range at three different mobile phase pH values (pH 6.2, 6.6, and 7.0) at a constant temperature. The predicted retention times were also in excellent agreement with the experimental times. When mobile phase pH was optimized, the overall average retention time error was under 2% (minimum error was 0.44% and maximum was 8.66%). It was also concluded that the accuracy of computer prediction depends on the applied pressure (flow rate).[6]

In addition to gradient optimization, many others also reported using the software for mobile phase pH optimization. In order to obtain a 2-D resolution map, three combinations of parameters were considered: gradient time (t_G) versus eluent composition of acetonitrile (ACN) and H_2O, t_G versus column temperature, and eluent composition versus pH.[7] Analyte separation was further optimized using DryLab software with experimental data input from two linear gradient runs: t_G = 30 min and t_G = 60 min; %B run from 5 to 50% at column temperature of 30 °C and aqueous mobile phase pH of 5.5 (analyte pK_a of 6.6–9.0). The overall difference between predicted and experimental retention time was 0.2%.[7]

There have been many success stories utilizing the software to achieve optimal separation. However, within the literature, there are limited discussions on the limitation and capability of the approach.

In one study,[8] multiple classes of compounds such as steroids, pesticides, algal pigments, fatty acid methyl esters, and acrylate monomers were chosen to examine the effectiveness of varying column temperature and gradient steepness via DryLab. They achieved optimal separation and good prediction accuracy for most analytes. However, in the case of testosterones, isomers of monohydroxytestosterone were not adequately resolved (maximum resolution <0.5) by any choice of gradient-time or temperature modulation. During the same study, for a group of toxicological compounds in body fluids, accurate retention time prediction of seven basic compounds was not feasible due to early elution.[8] It was also mentioned that the extent of extrapolation based on input data should be carefully chosen.

In another study,[9] several classes of compounds were also selected to elucidate the effectiveness of the combined use of column temperature and gradient steepness. An anomalous retention time–temperature relationship was observed in which tripelennamine exhibits an increase in retention as column temperature increases. Similar behavior was noted for aniline at pH 3.6 but not at other pH values. Although this sort of retention behavior is unusual, the software was able to provide accurate prediction as long as retention times varied linearly with column temperature. In addition, it was also suggested that prediction accuracy could be improved with interpolation of experimental parameters and also with use of more initial data points to define a suitable function, e.g., quadratic function of the parameter.

The selection of proper range of the gradient steepness for the input runs is recommended by the software to be a factor of 3,[10] e.g., gradient run times of 7 and 21 min with a linear gradient of change in organic composition as input runs. Further elucidation of the effect and importance of the gradient range selection on accuracy prediction is required.

The goal of the current paper is to further illustrate the impact of a key parameter: range of the gradient slopes from input experimental data on predicted results. Secondly, it also focuses on the optimal selection of gradient steepness range to improve prediction accuracy.

Experimental

Reagents and chemicals

HPLC-grade ACN was obtained from **Fisher Scientific** (Pittsburgh, PA). Water was collected from a Milli-Q system (**Millipore**, Billerica, MA). All other chemicals were of analytical grade and were obtained from **Sigma-Aldrich** (St. Louis, MO).

Table 1 Experimental gradients

	Experiment no.	%B (initial)	Isocratic hold (min)	%B (end)	Time range (min)	Gradient slope (%/min)
Initial (scouting)	1	5	0	95	10	9
	2	5	0	95	20	4.5
	3	5	0	95	40	2.25
	4	5	0	95	80	1.125
	5	5	0	95	120	0.75
	6	5	0	95	180	0.5
	7	5	0	95	240	0.375
Verification	8	5	1	95	5	18
	9	5	1	95	10	9
	10	5	1	95	30	3
	11	5	1	95	300	0.3

Instrumentation

All experiments were performed on an Alliance® 2695 HPLC system (**Waters Corp.**, Milford, MA). Data acquisition, analysis, and reporting were performed by Empower™ 1 chromatography software (**Waters Corp.**).

Chromatographic conditions and sample preparation

All experiments were performed on an X-Bridge™ C18 column (**Waters Corp.**) (50 × 4.6 mm, 3.5-µm particle size). Mobile phase A was an aqueous buffer solution containing 10 mM ammonium acetate (adjusted to pH 9 with ammonium hydroxide), and mobile phase B was ACN. A flow rate of 1.5 mL/min was used for all studies. The column temperature was 45 °C. The injection volume was 10 µL and the detection wavelength was set at 254 nm. *Table 1* lists all of the gradient programs used in the initial experiments and confirmatory experiments. The sample diluent was a water/acetonitrile mixture (50/50, v/v). All samples were prepared at appropriate concentrations for reliable tracking of analyte peaks (one compound per sample: 2-ethylpyridine; 3-ethylpyridine; 4-ethylpyridine; 3,4-lutidine; 2,4-lutidine; 2,6-lutidine; and 3,5-lutidine) and were injected into the HPLC immediately.

DryLab simulation

To simulate and optimize chromatographic separations using DryLab software, data from two or more initial runs on LC were entered to "calibrate" the software. However, in the case of mobile phase gradient optimization, only two initial experiments can be imported for the calibration, since the linear-solvent-strength model[11] is the basis for the DryLab simulation of gradient. These two initial experiments need to be carried out at two gradient slopes, usually one steep and one shallow, respectively, and the difference in the slopes is recommended to be within a factor of 3 of the other.[10]

To evaluate how the choice of initial slopes can impact the prediction accuracy, seven initial experiments were performed (see Table 1), and the data from these experiments were paired up for the simulation. Four verification experiments were then performed and the chromatographic data were compared with the simulation results.

Results and discussion

All of the experiments were performed on an X-Bridge C18 4.6 × 50 mm, 3.5-µm column with pH 9 mobile phase at a constant temperature of 45 °C. The set of seven compounds had pK_a values (pK_a ~ 6) less than two pH units below the pH of the aqueous mobile phase. Mobile phase A was an aqueous buffer solution containing 10 mM ammonium acetate (adjusted to pH 9 with ammonium hydroxide), and mobile phase B was ACN. A flow rate of 1.5 mL/min was used for all studies. The initial experiments consisted of gradient runs using a mobile phase linear gradient of 5–95% ACN in 10, 20, 40, 60, 120, 180, and 240 min. These gradients corresponded to slopes, as shown in Table 1. DryLab allows the use of only two initial gradient runs with different slopes as input data for predicting the retention time of the analyte in the mixture at

Table 2 Comparison of predicted retention times vs experimental retention times obtained for slope 0.3% ACN per minute

		Predicted RT for slope 0.3% based on initial slopes					
Peak name	**Experimental RT on slope 0.3%**	**9% and 4.5%**	**9% and 2.25%**	**9% and 1.125%**	**9% and 0.75%**	**9% and 0.5%**	**9% and 0.375%**
2-Ethylpyridine	9.393	28.26	20.52	10.59	11.65	10.55	10.03
3-Ethylpyridine	11.827	36.47	24.88	12.75	14.5	13.11	12.49
4-Ethylpyridine	11.953	3.79	25.45	12.97	14.64	13.29	12.6
3,4-Lutidine	10.907	3.68	25.19	12.65	13.93	12.35	11.62
2,4-Lutidine	10.36	3.61	24.07	12.16	13.28	11.77	11.08
2,6-Lutidine	8.47	3.34	3.78	10.86	11.35	9.92	9.25
3,5-Lutidine	12.72	3.73	26.83	13.79	15.77	14.12	13.42
Overall % error between experimental and predicted results		105.74	110.19	14.15	26.14	12.76	6.57
Peak name	**Experimental RT on slope 0.3%**	**4.5% and 2.25%**	**4.5% and 1.125%**	**4.5% and 0.75%**	**4.5% and 0.5%**	**4.5% and 0.375%**	
2-Ethylpyridine	9.393	13.86	8.52	10.29	9.9	9.7	
3-Ethylpyridine	11.827	16.37	10.16	12.77	12.31	12.11	
4-Ethylpyridine	11.953	16.53	10.24	12.83	12.44	12.2	
3,4-Lutidine	10.907	15.31	9.54	11.88	11.4	11.16	
2,4-Lutidine	10.36	15.08	9.22	11.36	10.87	10.64	
2,6-Lutidine	8.47	12.75	7.82	9.4	8.99	8.78	
3,5-Lutidine	12.72	17.7	10.82	13.82	13.21	13	
Overall % error between experimental and predicted results		42.84	11.98	9	4.71	2.66	
Peak name	**Experimental RT on slope 0.3%**	**2.25% and 1.125%**	**2.25% and 0.75%**	**2.25% and 0.5%**	**2.25% and 0.375%**		
2-Ethylpyridine	9.393	7.28	9.74	9.63	9.56		
3-Ethylpyridine	11.827	8.69	12.19	12.03	11.97		
4-Ethylpyridine	11.953	8.74	12.23	12.16	12.06		
3,4-Lutidine	10.907	8.15	11.32	11.13	11.03		
2,4-Lutidine	10.36	7.83	10.77	10.58	10.5		
2,6-Lutidine	8.47	6.66	8.87	8.73	8.64		
3,5-Lutidine	12.72	9.18	13.18	12.9	12.85		
Overall % error between experimental and predicted results		24.97	3.59	2.09	1.34		
Peak name	**Experimental RT on slope 0.3%**	**1.125% and 0.75%**	**1.125% and 0.5%**	**1.125% and 0.375%**	**0.75% and 0.5%**	**0.75% and 0.375%**	**0.5% and 0.375%**
2-Ethylpyridine	9.393	13.33	10.53	9.93	9.54	9.51	9.5
3-Ethylpyridine	11.827	17.33	13.27	12.45	11.9	11.92	11.92
4-Ethylpyridine	11.953	17.32	13.43	12.54	12.1	12.02	11.98
3,4-Lutidine	10.907	15.89	12.23	11.45	10.98	10.95	10.93
2,4-Lutidine	10.36	14.99	11.61	10.9	10.43	10.43	10.42
2,6-Lutidine	8.47	12.09	9.53	8.97	8.61	8.58	8.55
3,5-Lutidine	12.72	18.91	14.26	13.37	12.69	12.78	12.82
Overall % error between experimental and predicted results		45.02	12.21	5.3	0.95	0.78	0.67

a selected target gradient slope. Different pairs of the seven initial gradients (scouting slopes) were used in the software to predict retention time at four selected verification (target) gradient slopes. In order to compare experimentally obtained retention time for pyridines and lutidines at the target gradient slopes with the predicted retention times, four verification runs were performed. Verification runs were performed using a mobile phase gradient of 5–95% ACN in 5, 10, 30, and 300 min (corresponding to slopes 18%, 9%, 3%, and 0.3% per minute, respectively), as shown in Table 1. The purpose of these experiments was to find the optimal strategy for selecting the initial slopes that can be used for gradient optimization. The comparative results of the predicted retention times versus experimentally obtained retention times when using slopes of 0.3% and 18% are presented in *Tables 2* and *3*, respectively.

Table 3 Comparison of predicted retention times vs experimental retention times obtained for slope 18% ACN per minute

		Predicted RT for slope 18% based on initial slopes					
Peak name	Experimental RT on slope 18%	9% and 4.5%	9% and 2.25%	9% and 1.125%	9% and 0.75%	9% and 0.5%	9% and 0.375%
2-Ethylpyridine	3.353	3.55	3.353	3.66	3.66	3.66	3.66
3-Ethylpyridine	3.497	3.64	3.497	3.77	3.77	3.77	3.78
4-Ethylpyridine	3.497	3.22	3.497	3.77	3.76	3.77	3.77
3,4-Lutidine	3.39	3.26	3.39	3.68	3.68	3.69	3.69
2,4-Lutidine	3.353	3.31	3.353	3.66	3.65	3.66	3.66
2,6-Lutidine	3.227	3.28	3.227	3.55	3.55	3.56	3.56
3,5-Lutidine	3.497	3.2	3.497	3.77	3.77	3.77	3.78
Overall % error between experimental and predicted results		4.73	6.6	8.61	8.53	8.7	8.78

Peak name	Experimental RT on slope 18%	4.5% and 2.25%	4.5% and 1.125%	4.5% and 0.75%	4.5% and 0.5%	4.5% and 0.375%
2-Ethylpyridine	3.353	3.74	3.81	3.78	3.78	3.79
3-Ethylpyridine	3.497	3.87	3.97	3.92	3.93	3.93
4-Ethylpyridine	3.497	3.87	3.97	3.92	3.93	3.93
3,4-Lutidine	3.39	3.8	3.88	3.84	3.85	3.85
2,4-Lutidine	3.353	3.76	3.83	3.8	3.81	3.81
2,6-Lutidine	3.227	3.66	3.72	3.69	3.7	3.7
3,5-Lutidine	3.497	3.88	3.97	3.92	3.93	3.93
Overall % error between experimental and predicted results		11.64	14.02	12.85	13.1	13.14

Peak name	Experimental RT on slope 18%	2.25% and 1.125%	2.25% and 0.75%	2.25% and 0.5%	2.25% and 0.375%
2-Ethylpyridine	3.353	4.03	3.85	3.86	3.86
3-Ethylpyridine	3.497	4.23	3.99	4	4
4-Ethylpyridine	3.497	4.23	4	4	4
3,4-Lutidine	3.39	4.11	3.91	3.91	3.92
2,4-Lutidine	3.353	4.05	3.87	3.88	3.88
2,6-Lutidine	3.227	3.9	3.76	3.76	3.77
3,5-Lutidine	3.497	4.23	3.99	4	4
Overall % error between experimental and predicted results		20.85	14.95	15.12	15.21

Peak name	Experimental RT on slope 18%	1.125% and 0.75%	1.125% and 0.5%	1.125% and 0.375%	0.75% and 0.5%	0.75% and 0.375%	0.5% and 0.375%
2-Ethylpyridine	3.353	3.56	3.67	3.7	3.89	3.89	3.9
3-Ethylpyridine	3.497	3.61	3.75	3.79	4.04	4.03	4.03
4-Ethylpyridine	3.497	3.61	3.75	3.79	4.02	4.03	4.05
3,4-Lutidine	3.39	3.57	3.7	3.74	3.96	3.96	3.97
2,4-Lutidine	3.353	3.56	3.68	3.71	3.92	3.92	3.92
2,6-Lutidine	3.227	3.51	3.61	3.63	3.8	3.81	3.82
3,5-Lutidine	3.497	3.6	3.75	3.79	4.06	4.05	4.02
Overall % error between experimental and predicted results		5.12	8.85	9.85	16.29	16.3	16.39

As was pointed out by Snyder et al.,[9] the retention-time predictions made within the initial slopes region (the interpolated prediction between the two initial slopes used for simulation) are in better agreement with the experimental data compared to those predictions made outside the initial slopes region (extrapolated prediction).

In the authors' study, DryLab predictions obtained for the interpolated and extrapolated gradient slopes were directly compared. Extrapolated predictions refer to the circumstance when the analytes' retention times are predicted for a slope that is not between the initial scouting slopes used for simulation (outside of interpolated area). The overall percent error of predictions using slopes within the interpolated slope area was significantly smaller than that for the extrapo-

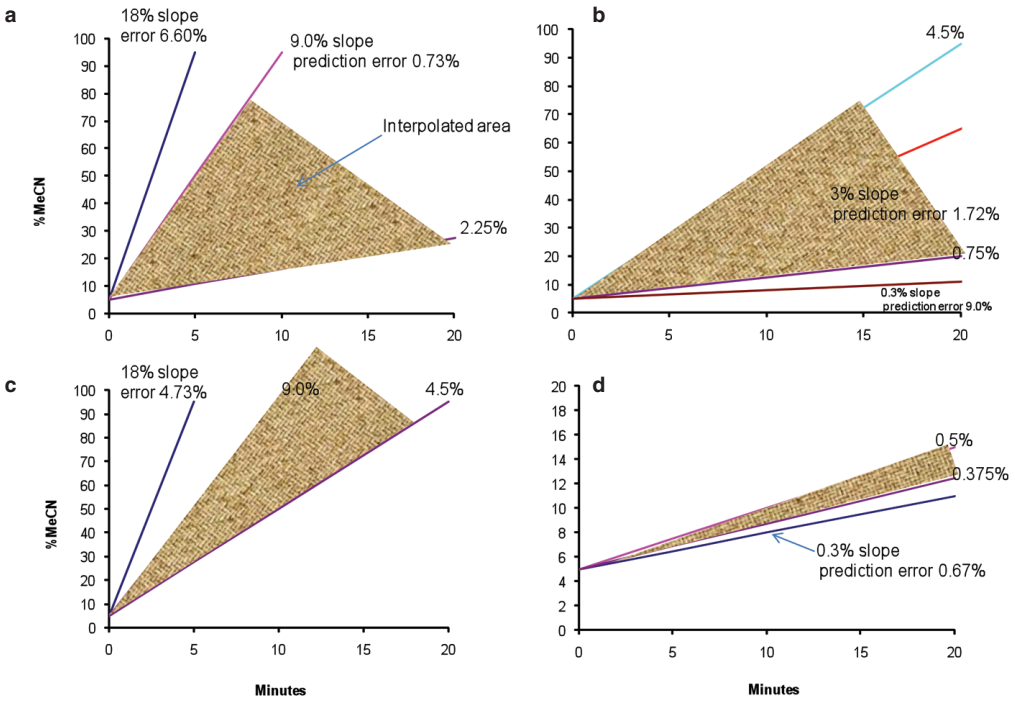

Figure 1 Overall percent-error prediction for interpolated slopes and extrapolated slopes (B%/min). a) Initial slopes 2.25% and 9%, b) initial slopes 0.75% and 4.5%, c) initial slopes 4.5% and 9%, d) initial slopes 0.5% and 0.375%.

lated slopes, as shown in *Figure 1*. Indeed, the overall prediction error for retention times on an interpolated slope of 9%, when initial slopes used for simulation are 9% and 2.25%, is only 0.73% (Figure 1*a*). At the same time, the prediction for the extrapolated steep slope of 18% (B%/min) using the same initial slopes has 6.60% overall error (Figure 1*a*). A similar trend is observed for the shallow extrapolated slope of 0.3% when the initial slopes used for simulation were 4.5% and 0.75% (B%/min) (Figure 1*b*). The predicted retention times on the interpolated 3% slope had only 1.72% overall error, and overall error was 9% for the extrapolated 0.3% slope (B%/min) using the same initial scouting slopes (Figure 1*b*).

For the extrapolated slope predictions, the overall percent error decreased when initial slopes used for simulation were closer to the area of extrapolation (Tables 2 and 3). The overall percent error increased when the area of extrapolation was farther away from the initial slopes. Table 3 demonstrates this trend using an 18% slope as the target slope for prediction. For the steep extrapolated 18% slope, the best prediction was obtained by simulation using the initial slopes at 9.0% and 4.5% (Table 2); the overall percent error for predicted versus experimental retention times was only 4.73%, or less than 0.16 min (Figure 1*c*). The same trend was observed for the extrapolated simulation of shallow gradient at 0.3% slope. The best simulation was obtained when initial gradients were closest (initial slopes 0.5% and 0.375%) to the target (slope 0.3%), as shown in Table 2. The overall percent error was only 0.67% when compared to the simulated retention times for the 0.3% slope based on prediction using initial slopes of 0.5% and 0.375% (Figure 1*d*).

Figure 2 illustrates the natural logarithmic function of the retention factor versus organic gradient slope for pyridines and lutidines. It was noticed that using a wider interpolated area (i.e., increasing the difference between the initial slopes) generally leads to better prediction if the extrapolated target slope is roughly on the same line as the initial pair of slopes on the function shown on Figure 2. The extrapolated predictions, obtained when the initial pair of slopes used for simulation were on the curved part of the function shown on Figure 2, were less reliable based on overall percent error between predicted versus experimental retention times. *Figure 3* demonstrates the overall retention time prediction error (from Table 2) versus ratio of the initial input slope used in DryLab for prediction to the extrapolated 0.3% target slope. For example, the input retention times used for simulation were obtained from the initial gradient slope of 9%; based on that, the retention times were predicted for the extrapolated 0.3% target slope, making the ratio 9% ÷ 0.3% = 30 (Figure 3). This ratio indicates the difference between the target slope and the initial slope used for prediction. Based on the data presented in Tables 2 and 3, in order to achieve better prediction accuracy, the initial pair of slopes should be close to the targeted extrapolated slope (within the range of a factor of 2) (Figure 3). Moreover, the general recommendation from the software description, that the two initial slopes should differ by no more than a factor of approx. 3, is well-supported by experimentally obtained results.

Conclusion

The optimal strategy for choosing the initial pair of gradient slopes for chromatography simulation is to have the intended gradient slope for the final separation in between the initial pair of slopes used in the simulation. When an interpolated simulation is not possible, the initial pair of slopes should be chosen close to the target extrapolated slope (within a factor of 2) and the two initial

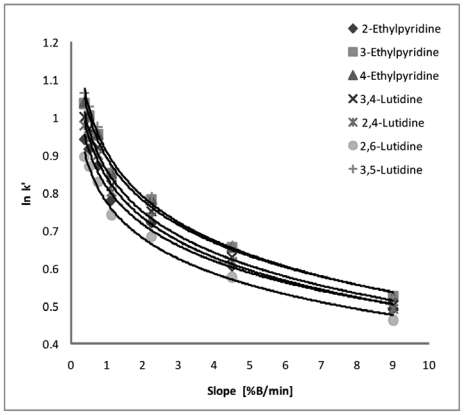

Figure 2 Pyridine and lutidine retention factor at different acetonitrile gradient slopes at constant temperature.

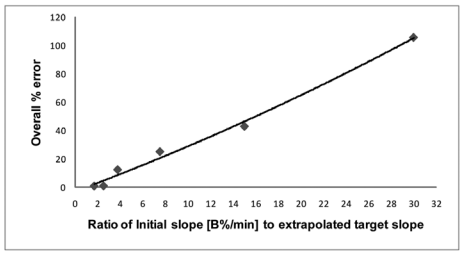

Figure 3 Overall percent-error retention time prediction for 0.3% gradient slope versus ratio of initial input slope used for prediction to extrapolated target slope of 0.3%.

slopes should differ by no more than a factor of approx. 3. The interpolated simulation as well as the extrapolated simulation results, when the initial slopes were in close range to the target slope, provided very good prediction with overall percent-error difference of experimental retention times: less than 1%. This provides the basis for further methods optimization and the development of robust stability-indicating methods.

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