Rapid Method Assessment of Nonsteroidal Anti-Inflammatory Separations using Agilent ZORBAX Rapid Resolution High Throughput Columns with the Agilent 1200 Series Method Development Solution Controlled by ACD/Labs AutoChrom for ChemStation

Application Note

Pharmaceutical

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Abstract

Two samples consisting of various nonsteroidal anti-inflammatory drugs (NSAID) are separated using methods developed on an Agilent 1200 Series Method Development Solution controlled by ACD/Labs AutoChrom for ChemStation software. The chromatograph while under control of AutoChrom can screen up to seven columns, thirteen 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 four columns (Agilent ZORBAX StableBond SB-C18, Agilent ZORBAX Eclipse Plus C18, Agilent ZORBAX Bonus RP and Agilent ZORBAX Eclipse Plus Phenyl-Hexyl) are screened using five mobile phase modifiers. Twenty solvent column experiments are initially screened for each sample set.

Agilent Technologies

Introduction

Using short 1.8 µm Agilent ZORBAX Rapid Resolution High Throughput (RRHT) columns for screening of different selectivity modifiers is attractive due to the possible time and solvent savings. 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. RRHT columns are designed to yield comparable separations of 150 mm, 5 µm columns with 50 mm, 1.8 µm columns. Equivalent resolution can be achieved at higher flow rates allowing separations in one-third to one-fifth of the time. [1,2,3]

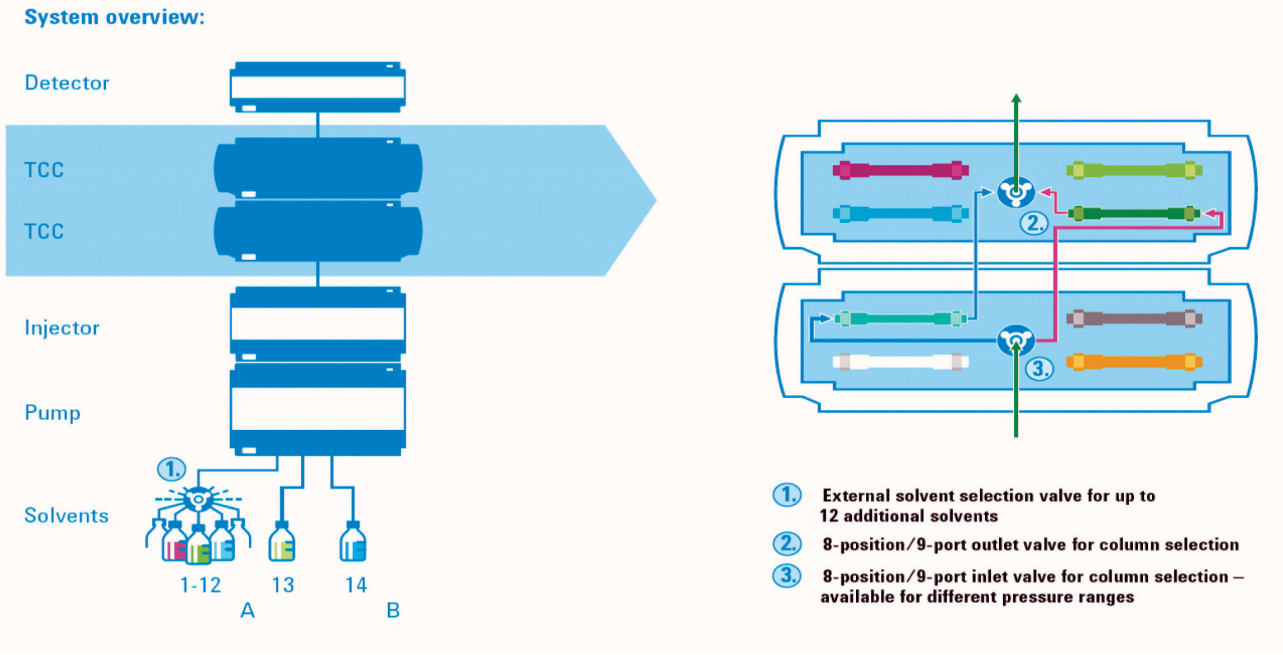
Method development involves maximizing the amount of resolution and is generally achieved by exploiting selectivity achievable through a judicious choice of mobile phase and column. This is a challenging and time-consuming activity. It requires experiment planning, multiple mobile phase preparations, method transcription into chromatographic software and data analysis. The Agilent 1200 Series LC Method Development Solution is a flexible system that can be used for up to eight columns with lengths up to 250 mm. It can be used with a sequence of different methods allowing automated column and solvent changes. [4,5] Using an Agilent 1200 Method Development Solution based on an Agilent 1200 Series RRLC module with AutoChrom for ChemStation, a method for Salicylic Acid and process impurities was developed in approximately 20 hours with minimal user intervention. [6]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most common pain relief medicines in the world. Most people are familiar with over-the-counter, nonprescription NSAIDs, such as aspirin and ibuprofen but many others are available by prescription. Every day more than millions of people use them to treat headaches, sprains, and arthritis symptoms. NSAIDs also help reduce inflammation and lower fevers. They prevent blood from clotting, which is good in some cases but not so beneficial in others. For example, because they reduce clotting action, some NSAIDS, especially aspirin, may have a protective effect against heart disease. However, you may bruise more easily. NSAIDs can increase the risk of developing nausea, an upset stomach, or an ulcer. They also may interfere with kidney function. All NSAIDs should be used with caution. All can have dangerous side effects, especially stomach ulcers and gastrointestinal bleeding. The FDA has warned that prolonged use at high doses of any NSAID may raise the risk of heart attack or stroke. NSAIDs (except low–dose aspirin) may not be appropriate for people already at risk of heart disease or stroke [7].

In this work RRHT columns, AutoChrom for ChemStation and the Agilent 1200 Method Development Solution is used to quickly evaluate method development choices. The short column length and high efficiency allow short analysis times and rapid equilibration leading to quick investigations into selectivity.

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 semimicro 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 maintenance more convenient. Valve 1 acts as an entrance to the columns whereas valve 2 acts as an 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. Ports 1 on both valves are connected to the ZORBAX StableBond C18 column, and port 2 on both valves are connected to ZORBAX Eclipse Plus C18. Port 3 is connected to ZORBAX Bonus RP, port 4 is connected to ZORBAX Eclipse Plus Phenyl-Hexyl and port 8 is connected to a bypass-connecting capillary. The solvent passing into each column is heated using one of seven individual low dispersion heat exchangers. A G1160 12-solvent selection valve is connected to valve position A1 on the G1312B. Up to 15 solvents can be screened using this system and the internal solvent selection valve of the Binary SL pump. However, this work limited the solvents 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 is used as a final weak solvent and a rinse for the modifiers from the columns to 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 2 ml/min. Agilent ChemStation B0 4.01 SP1 was used to control the liquid chromatography together with AutoChrom for ChemStation Version 12.01 from Advanced Chemistry Development, Inc. (Toronto, Canada).



The 1200 Method Development Solution LC can hold up to eight columns (but only seven when AutoChrom software is used). Automated switching between 12 buffers or two organic modifiers is typical.

Figure 1. System overview and valve diagram.

Four Agilent ZORBAX columns were used in this work:

- Agilent ZORBAX RRHT StableBond SB-C18, 4.6 mm × 50 mm, 1.8 µm, p/n 827975-902
- Agilent ZORBAX RRHT Eclipse Plus C18, 4.6 mm × 50 mm, 1.8 µm, p/n 959941-902
- Agilent ZORBAX RRHT Bonus-RP, 4.6 mm × 50 mm, 1.8 µm, p/n 827668-901
- Agilent ZORBAX RRHT Eclipse Plus Phenyl-Hexyl, 4.6 mm × 50 mm, 1.8 µm, p/n 959941-912

The columns were chosen to enhance selectivity in the separation. The columns chosen include a highly end-capped column recommended as a first choice in method development (Eclipse Plus C18), a nonend-capped C18 that will allow selective interaction with silanol groups, and imbedded amine column and a phenyl column. The Eclipse Plus C18, the Bonus RP and the Eclipse Plus Phenyl Hexyl columns are bundled together as the Rapid Resolution HT (RRHT) Selectivity Method Development Kit (p/n 5190-1433) or separately using the individual part numbers provided. StableBond C18 was chosen to provide an alternative C18 selectivity using the neutral to low pH mobile phases typically indicated for these samples. The following compounds were examined in this work. In the first group, seven common analgesic compounds were chosen. These include salicylic acid, acetyl salicylic acid, acetaminophen, caffeine; phenacetin, acetanilide and 2-acetamidophenol. They were all were purchased from Sigma Aldrich. In the second group nine analgesics: ketoprofen, celecoxib (Celebrex), sulindac, diclofenac, tolemetin, diflusinal, piroxicam, and duplicating from the first set, acetaminophen and phenacetin. These were also purchased from Sigma Aldrich with the exception of celecoxib, which was obtained using a commercial preparation. Structures are shown in Figure 1a and 1b. The pKa's of these compounds range from 2.9 to 11. TFA, formic acid, acetic acid ammonium acetate, were also from Sigma Aldrich. Acetonitrile was purchased from Honeywell. Milli-Q 18 M-Ohm water was used. All samples were prepared at 1 mg/ml in 50/50 water/acetonitrile and were combined in equal portions.

A method development strategy is outlined in the screenshot from AutoChrom in Figure 2. The plan is to screen columns and buffers across the maximum operating range of each col-

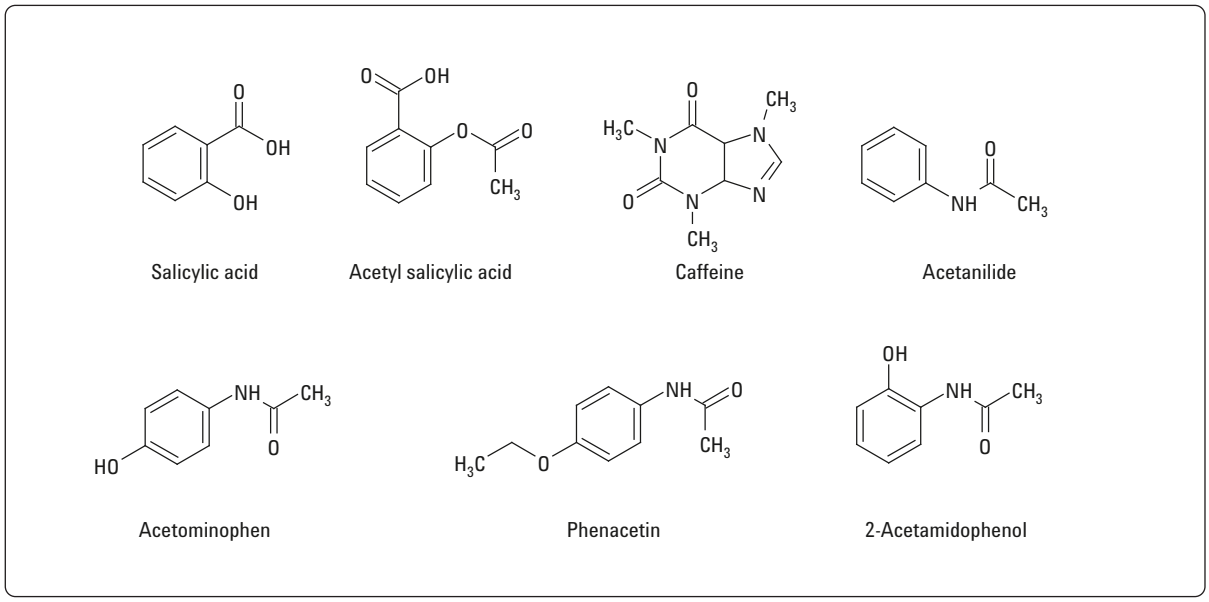


Figure 2a. NSAIDs.

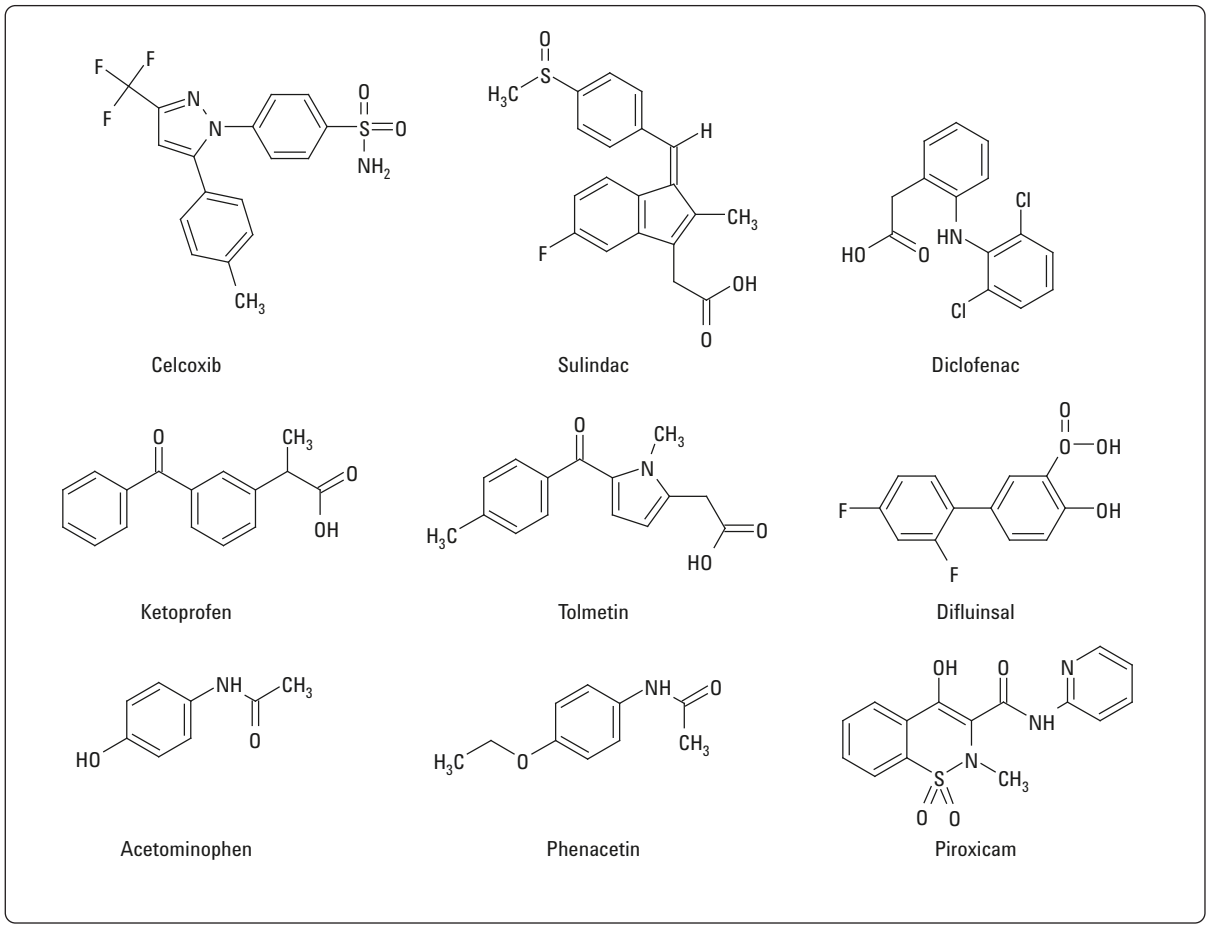


Figure 2b. Common and prescription NSAIDs.

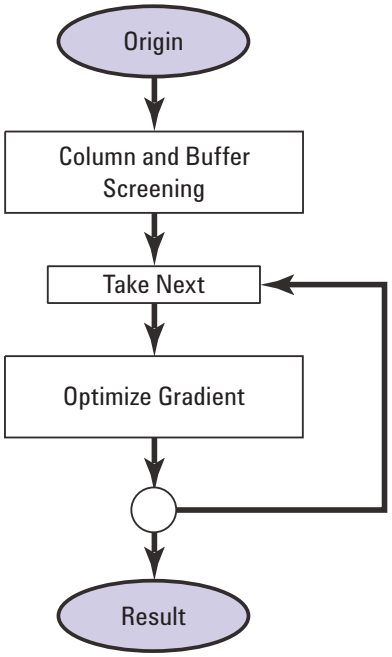
umn, select the best column and buffer combination, and then develop an isocratic separation. For the screening experiments, different gradients are selected for each column; 5 % to 100 % organic for the StableBond SB-C18, 8-100 % for the Eclipse Plus C18 and 0-100 % for the Bonus RP and the Eclipse Plus Phenyl Hexyl. 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. [8] In other cases mobile phase pH may be too high or too low for a given column; AutoChrom uses the user-defined ranges for each column to build scouting gradients across each columns' entire useful 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 summary, 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 260 nm detection wavelength. UV spectra are collected from 220 nm to 400 nm.

Discussion

One of the major obstacles in column and buffer screening is examining and reducing the data. In this case over 20 screening experiments are examined. AutoChrom acts as a method development assistant, suggesting mobile phase conditions, identifying and tracking components, and finally summarizing experiments and data. In a first step, peaks are integrated by



4 Columns

- SB-C18
- Eclipse Plus C18
- Bonus RP
- Eclipse Plus Phenyl Hexyl

6 Buffers

- 0.1% TFA (pH 1.8)
- 0.1% Formic Acid (pH 2.8)
- 0.1% Acetic Acid (pH 3.8)
- 10 mm CH_3COONH_4 (pH 4.8)
- 10 mM CH3COONH$_4$ (pH 6.5)
- Water

24 Scouting Experiments

- 2 mL/min 4.6 mm × 50 mm RRHT 25 °C
- 1200 Method Development Solution
- Acetonitrile
- 3 min scouting gradient

Method Development Scheme evaluates in manual method development; Evaluate Eclipse Plus C18 at low pH early as it has a high probability of success.

Figure 3. AutoChrom strategy screenshot.

evaluating all peaks with a minimum (and user determined) k' and a percentage of maximum peak height. All peaks integrated and identified then tracked using the compounds UV or Mass Spectra. In this case seven UV spectra are identified and will be used. Figure 3 shows the variety of UV spectra of the compounds in this work. In a separate experiment individual compounds were injected and correlated to the UV spectra.

AutoChrom "scores" the individual experiments using the method suitability requirements. A minimum k' of 1, resolution of 2, run time of less than 6 minutes. After the peaks are integrated for all screened combinations the data are available in numerical form and as chromatograms.

As can be seen in Figure 4 seven components are identified but are not resolved in all screening experiments. A failure to resolve and detect all components in the mixture will lead to a low "score". If all the peaks are found and resolved, the score will be higher. In addition, the minimum resolution between all peaks is calculated and compiled. The analyst is directed to choose the screening choice with the highest score and best resolution. However, several options can be evaluated if necessary. Only nine of the 20 chromatograms resolve all seven compounds. Low pH mobile phases are the most commonly successful buffer. TFA and formic acid yield similar separations for both the StableBond SB-C18 and the Eclipse Plus C18. Acetic acid yields a peak order change for the last three peaks. In this screening StableBond SB-C18, Eclipse Plus C18 and Eclipse Plus Phenyl-Hexyl all yield successful screenings. Table 1 summarizes the resolution scores, minimum resolution scores and retention times for the successful screenings shown in Figure 4. Based on the minimum resolution score of 3.2, the Eclipse Plus C18 /TFA pair is chosen for further development. Based on the solvent eluting the first and last peaks, two additional gradients are proposed and implemented.

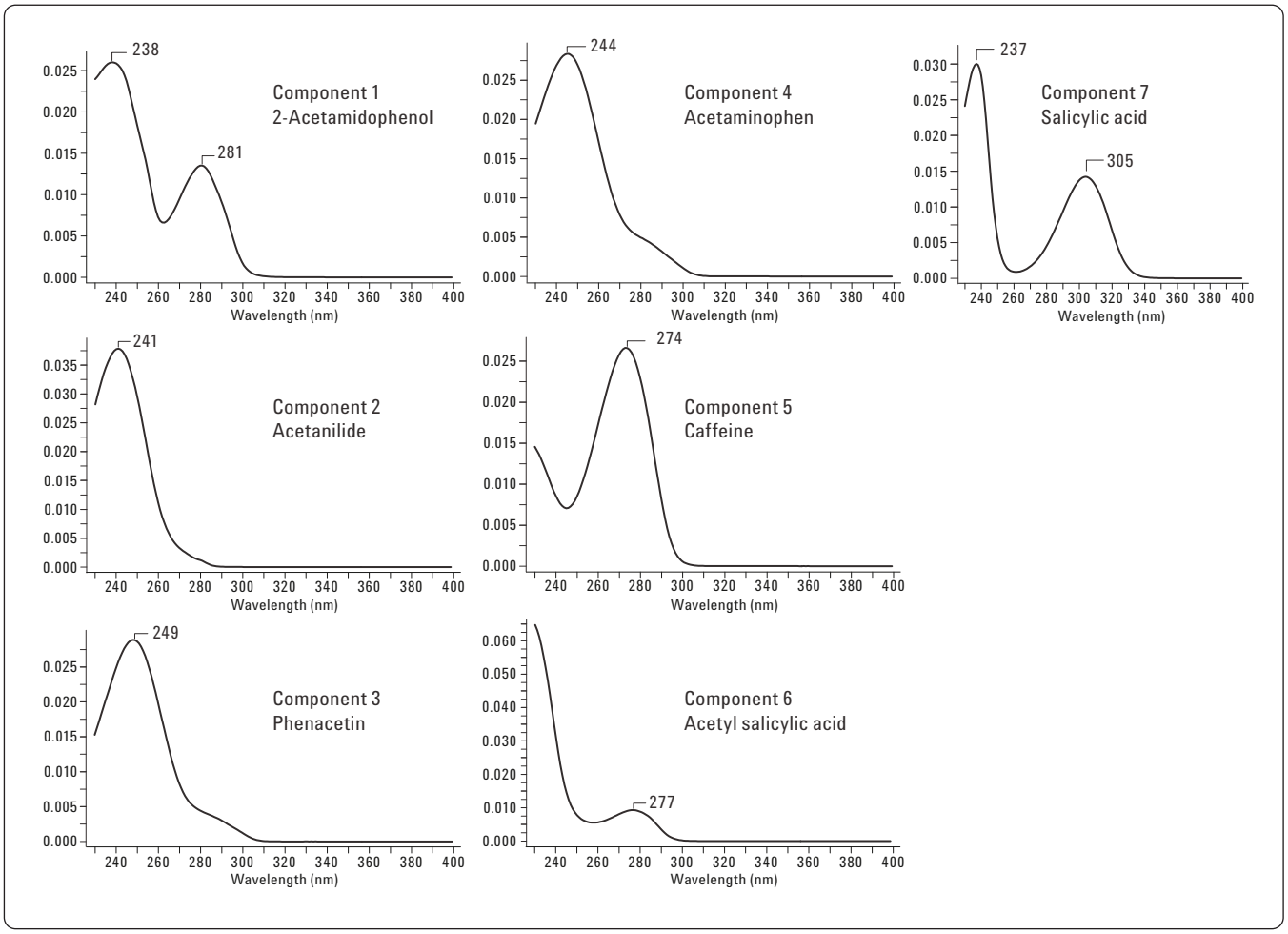


Figure 4. UV spectra of common NSAIDs.

When the proposed experiment is accepted, the conditions are seamlessly transferred to the ChemStation. Figure 5 shows the two new chromatograms produced as well as the initial screening chromatogram. Finally the chromatographic data is transferred to the optimization software within AutoChrom, and calculations are then performed. A resolution map is developed illustrating that a gradient with maximum solvent B conditions between 25 and 55 % produces a separation that meets our method suitability requirements (Figure 6). While maximum resolution can be achieved at approximately 30 % B, the steep slope in the resolution map indicates that this may not be a very robust condition to use. The software proposes a method using a gradient between 8 and 40.1 % B over 2 minutes. In this case, however, the proposed experiment has already been performed. We have already met the initially proposed analysis criterion, and have achieved separation of our seven-compound mixture in 2 minutes with a minimum resolution of 4 as shown in Figure 7.

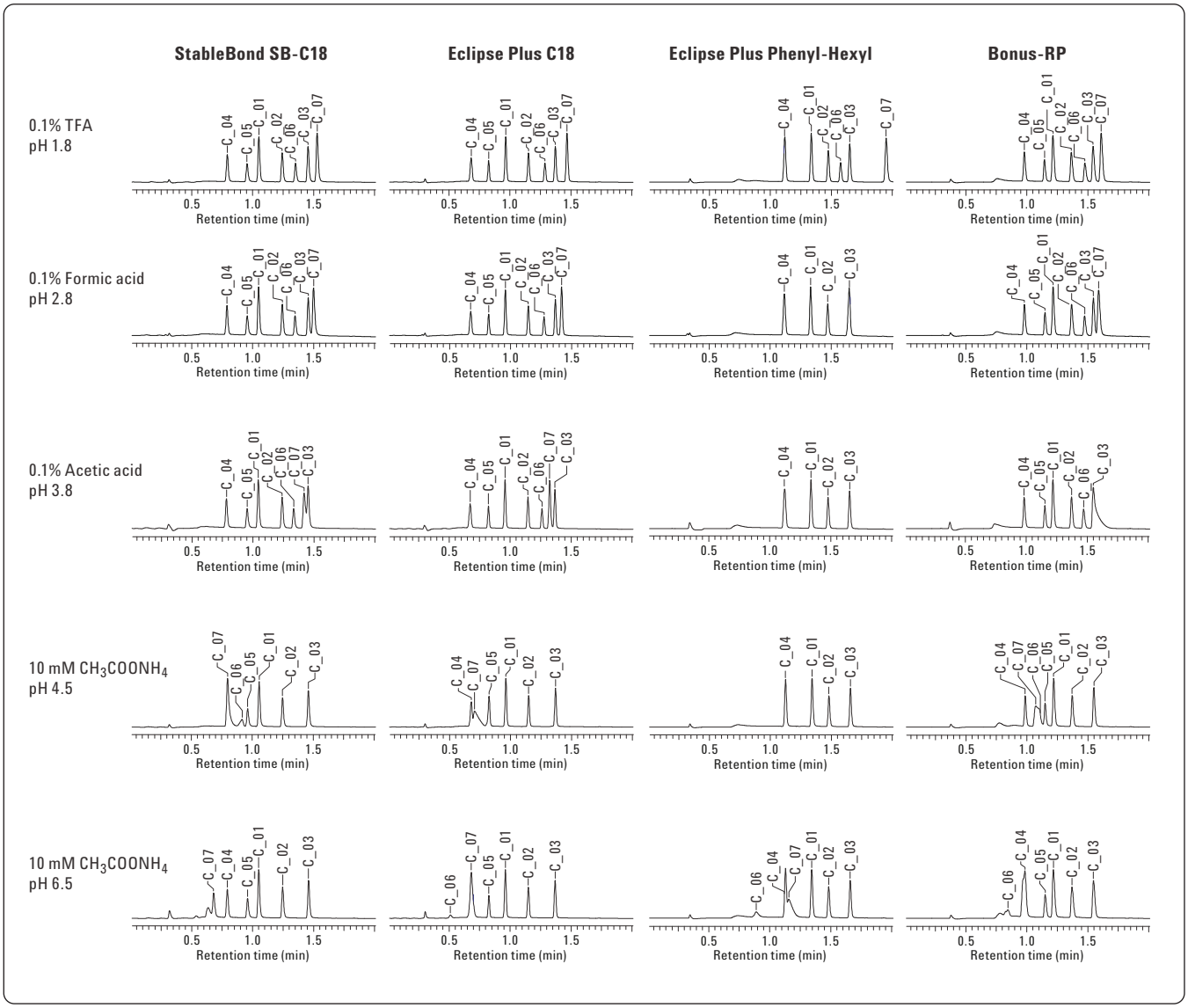


Figure 5. NSAID column and buffer screening.

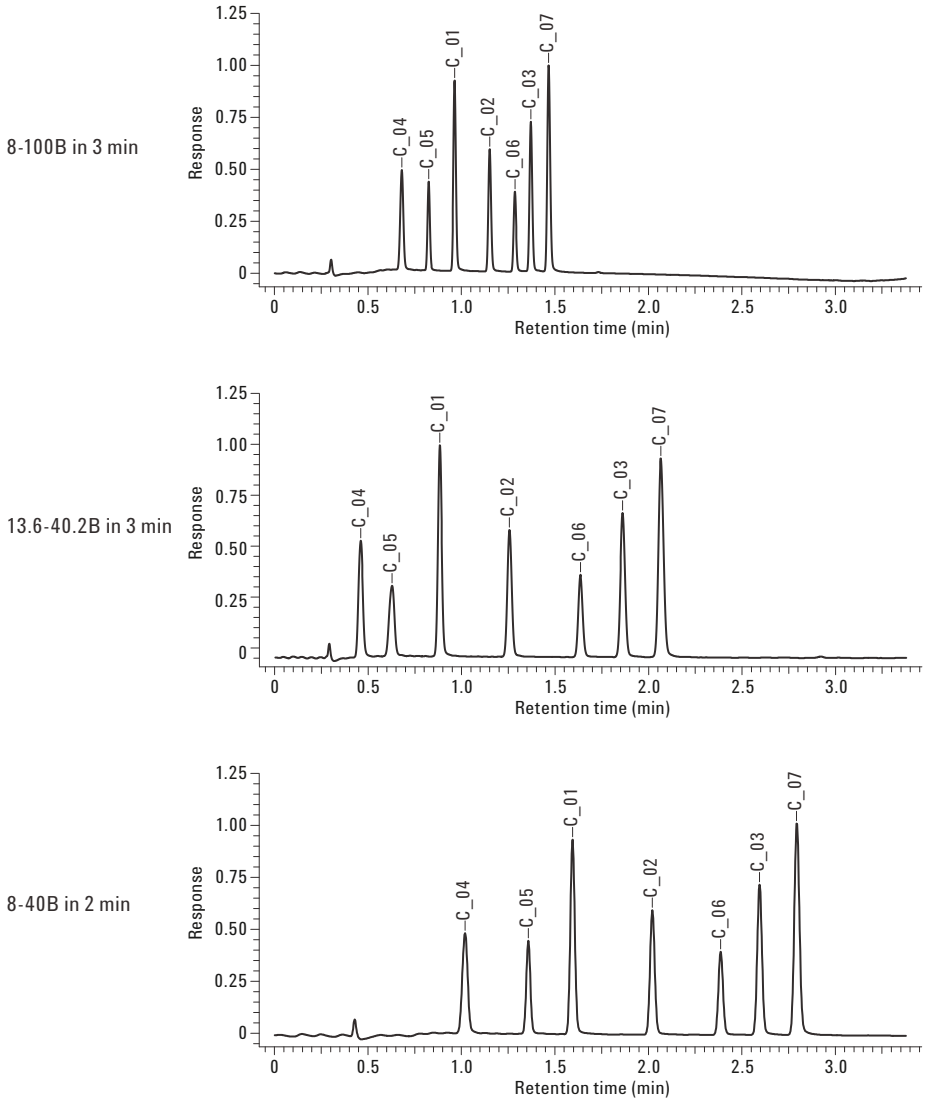
Table 1. Best Choices Column and Buffer Scouting

Experiment	Rs score	Min Rs	C_01	C_02	C_03	C_04	C_05	C_06	C_07
SB-C18 and 0.1% TFA	1	2.456	1.051	1.242	1.454	0.793	0.954	1.352	1.529
SB-C18 and 0.1% Formic Acid	0.979	1.437	1.05	1.243	1.457	0.79	0.957	1.348	1.5
SB-C18 and 0.1% Acetic Acid	0.833	0.954	1.048	1.242	1.456	0.786	0.956	1.337	1.423
Eclipse Plus C18 and 0.1% TFA	1	3.288	0.965	1.151	1.371	0.681	0.825	1.286	1.466
Eclipse Plus C18 and 0.1% Formic Acid	1	1.921	0.962	1.149	1.37	0.677	0.825	1.279	1.422
Eclipse Plus C18 and 0.1% Acetic Acid	1	1.623	0.958	1.146	1.368	0.673	0.823	1.261	1.325
Eclipse Plus Phenyl Hexyl and 0.1% TFA	1	1.846	1.214	1.364	1.543	0.979	1.144	1.476	1.609
Eclipse Plus Phenyl Hexyl and 0.1% Formic Acid	0.915	1.246	1.215	1.367	1.545	0.98	1.149	1.474	1.589
Eclipse Plus Phenyl Hexyl and ph 4.8	1	1.518	1.22	1.371	1.55	0.986	1.15	1.111	1.071

First choice	ZORBAX Eclipse Plus C18 w/0.1% TFA
Second choice	ZORBAX SB-C18 w/0.1% TFA
Third choice	ZORBAX Eclipse Plus C18 w/0.1% Formic Acid
Fourth choice	ZORBAX Eclipse Plus Phenyl-Hexyl w/0.1% TFA

The final chromatogram is shown with peaks numbered in elution order rather than by the components identified with the peak-matching algorithm.

A second set of NSAIDs shown in Figure 1b, were screened by the system. This group is more diverse than the first group and contains more compounds that require physician permission to obtain. The pKa of these compounds range from 2.9 for diflusinal to 11.1 for celecoxib. The results of the initial solvent and column screening indicate that the best results are achieved with low pH, 0.1 % TFA. In fact, interesting results were achieved on each of the screened columns and are depicted in Figure 8a. As can be seen the elution order is the same for three of the four columns with ZORBAX Bonus RP being the exemption. Peak order changes are be useful in identity confirmation or impurity analysis. Figure 8b shows the screening in 0.1 % formic acid. Excellent resolution for all compounds is still found on the ZORBAX StableBond C18 and the ZORBAX Eclipse Plus C18 columns but more compound overlap is found on the ZORBAX Bonus RP and ZORBAX Eclipse Plus Phenyl-Hexyl columns at this slightly higher pH. Since the bulk of the screening occurred, several good analysis choices can be evaluated. These results are shown in Figure 9 and Table 2. Several critical points are revealed. In most cases the original screening method gave excellent results, and all method suitability criterion were met. Retention Index (k') was longer and peak elution order was different on ZORBAX Bonus RP than other columns screened. This result was not surprising as it had been identified as a column that can be used to develop orthogonal separation to C18 columns [9]. ZORBAX Eclipse Plus C18 gave similar results to ZORBAX StableBond C18 at low pH where silanol effects would be masked. The ZORBAX Eclipse Plus Phenyl-Hexyl exhibited separation capabilities similar to the C18 columns examined. This is expected when using acetonitrile, because the π- π effects might be masked. [10] In summary several good method options are found in less than 24 hours.

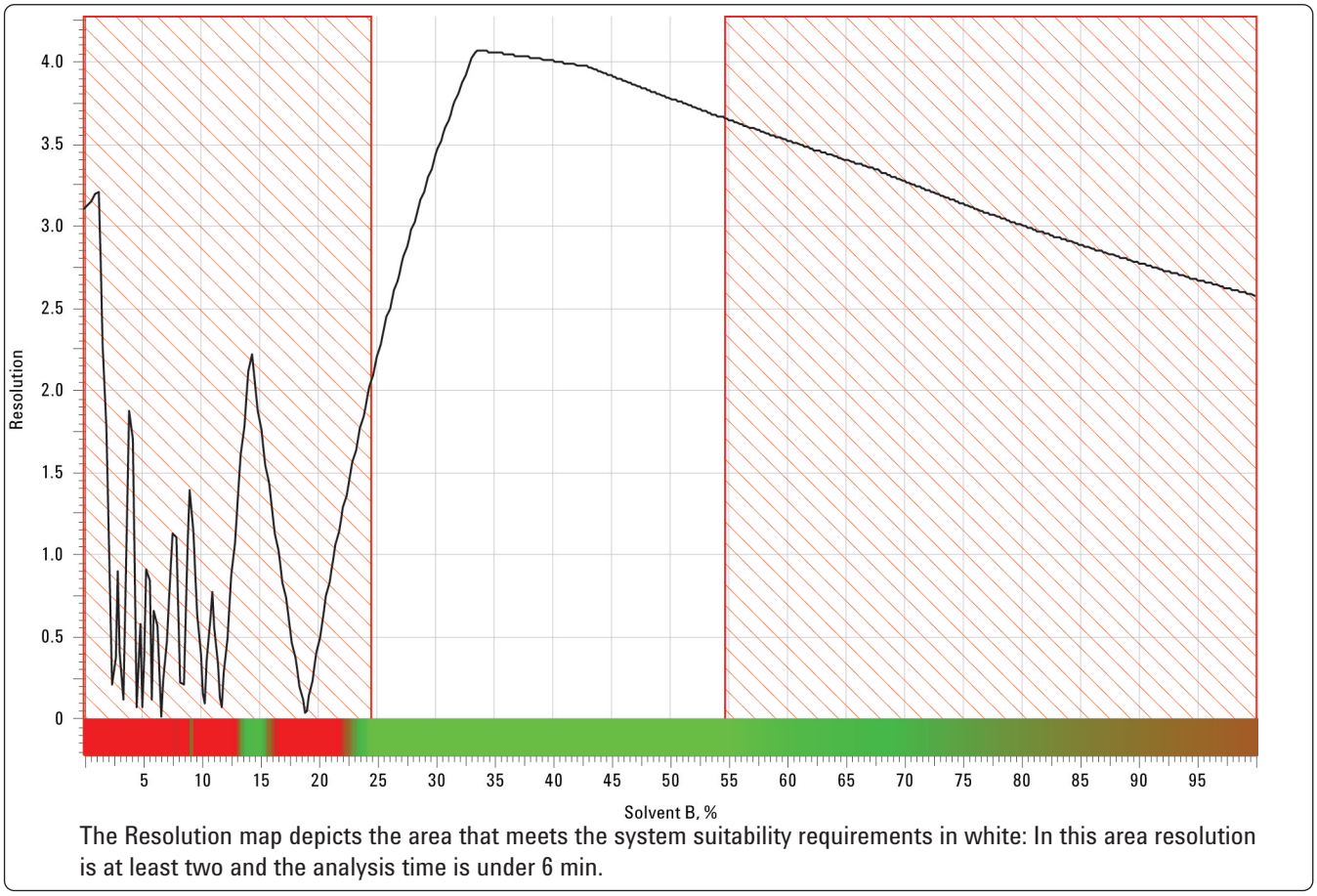


Suit.	Gradient
0.7105	8-100B (3 min)
0	13.6-40.2B (3 min)
1	8-40.08B (2 min)

2 mL/min
Water with 0.1% TFA
1200 SL Method Development Solution LC
RRHT Eclipse Plus C18
5 µL Injection
Approx 0.2 mg/mL each component

1. Acetominophen (C_04)
2. Caffeine (C_05)
3. 2-Acetamidophenol (C_01)
4. Acetanalide (C_02)
5. Acetyl Salicylic Acid (C_06)
6. Phenacetin (C_03)
7. Salicylic Acid (C_07)

Figure 6. Optimization of Eclipse Plus C18 for seven common NSAIDs.



The Resolution map depicts the area that meets the system suitability requirements in white: In this area resolution is at least two and the analysis time is under 6 min.

Figure 7. Resolution map for Eclipse Plus C18 separation of common NSAIDs.

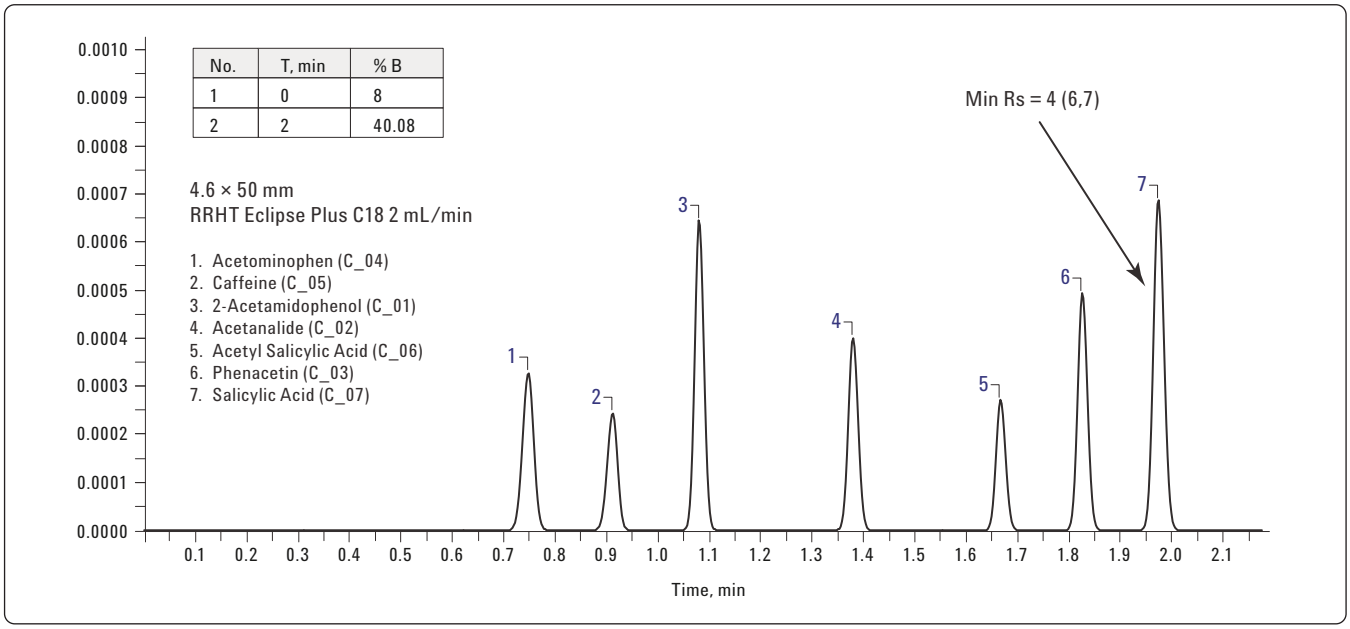


Figure 8. Final resulting separation of common NSAIDs on Eclipse Plus C18.

Conclusions

With the assistance of AutoChrom, a 2-min gradient method for the seven-component analgesic mixture was developed in approximately 20 hours. Most of this time however was spent in data acquisition for screening the 20 initial conditions. The separation of the nine-component analgesic mixture was carried out using similar methodology. Determining optimized separations between 2.5 and 3.5 minutes for each of the four columns took approximately three additional hours. Using the Agilent 1200 Series Method Development Solution, the process of column and buffer screening was accomplished overnight. AutoChrom software managed and documented all chromatographic conditions used in the development of this method. Method development time was 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 can do other tasks instead of continuously interacting with the HPLC system. The importance of selectivity in method development, whether generated by choice of column or mobile phase additive (pH), is proven to be critical.

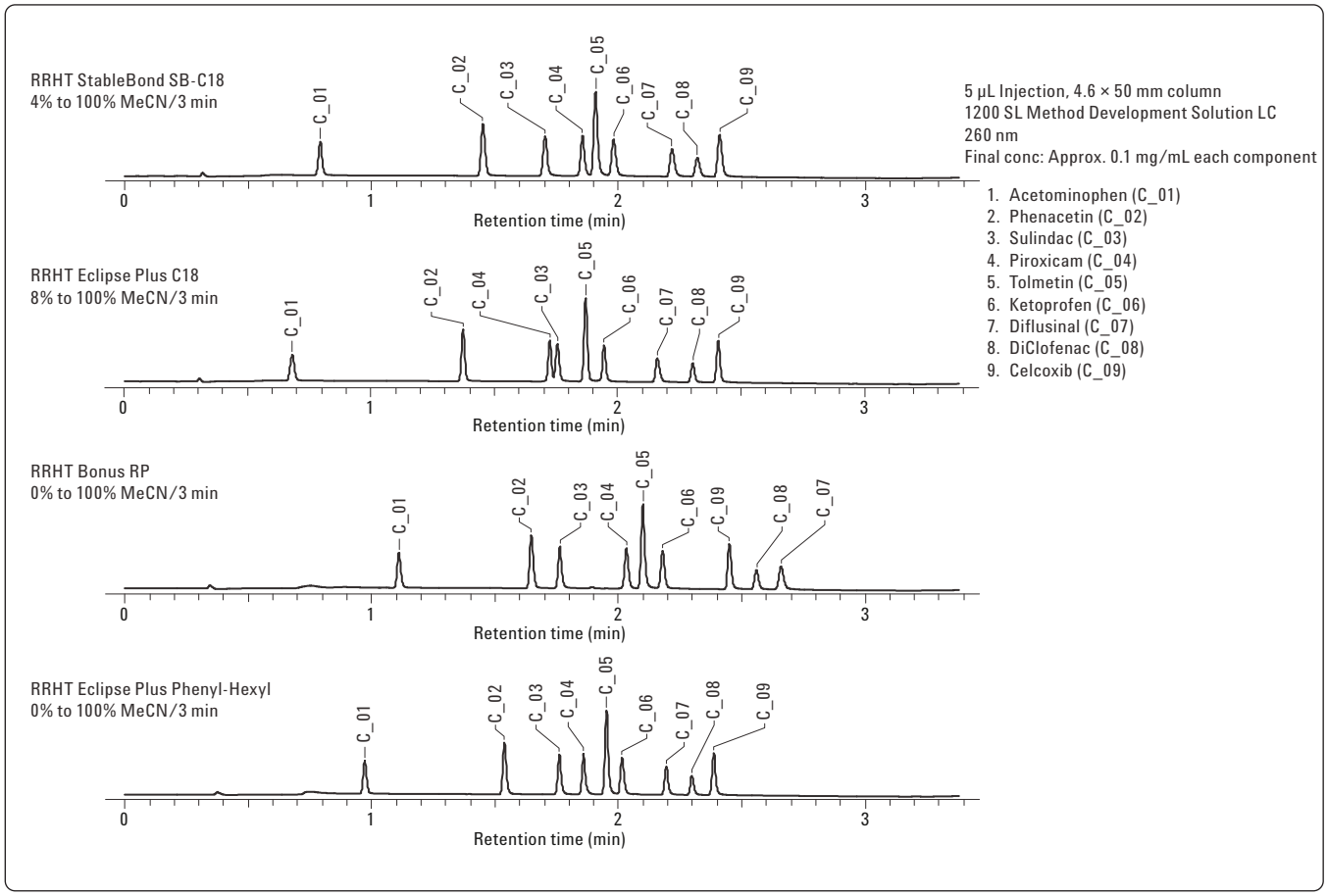
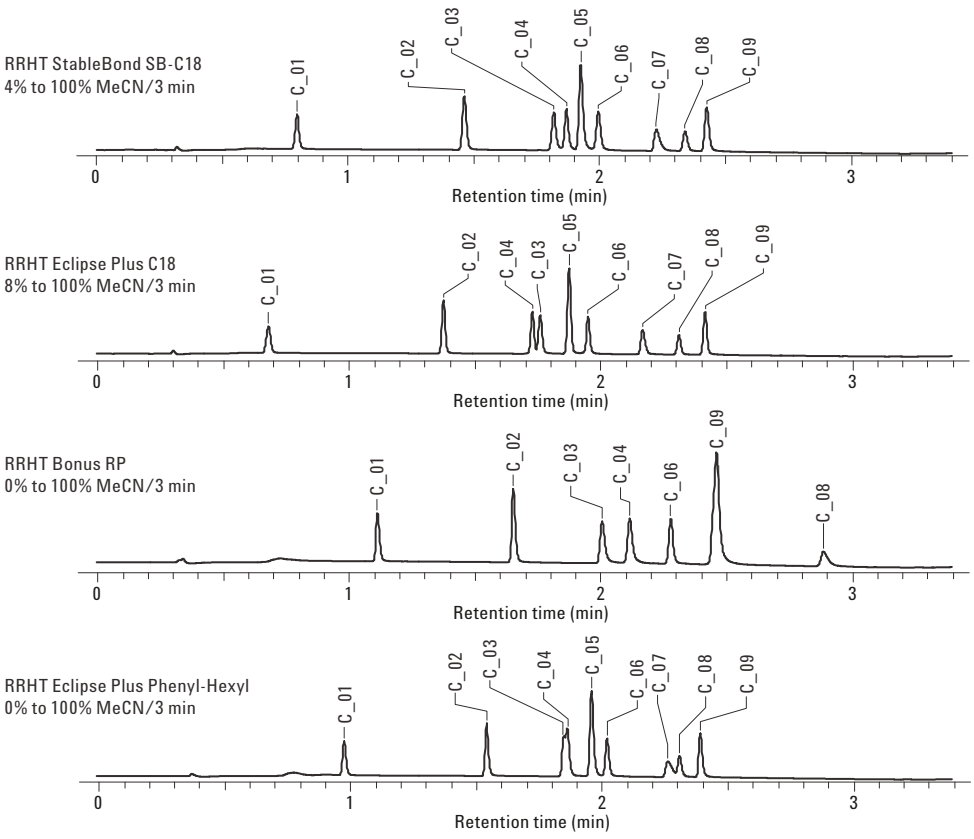


Figure 9a. Column screening 0.1% TFA in water.

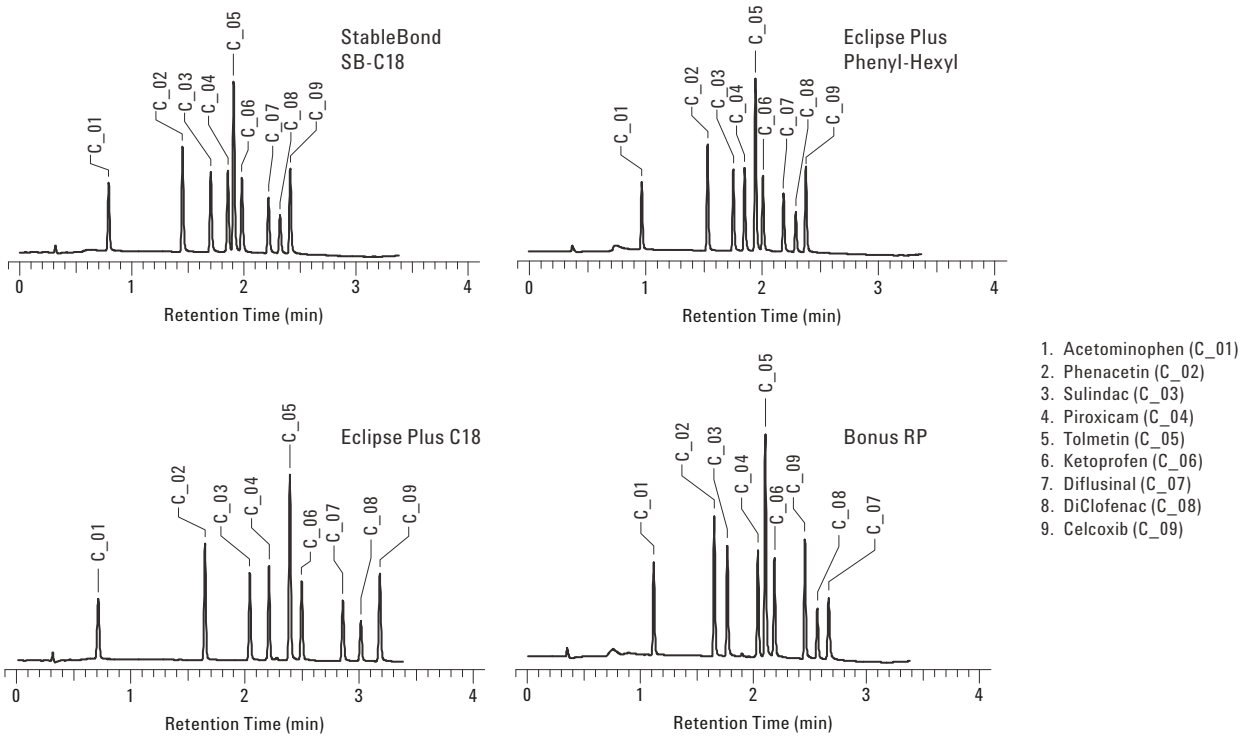


5 µL Injection, 4.6 × 50 mm column
1200 SL Method Development Solution LC
260 nm
Final conc. Approx. 0.1 mg/mL
each component

1. Acetominophen (C_01)
2. Phenacetin (C_02)
3. Sulindac (C_03)
4. Piroxicam (C_04)
5. Tolmetin (C_05)
6. Ketoprofen (C_06)
7. Diflusinal (C_07)
8. DiClofenac (C_08)
9. Celcoxib (C_09)

Best separations for all 4 column are found using 0.1% TFA, however a peak order change is found when using the Bonus RP column (last 3 peaks) Formic Acid gives good results for Eclipse Plus C18 and StableBond SB-C18.

Figure 9b. Column screening 0.1 % formic acid in water.



Several good separation options are found although the Agilent ZORBAX Eclipse Plus C18 gives the highest resolution.

Figure 10. Optimization with different phases.

Table 2. Summary Gradient Final Analysis Conditions

Column	Gradient (% MeCN)	Solvent used (mL)	Minimum resolution	Minimum k'	Run time (min)
StableBond SB-C18	4–100	4.91	2.01 (peaks 4, 5)	1.16	2.45
Eclipse Plus C18	8–70	6.44	3.42 (peaks 5, 6)	1.08	3.22
Eclipse Plus Phenyl-Hexyl	0–100	4.87	2.38 (peaks 5, 6)	1.62	2.43
Bonus RP	0–-100	5.39	2.47 (peaks 4, 5)	2.02	2.70

All columns above are RRHT, 4.6 x 50 mm, 1.8 µm. Solvents used are water with 0.1% TFA and acetonitrile.

References

1. A. D. Broske, R.D. Ricker, B.J. Permar, W. Chen, and M. Joseph, "The Influence of Sub-Two Micron Particles on HPLC Performance", 5989-9251EN, May 2003.
2. W. J. Long and J. W. Henderson Jr., "High-Resolution Analysis of Taxanes Using Rapid Resolution HT (1.8 µm) Agilent Eclipse Plus Phenyl-Hexyl Columns", 5989-9340EN, August 28, 2008.
3. J. W. Henderson Jr. and W. J. Long, "Exploiting RRHT Columns with Different C18 Selectivities to Quickly Develop Methods for Endocannabinoids", 5989-6128EN, January 19, 2007.
4. L. R. Snyder, J. J. Kirkland, J. L. Glajch, "Practical HPLC Method Development", 2nd ED. Wiley-Interscience, New York, 1997.
5. "Agilent 1200 Series LC Method Development Solution-Automated method development with up to eight columns". Technical Note, 5989-9338EN, October 1, 2008.
6. W. Long, M. Antler, A. Vazhentsev, "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", 5990-4809EN, October 17, 2009 .
7. http://en.wikipedia.org/wiki/Nonsteroidal_anti-inflammatory_drug.
8. B.A. Bidlingmeyer, A.D. Broske, "The role of pore size and stationary phase composition in preventing aqueous-induced retention time loss in reversed-phase HPLC". *J. Chromatogr. Sci.* 2004 Feb; 42 (2):100-6.
9. J. W. Dolan, L. R. Snyder, "Selecting an "orthogonal" column during high-performance liquid chromatographic method development for samples that may contain non-ionized solutes", *Journal of Chromatography A*, 1216 (2009) 3467–3472.
10. William J. Long and Anne E. Mack, "Comparison of Selectivity Differences Among Different Agilent ZORBAX Phenyl Columns using Acetonitrile or Methanol", 5990-4711EN, October 6, 2009.

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Printed in the USA
May 20, 2010
5990-4976EN

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