Fast Method Development of Salicylic Acid Process Impurities using Agilent ZORBAX Rapid Resolution High Throughput Columns with the Agilent 1200 Series Method Development Solution Controlled by AutoChrom Version 12.01

Application Note

Pharmaceutical

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Abstract

A sample consisting of salicylic acid and its published process impurities are separated using an analytical method developed on an Agilent 1200 Series Method Development Solution controlled by ACD/AutoChrom software, version 12.01. The chromatograph while under control of AutoChrom can screen up to seven columns, 13 buffers and two organic solvents with columns held in four temperature-controlled zones. The software helps plan the next best experiment to perform, allowing the analyst to focus on quickly developing methods using conditions with the best likelihood of success. In this work three columns (Agilent ZORBAX StableBond SB-C18, ZORBAX Eclipse Plus C18 and StableBond SB-Aq) are screened using five mobile phase modifiers. Temperature is maintained at 25 °C throughout the experiment. Fifteen solvent column experiments are initially screened. Several experiments follow to construct a retention model. A solution is achieved with a final in under 3 min isocratic separation.

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Introduction

Rapid Resolution High Throughput (RRHT) columns are designed to yield separations of 150 mm, 5 µm columns with 50 mm, 1.8 µm columns. Equivalent resolution can be achieved at higher flow rates, 3 to 5 times faster. [1,2,3,4]

Analytical method development is a challenging and time consuming activity. It requires planning experiments, preparing multiple mobile phases, transcribing numerous methods into the chromatographic software and data analysis. Small changes in mobile phase composition can affect the elution order, so peak tracking throughout the method development process is also an important task. [5,6,7]

Selectivity is an important parameter in analytical method development. Using short 1.8 µm columns rapid screening of different selectivity modifiers is attractive due to the time and solvent savings that are possible. Separations that are developed on these RRHT columns can be easily transferred to a variety of other instruments with capabilities across the 400 to 1200 bar range. In general using 4.6 × 50 mm RRHT columns, many analyses may be completed in one third to one tenth of the time required with a 4.6 × 150 mm, 5 µm column. More method development options can be explored in less time.

Salicylic acid, also known as 2-hydroxybenzoic acid is one of several beta hydroxy acids. It is the key additive in many skin-care products. It is also found in many plants that are used in traditional medicine. (8,9) Sodium salicylate is commercially prepared from sodium phenoxide and carbon dioxide at high pressure and temperature in the Kolbe-Schmitt reaction. It is acidified to give the desired salicylic acid. [10] In this work RRHT columns, Autochrom and the Agilent 1200 SL Method Development Solution will be used to quickly evaluate method development choices.

Experimental

An Agilent 1200 Series Method Development Solution based on the Agilent 1200 Series Rapid Resolution LC components was used for this work. This system consisted of a G1312B Binary Pump SL, capable of delivering up to 600 bar; two G1316C Thermostatted Column Compartments (TCC), a G1376D High Performance Autosampler SL+, a G1315C SL Diode Array Detector equipped with a semi-micro flow cell with a 6-mm path length. Both TCC's are equipped with an 8-position/9-port selection valve. The valves are new QuickChange Valves that are mounted on a slide-out rail to make plumbing and maintance more convenient. Valve 1 acts as an entrance to the columns whereas valve 2 acts as an

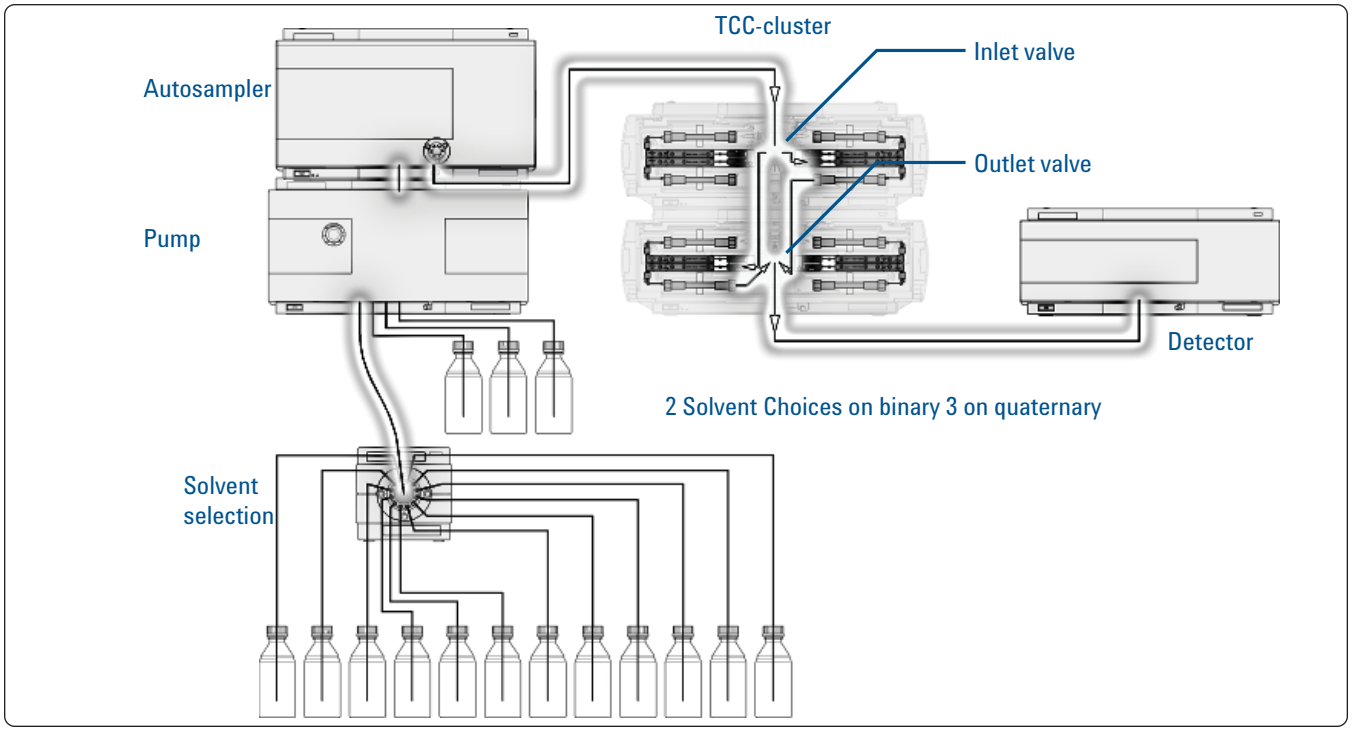

Figure 1. Instrument diagram.

exit. The center port on valve 1 was connected to the auto sampler and the center port on valve 2 was connected to the flow cell in the detector. Port 1 on both valves were connected to the StableBond C18 column, and port 2 on both valves were connected to Eclipse Plus C18. Port 7 was connected to StableBond Aq and Port 8 was connected to a bypass connecting capillary. The solvent passing into each column was heated using one of seven individual low dispersion heat exchangers. A G1160 12-solvent selection valve was connected to valve position A1 on the G1312B. Together with the internal solvent selection valve of the Binary SL Pump, up to 15 solvents can be screened using this system, although in this work it was limited to six. The following mobile phase modifiers and buffers were used: 0.1% trifluoroacetic acid (TFA), 0.1% formic acid (FA), 0.1% acetic acid, 10 mM ammonium acetate titrated to pH 4.8 with acetic acid, and 10 mM ammonium acetate titrated to pH 6.5 with acetic acid. Water was used as a final weak solvent, to rinse the modifiers from the columns, and allow proper column storage. All modifiers were purchased from Sigma Aldrich except acetic acid which was purchased from EM Science. Acetonitrile was used throughout as a strong solvent. Temperature was controlled at 25 °C, flow rate was set at 1.49 mL/min. Agilent Chemstation B0 4.01 SP1 was used to control the liquid chromatograph together with AutoChrom Version 12.01 from Advanced Chemistry Development, Inc. (Toronto, Canada).

Three Agilent columns were used in this work:

- ZORBAX RRHT StableBond SB-C18, 4.6 mm × 50 mm, 1.8 µm, p/n 822975-902
- ZORBAX RRHT Eclipse Plus C18, 4.6 mm × 50 mm, 1.8 µm, p/n 959941-902
- ZORBAX RRHT StableBond SB-Aq, 4.6 mm × 50 mm, 1.8 µm, p/n 827900-914

The following compounds were examined in this work: salicylic acid (SA), three impurities: 4-hydroxybenzoic acid (4HBA), 4-hydroxyisophthalic acid (4HIPA), phenol (PHE), and two metabolites: gentisic acid (GA), salicyiglycine (SG) were all were purchased from Sigma Aldrich, or ARCOS. Structures, and pKa values are shown in Figure 2. TFA, formic acid, acetic acid, and ammonium acetate, were also from Sigma Aldrich. Acetonitrile was purchased from Honeywell. Milli-Q 18 M-Ohm water was used.

A method development strategy is outlined in the screenshot from Autochrom in Figure 3. The plan is to screen columns

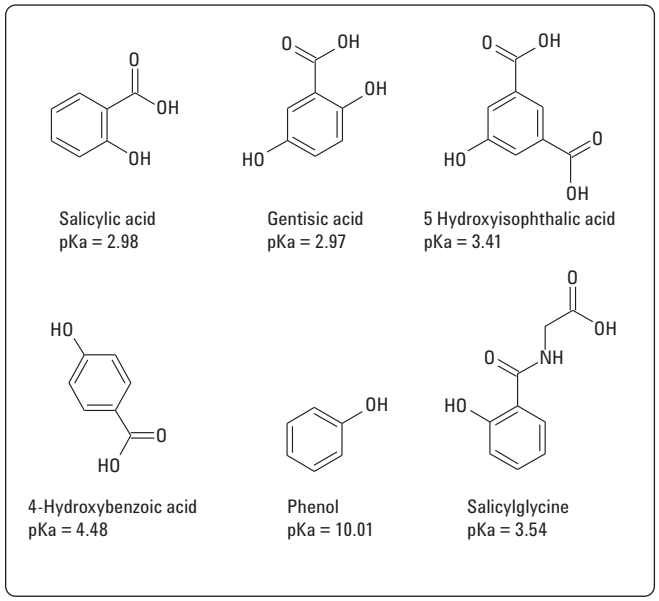

Figure 2. Structures and pKa values of the mixture components.

and buffers across the maximum operating range of each column, to select the best column and buffer combination. Then different gradients are selected for each column; 5% to 100% organic for the StableBond SB-C18, 8-100% for the Eclipse Plus C18 and 0% to 100% for the StableBond Aq. While gradients can be achieved for the two C18 columns starting at lower organic content, use of these columns at 0% organic can lead to phase collapse [11]. AutoChrom manipulates the user defined ranges for each column to build scouting gradients across each column's entire effective range.

The time allotted to each scouting run is user controlled, but a default analysis consisting of 20 column volumes across the gradient range is calculated. The calculation is based on the column dimensions, column void volume and desired flow rate. In addition to the analysis runs, columns are equilibrated and stored. A purge run is also programmed where solvents are directed through a bypass capillary, preventing incompatible solvents from damaging the columns. In short, five methods are created, transcribed, and executed for each column solvent scouting pair.

A ChemStation acquisition method found in AutoChrom is edited to achieve good chromatographic response to the mixture. In this case good response for all compounds is found at 230 nm detection wavelength. UV spectra are collected from 220 nm to 400 nm.

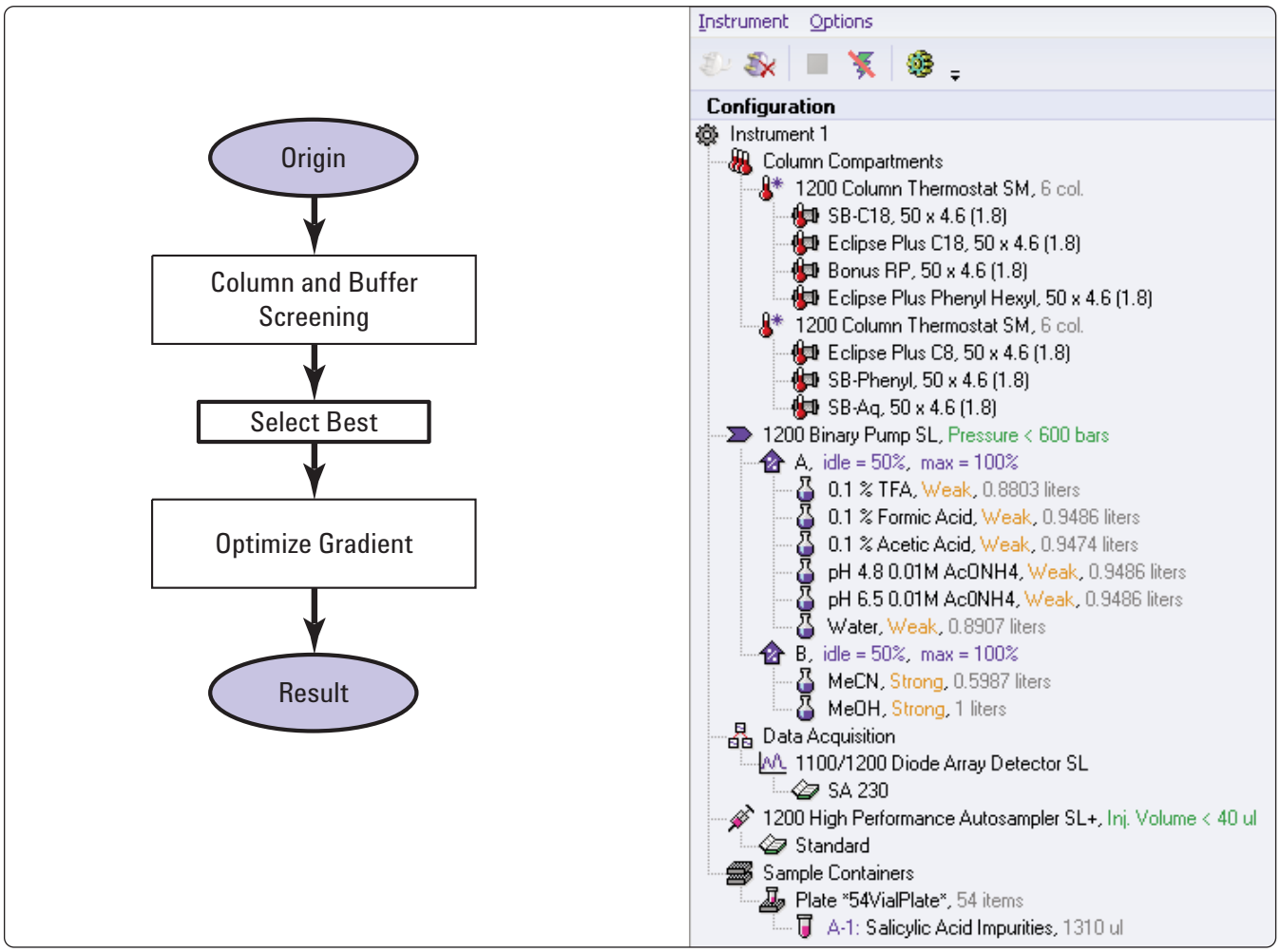

Figure 3. Autochrom strategy screenshot.

Discussion

Chromatographic peak tracking can be a time consuming step in analytical method development. With simple mixtures it is frequently accomplished by injecting individual standards of each component using each chromatographic condition. As more compounds or conditions are added this is no longer a practical methodology. The UV spectra can also be used to track components manually. Many analysts heavily involved in method development create elaborate spreadsheets containing
retention times and uv peak information.

ACD/AutoChrom combines instrument control for Agilent 1100 and 1200 Series LC, and LC/MS systems with software for logical method development. After the analyst defines the method development goal, AutoChrom generates the method files for Agilent Chemstation software, executes the experiments, guides the analyst through the data processing, and assists the analyst in selecting the next experiment.

AutoChrom will run column, buffer, temperature and solvent screening experiments, find and track all peaks in the samples, and select the best result. Peak tracking is based on UV or MS spectral similarity. (This work demonstrates UV matching only.) The UV peak-tracking utility, UV-MAP, extracts pure spectra for each component detected. The spectra are then compared across each injection. Peaks that are "best" matches are assigned first. Weaker matches are assigned later.

AutoChrom guides the analyst through method optimization. When AutoChrom's suggestion for the next experiment is accepted by the user, the software will execute the next experiment automatically. The software provides an overview of the experiments, and allows access to the original data when necessary. Experiments are summarized in a peak table.

While the Agilent 1200 Series Method Development Solution is capable of screening up to seven columns when used with AutoChrom, only three are used in this work. Since the USP method for the analysis of these compounds specifies the use

of a C18 column, StableBond SB-C18 and Eclipse Plus C18 were evaluated.[11] In previous work SB-Aq was evaluated, so it was added to the screening.[10] Further, the column is placed in position 7 on the column selection valves. This demonstrates the ability of the system to pick and choose conditions to be evaluated.

AutoChrom divides the experiments into "waves." Each wave is a planned group of experiments. In this work, two waves of experiments were executed; initial column and buffer screening, followed by gradient optimization. At the end of each wave, the software suggests the next experiment to perform, but the operator must accept the suggestion about how (or if) to continue method development. If the chromatographer does not accept the experiment suggested by the software, they may enter their own experiment to execute next. This allows the operator to control the method development process.

In the first wave all three columns were screened with each of the five solvents on the G1160 valve and then washed with water and stored in a 50:50 mixture of acetonitrile and water. The results of this initial screening are summarized in Table 1. This table lists the experiments, columns and resulting "grading" of the separation. In addition, the components are tracked by UV spectral similarity. Figure 4 shows the 15 corresponding chromatograms from these initial screenings. As can be seen in Table 1 and Figure 4, the StableBond SB-C18 and Eclipse Plus C18 using 0.1% TFA produce the best separations. AutoChrom suggests that the Eclipse Plus C18

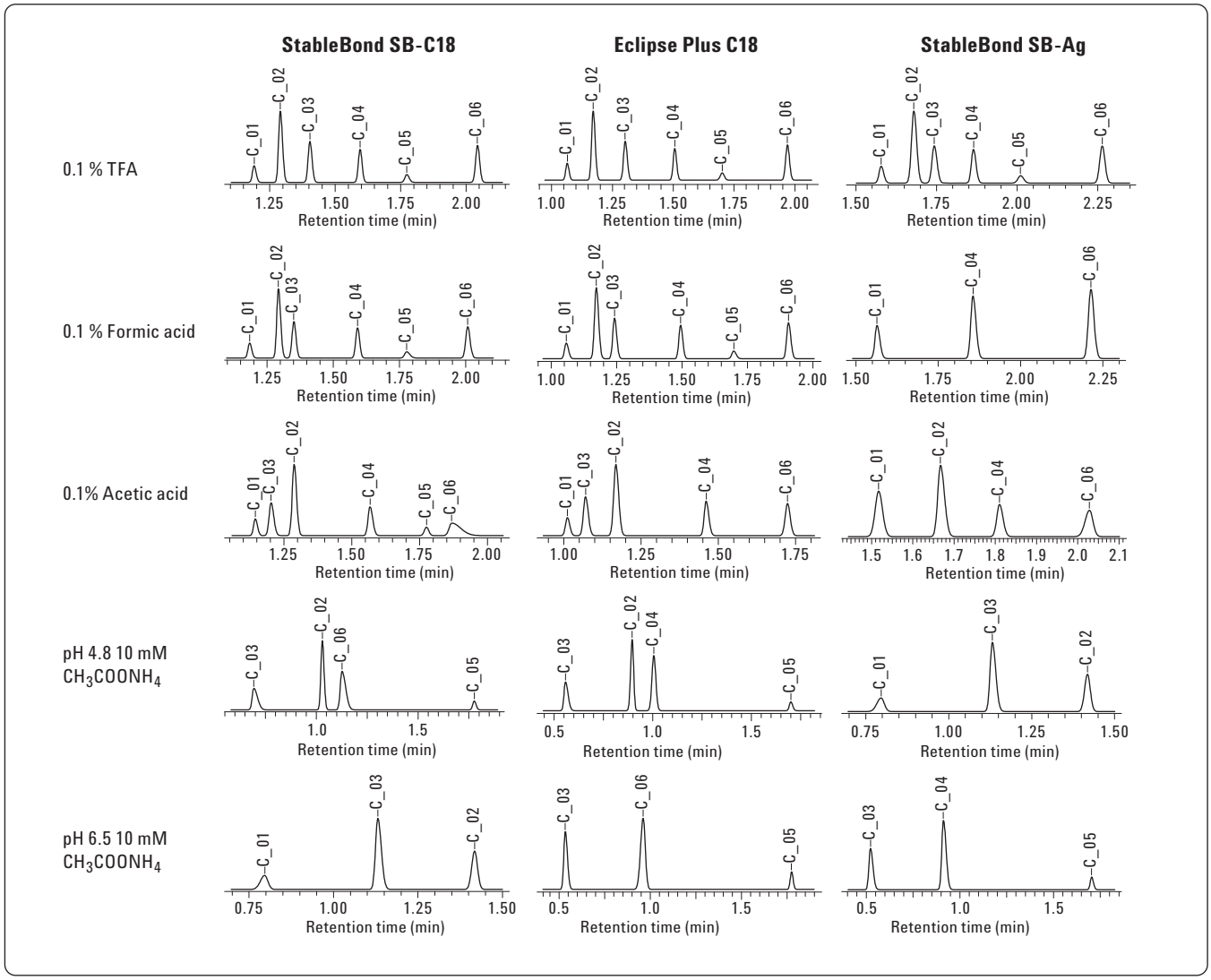

Figure 4. Fifteen Chromatograms from wave 1.

Table 1. Wave 1 Solvent and Column Screening Results

Column and Buffer	Resolution score	Minimum resolution	Peaks found/ total peaks	Retention time					
				Peak_01	Peak_02	Peak_03	Peak_04	Peak_05	Peak_06
SB-C18 and 0.1% TFA	1	3.629	6/6	1.19	1.29	1.404	1.595	1.774	2.044
Eclipse Plus C18 and 0.1% TFA	1	3.982	6/6	1.064	1.171	1.302	1.506	1.701	1.969
SB-Aq and 0.1% TFA	1	2.067	6/6	1.577	1.679	1.742	1.864	2.011	2.264
SB-C18 and 0.1% formic acid	1	1.947	6/6	1.184	1.292	1.35	1.59	1.775	2.005
Eclipse Plus C18 and 0.1% formic acid	1	2.297	6/6	1.056	1.171	1.241	1.494	1.698	1.908
SB-Aq and 0.1% formic acid	0.4	–	3/6	1.564			1.855		2.214
SB-C18 and 0.1% acetic acid	1	1.961	6/6	1.144	1.287	1.202	1.567	1.776	1.869
Eclipse Plus C18 and 0.1% acetic acid	0.8	–	5/6	1.011	1.168	1.069	1.459		1.723
SB-Aq and 0.1% acetic acid	0.6	–	4/6	1.517	1.667		1.81		2.029
SB-C18 and pH 4.8	0.6	–	4/6		1.029	0.693		1.776	1.126
Eclipse Plus C18 and pH 4.8	0.6	–	4/6		0.894	0.554	1.004	1.702	
SB-Aq and pH 4.8	0.4	–	4/6	0.796	1.418	1.131			
SB-C18 and pH 6.5	0.4	–	4/6			0.533		1.778	0.961
Eclipse Plus C18 and pH 6.5	0.4	–	4/6			0.522	0.912	1.704	
SB-Aq and pH 6.5	0.4	–	4/6			1.034	1.31	2.015	

with 0.1% TFA column and buffer combination is slightly better than the SB-C18 column, and should be used for further method development. The selection of the best experiment is based on the number of components detected, and the resolution score, which is the average value of normalized resolutions between all peaks on the chromatogram. However, there may be additional considerations when selecting the best method from the screening set.

Figure 2 lists the pKa of each analyte. As can be seen the pKa's are mostly between 2.97 and 5. Phenol can also be classified as a weak acid. Acidic compounds are best retained in mobile phases where the compounds are fully protonated. The 0.1% TFA (pH 2) and the 0.1% formic acid (pH 2.7) mobile phases fully meet this criterion. The acetic acid mobile phase (0.1% pH 3.8), show peak order changes and peak broadening, evident on the two StableBond columns (SB-C18 and SB-Aq). This is probably due to the exposed silanol groups on the non-end-capped StableBond column. Two analytes that have pKa's at or below 3 could be interacting with these groups. It is possible to use these silanol groups to the analysts' advantage by judiciously choosing mobile phase conditions to control the charge of these silanol groups. However in this case the work is performed at a pH where the silanol groups on the silica are uncharged. StableBond has been shown to possess very good long term stability in mobile phases containing TFA. [12]

The software suggests the best experiment, but the analyst must accept the decision, or choose another. After the analyst chooses the best separation based upon the chromatographic grading or other reasoning, the software will move on to the second wave of experiments for method optimization. In this case, although the Eclipse Plus C18 column is suggested by the software, StableBond SB-C18 is chosen for further experimentation. By including the analyst in the decision process, other factors such as column stability can be considered in the method development process.

The next step is gradient optimization. The resulting chromatogram from the screening run is processed by AutoChrom, which proposes two new sets of gradient conditions. These conditions are chosen to yield gradients with varied slopes in order to build a chromatographic retention model. Initial starting conditions, and earliest and latest elution compositions are used to construct these gradients.

The results of these two experiments are collected and summarized with the best first experiment. Table 2 shows the collected retention data and summarizes the experiments. Figure 5 shows the three chromatograms from this second wave. In this case no model was created but a new condition was proposed by the system. The experimental data is combined with predicted retention times, and a predicted chromatogram is shown in Figure 6.

Table 2. Gradient Optimization Results

	Retention Times					
Gradient Program	**Peak_01**	**Peak_02**	**Peak_03**	**Peak_04**	**Peak_05**	**Peak_06**
5% to100% (4 min)	1.19	1.29	1.404	1.595	1.774	2.044
17.5% to 40.1% (4 min)	0.592	0.775	0.94	1.299	1.632	2.405
5% to 40.7% (3 min)	1.479	1.592	1.772	2.15	2.315	2.91

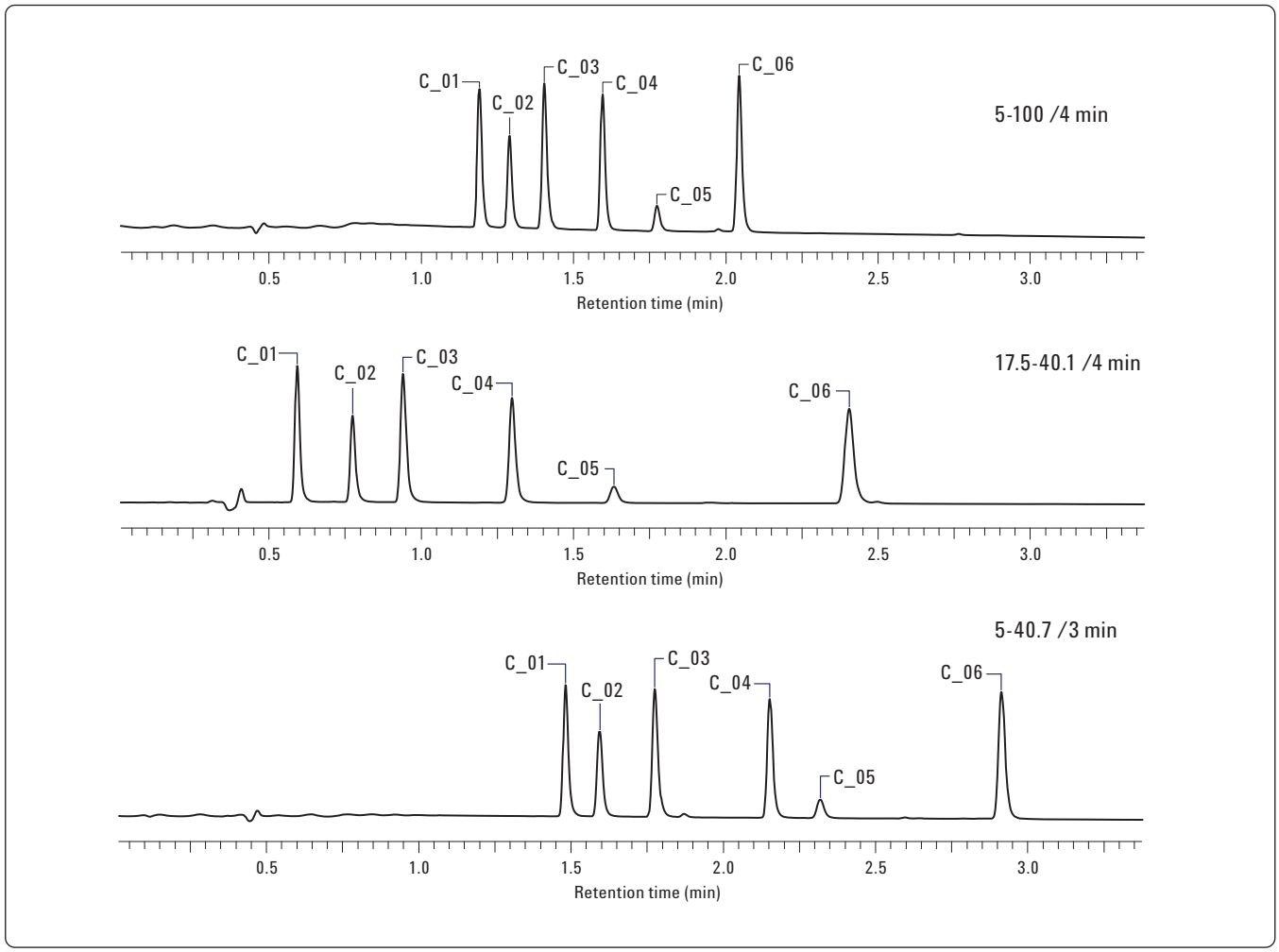

Figure 5. Experimental chromatograms from gradient optimization experiments.

	Gradient Program	Retention Times					
		Peak_01	Peak_02	Peak_03	Peak_04	Peak_05	Peak_06
Experimental	5% to 100% (4 min)	1.19	1.29	1.404	1.595	1.774	2.044
	17.5% to 40.1% (4 min)	0.592	0.775	0.94	1.299	1.632	2.405
	5% to 40.7% (3 min)	1.479	1.592	1.772	2.15	2.315	2.91
Predicted	15%	0.716	0.943	1.198	2.011	2.295	5.37

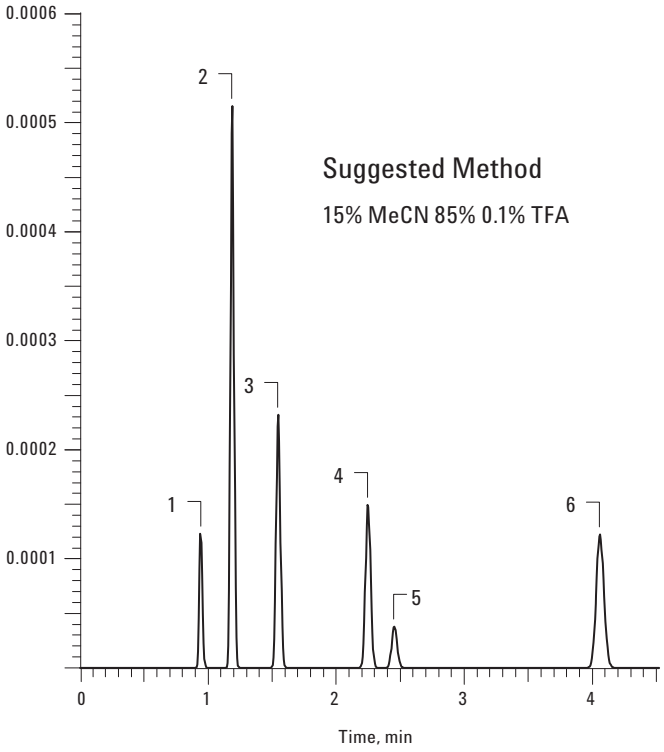

Figure 6. First three chromatograms and system suggested analysis (no model built, more data needed).

The three chromatograms from the gradient optimization experiments are then transferred into LC Simulator, a program included in AutoChrom. This program calculates an optimal separation based upon a chromatographic model, and user-defined criteria for a suitable method, which can include k', run time, resolution, robustness, and column stability criteria. LC Simulator calculates a model for the separation based on the experimental data, and then uses the model to determine optimal chromatographic conditions (targeting an isocratic solution). A resolution map is created, which allows the analyst to see how changing the gradient affects the

resolution of the critical pair. The resolution map is shown in Figure 7. If one can be found it is described graphically with the conditions transferred back to ChemStation and implemented at the analyst's direction. As can be seen in Figure 7, a simulator model is constructed. Based on this model, data conditions are chosen using the graphical interface, where a predicted chromatogram is generated. The accepted conditions are then sent directly to ChemStation. The data is collected for the experiment. The experimental chromatogram is similar to the LC Simulator predicted chromatogram, but the accuracy is not perfect, particularly for the last component. Accuracy may be improved by changing the equation used to build the model, and adding additional experimental data.

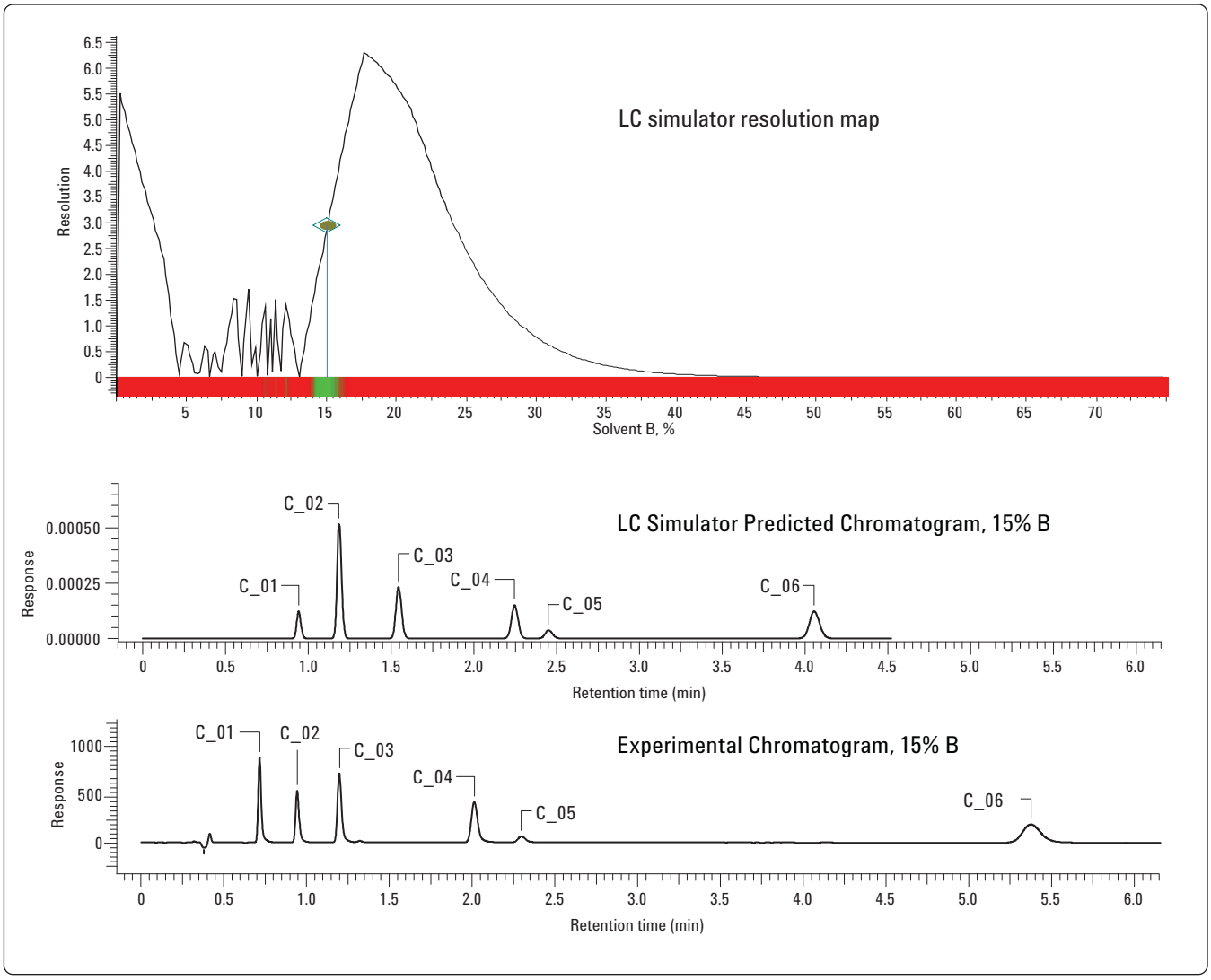

Figure 7. LC simulator resolution map and predicted chromatogram, compared with experimental chromatogram from ChemStation.

After the suggested experiment is executed, the analyst decides whether or not to continue method optimization. If the method is not acceptable, we can add the fourth experiment to our model, improving the accuracy, and allow the software to suggest the next experiment. In this case, we are satisfied with the method, so we simply stop method development at this point.

As a final step, the flow rate is optimized. Figure 8 shows four chromatograms run at different flow rates between 1.5 ml/min and 3 ml/min. As can be seen the efficiency of the method improves substantially at higher flow rates by a factor of nearly two. The optimal flow efficiency is achieved at 2.5 ml/min but increasing the flow rate to improve throughput is common. The efficiency is still 11,000.

Conclusions

With the assistance of AutoChrom, a fast isocratic method for salicylic acid and related compounds was developed in approximately 20 h. Most of this time was spent on data acquisition for screening the 15 initial conditions. Using the Agilent 1200 Series Method Development Solution, the process of column and buffer screening was accomplished overnight. The short (less than 5-min runs) led to chromatography development of an isocratic method under 3 min. AutoChrom software managed and documented all chromatographic conditions used in the development of this method. Method development time is dramatically reduced using the Agilent 1200 Series Method Development Solution with ACD/AutoChrom. Because of the unattended and automated switching of columns and solvents during the screening and optimization process, users are free to do more important tasks instead of continuously interacting with the HPLC system.

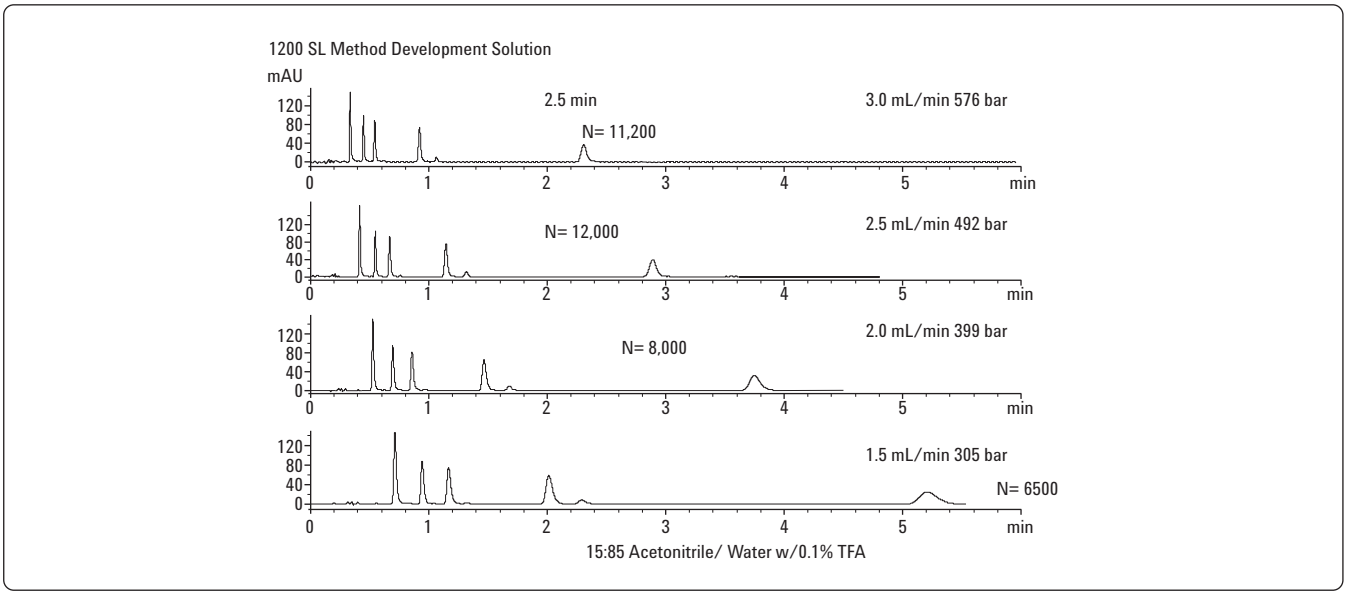

Figure 8. Four chromatograms run at different flow rates between 1.5 ml/min and 3 ml/min.

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