Agilent LC Method Development Applications Notebook

CREATING CONSISTENT, EFFICIENT, AND COST-EFFECTIVE ANALYTICAL LC METHODS

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OPTIMIZED LC WORKFLOWS MAXIMIZE METHOD DEVELOPMENT EFFICIENCY

Today's method development demands flexible, highly efficient initial development, screening, and method transfer across HPLC and UHPLC systems.

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- Screen hundreds of chromatographic conditions automatically—on a single system
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EC-C18	SB-C18	HPH-C18	Bonus-RP	SB-Aq
EC-C8	SB-C8	HPH-C8	PFP	EC-CN
Phenyl-Hexyl				HILIC

Use this general protocol to develop fast, robust LC methods

1. Test initial conditions: 10-95% gradient with ACN plus 0.1% formic acid (low pH) at 30 °C.
2. Adjust slope (% organic over time) to incorporate the first and last peak. Optimize for analytes of interest.
3. If ACN gives poor peak shape or retention, change the organic modifier to MeOH.
4. Adjust column temperature (up to allowable limits).
5. Try alternate column chemistries to vary retention and selectivity.

For help with choosing different selectivities, visit **www.hplccolumns.org**

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GENERAL ANALYTICAL METHOD DEVELOPMENT—PRINCIPLES

Instrument, Method, and Sample Optimizations to Get the Most from Agilent InfinityLab Poroshell 120, 1.9 µm Columns

Application Note

General Analysis

Author

Anne Mack
Agilent Technologies, Inc.

Abstract

Six compounds were analyzed through isocratic elution to demonstrate the effect of several instrument, method, and sample variables on column performance. A very efficient Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm column capable of generating nearly 14,000 plates was used in this work. The impact on column performance of system capillaries, detector flow cells, data collection rates, injection volumes, sample solvents, and sample concentrations was studied.

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Introduction

Superficially porous particle LC columns are a popular tool in liquid chromatography. These columns generate high efficiency at lower pressure compared to their totally porous particle column counterparts [1]. This is primarily due to a shorter mass transfer distance and substantially narrower particle size distribution of the particles in the column [2]. The current trend with superficially porous particles is to reduce particle size for further efficiency improvements. The higher efficiency can be used to speed up analysis, or improve results by increasing resolution and sensitivity.

Small-dimension LC columns packed with small particles deliver increased productivity with faster analyses or greater resolution, reduced solvent usage, and better LC/MS and ELSD compatibility, compared to larger bore columns with 4.6 or 3 mm internal diameters, which require faster flow rates for equivalent linear velocities. Simply swapping a larger id column for a smaller one can yield these benefits. However, to take full advantage of small dimension columns, the LC instrument, method, and sample must all be optimized.

Previous work shows the effect of extra-column volume on a variety of column dimensions and particle sizes. Extra-column volume is simplified in this experiment because the only variables are the diameter and length of the connecting capillary between the autosampler and column. We show that the effect of extra-column volume is dependent on column dimension, but is not dependent on particle size.

However, the impact of extra-column volume will be more noticeable with small particle columns. For a 2.1 × 50 mm, 1.8 µm column, efficiency begins to decrease with as little as 2 µL of additional volume. In addition, we determined that larger 4.6 mm id columns are not significantly affected by extra volume ranging from 1.2 to 9.1 µL. Further work shows that a 5 µm column exhibits similar decreases in efficiency compared to a same-dimension 1.8 µm column, when data are normalized to account for percent efficiency decrease as a function of additional system volume [3].

Finally, the impact of reducing the LC system volume with small-dimension totally porous sub-2 µm particle columns is demonstrated [4-5]. This work includes optimizing the LC instrument, method, and sample for use with a highly efficient superficially porous Agilent InfinityLab Poroshell 120 EC-C18, 1.9 µm column.

Experimental

An Agilent 1290 Infinity LC System was used in this experiment. Table 1 shows the configuration details. For some experiments, the instrument was modified from this configuration. This information is provided as necessary and appropriate throughout this work. One Agilent LC column was also used in this experiment, and is listed in Table 1.

The LC method parameters for most analyses are shown in Table 2. In several experiments performed throughout this work, one parameter was varied to demonstrate the effect on chromatography; the data are labeled to indicate where modifications were made.

Table 1. UHPLC System Configuration

Agilent 1290 Infinity LC System Configuration	
Agilent 1290 Infinity Binary Pump (G4220A)	35 µL Solvent mixer: Jet weaver, 35 µL/100 µL (G4220-60006)
Agilent 1290 Infinity High Performance Autosampler (G4226A)	Seat assembly, ultra low dispersion, for Agilent 1290 Infinity Autosampler (G4226-87030) Autosampler → Heater: Capillary, stainless steel, 0.075 × 220 mm, SV/SLV (5067-4784) Vial, screw top, amber with write-on spot, certified, 2 mL, 100/pk (5182-0716) Cap, screw, blue, PTFE/red silicone septa, 100/pk (5182-0717) Vial insert, 250 µL, glass with polymer feet, 100/pk (5181-1270)
Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)	Heat exchanger, low dispersion, 1.6 µL, double (G1316-60005) Heater → Column: Agilent InfinityLab quick-connect assembly, 105 mm, 0.075 mm (5067-5961) Column → Flow cell: Capillary, stainless steel, 0.075 × 220 mm, SV/SLV (5067-4784)
Agilent 1290 Infinity Diode Array Detector (G4212A)	Agilent Ultra-Low Dispersion Max-Light Cartridge Flow Cell, 10 mm (G4212-60038)
Agilent OpenLAB CDS ChemStation Edition Revision C.01.05 [35]	G4220A: B.06.53 [0013] G4226A: A.06.50 [003] G1316C: A.06.53 [002] G4212A: B.06.53 [0013]
Agilent LC Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm (699675-902)

All analyses used instrument configuration shown above, unless stated otherwise.

Table 2. UHPLC Method Parameters

Column	Mobile phase	Flow rate (mL/min)	Elution	Injection volume (µL)	Sample	Thermostated Column Compartment (°C)	Diode Array Detector
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm	20 mM sodium phosphate pH 7 in water with acetonitrile premixed 40/60	0.5	Isocratic	0.5	Uracil, butyl paraben, amitriptyline, naphthalene, dipropyl phthalate, acenaphthene see Table 3 for more information	25	254 nm, 80 Hz

All analyses used method parameters shown above, unless stated otherwise.

Six compounds were analyzed in this work, shown in Table 3. The test standard was prepared according to the concentrations in Table 3, unless stated otherwise. All analytes were purchased from Sigma-Aldrich. Sodium phosphate was also purchased from Sigma-Aldrich. Acetonitrile was purchased from Honeywell (Burdick and Jackson). Water was 0.2 µm filtered 18 MW from a Milli-Q system (Millipore).

Table 3. Sample Information

Compound	Classification	Concentration in mobile phase	Retention factor (k')
Uracil	Void marker	n/a	n/a
Butyl paraben	Weak acid	0.05 mg/mL	1.3
Amitriptyline	Base	0.25 mg/mL	2.2
Naphthalene	Neutral	0.25 mg/mL	3.3
Dipropyl phthalate	Polar neutral	0.5 mg/mL	3.8
Acenaphthene	Neutral	0.5 mg/mL	6.1

All analyses used the sample shown above, unless stated otherwise.

Results and Discussion

All optimization experiments were done with the same standard, method, and system, except where noted otherwise. For each experiment, one variable was changed at a time to demonstrate the effect of that parameter on the efficiency of an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm column. For each experiment, efficiencies for butylparaben (k′ = 1.3), naphthalene (k′ = 3.3), and acenaphthene (k′ = 6.1) are illustrated to show the impact on performance of an early, mid, and late-eluting compound.

Instrument optimizations

Low-volume instrument configurations should be used with small-volume columns. The system volume of an Agilent 1290 Infinity LC was optimized in two steps. First, standard red 0.12 mm id capillaries were replaced with black 0.08 mm id capillaries. Second, the standard 1.0 µL flow cell was replaced with a 0.6 µL flow cell. Table 4 shows the details regarding the differences in system configuration. Figure 1 shows the results. In Figure 1A, there is a small shift in retention time observed for all peaks when the capillary volume is reduced. When the flow cell volume was reduced, all peaks became taller, narrower, and more efficient. Efficiency values are plotted in Figure 1B. System volume has a large impact on the efficiency of early eluting compounds. Both the capillaries and the flow cell substantially contribute to the LC system's volume. Therefore, both can individually impact the performance of a small-volume, high-efficiency column such as the InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm.

Subsequent experiments in this work used the smaller LC system volume configuration in Table 4.

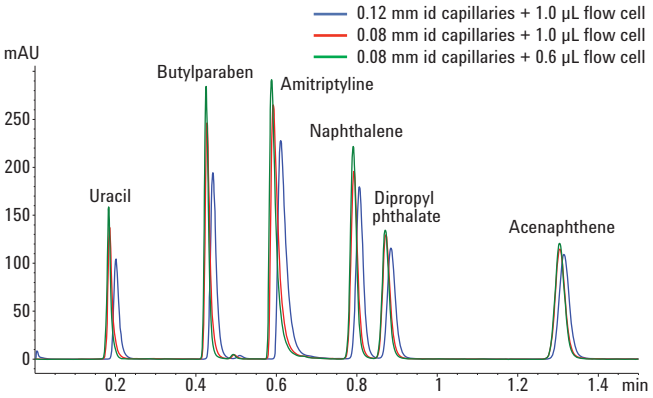

Figure 1A. The performance of an Agilent InfinityLab Poroshell 1.9 µm column is improved when LC system volume is reduced by using smaller internal diameter capillaries and a smaller volume detector flow cell.

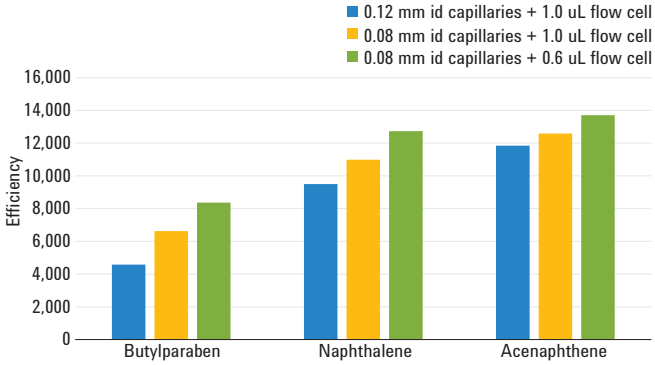

Figure 1B. The performance of an Agilent InfinityLab Poroshell 1.9 µm column is improved when LC system volume is reduced by using smaller internal diameter capillaries and a smaller volume detector flow cell.

Table 4. Instrument Modifications for Capillary and Flow Cell Comparisons

Agilent 1290 Infinity LC system configuration modifications	Larger system volume	Smaller system volume
Agilent 1290 Infinity High Performance Autosampler (G4226A)	Autosampler → Heater: Capillary, stainless steel, 0.12 × 340 mm (5067-4659)	Autosampler → Heater: Capillary, stainless steel, 0.075 × 220 mm, SV/SLV (5067-4784)
Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)	Heat exchanger, low dispersion, 1.6 µL, double (G1316-60005) Heater → Column: Agilent InfinityLab quick-connect assembly, 105 mm, 0.075 mm (5067-5961) Column → Flow cell: Capillary, stainless steel, 0.12 × 340 mm (5067-4659)	Heat exchanger, low dispersion, 1.0 µL, long, down (G1316-80012) Heater → Column: Agilent InfinityLab quick-turn fitting (5067--5966) Column → Flow Cell: Capillary, stainless steel, 0.075 × 220 mm, SV/SLV (5067-4784)
Agilent 1290 Infinity Diode Array Detector (G4212A)	Agilent Standard Max-Light Cartridge Flow Cell, 1.0 µL, 10 mm (G4212-60008)	Agilent Ultra-Low Dispersion Max-Light Cartridge Flow Cell, 0.6 µL, 10 mm (G4212-60038)

Method optimizations

After optimizing your LC system hardware, consideration should also be given to optimizing your method. When it comes to high efficiency columns such as the 1.9 µm InfinityLab Poroshell, detector data collection rate and its impact on column performance is commonly discussed. Data collection rate is the frequency at which the instrument takes measurements throughout an analysis. It is critical that a sufficient number of data points is measured across a chromatographic peak to accurately reflect the efficiency of that column. Too few data points will show artificially broad peaks, as shown in Figure 2A with butylparaben. In this example, we test the data collection rate of a diode array detector; however, other LC detectors also have a sampling rate that needs to be set in the method. Note that a default method in ChemStation has the diode array detector data collection rate set to 2.5 Hz; when building a new method for the 1.9 um Poroshell, it is imperative that the rate is increased to reflect the performance of the column. According to Figure 2B, this method should use a rate of at least 40 Hz; higher data collection rates could increase baseline noise and reduce method sensitivity.

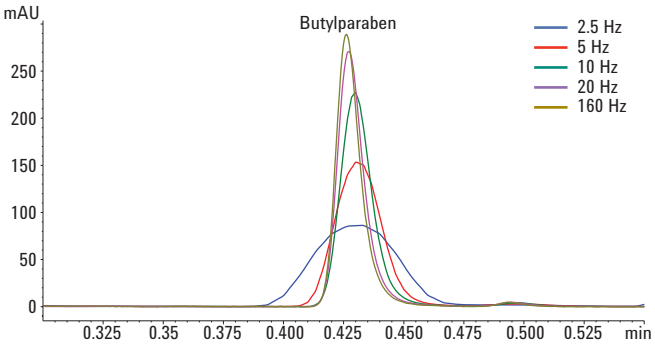

Figure 2A. Fast data collection rates must be used with Agilent InfinityLab Poroshell 1.9 µm columns to accurately measure the efficiency of the column, especially for early eluting compounds such as butylparaben (k′ = 1.3).

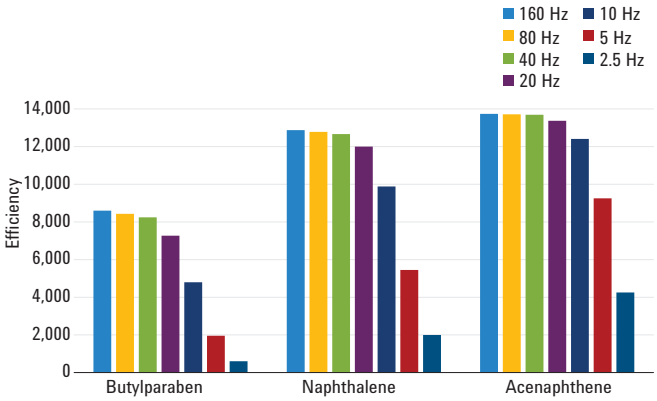

Figure 2B. Fast data collection rates must be used with Agilent InfinityLab Poroshell 1.9 µm columns to accurately measure the efficiency of the column, especially for early eluting compounds such as butylparaben (k′ = 1.3).

Another method parameter that can be used to optimize performance of a small-volume column is injection volume. The injection volume can be considered with the overall system volume, and should be kept as small as reasonably possible for your analysis. Figure 3A shows the effect that injection volume can have on chromatography. Comparing a 0.5 µL injection to a 16 µL injection with this 2.1 × 50 mm column shows a big difference in peak width and chromatographic performance. Efficiency values (Figure 3B) demonstrate the same trend that we saw with LC system volume. Larger injection volumes lead to broader peaks, and this effect is more pronounced with early eluting compounds. To isolate the impact of injection volume from possible effects of sample loading, the same amount of sample was injected onto the column throughout this experiment. The original sample was serially diluted with mobile phase, while the injection volume was appropriately scaled. Figure 4 illustrates that the same amount of sample was injected onto the column regardless of the injection volume being tested; this is shown through constant area counts for each compound.

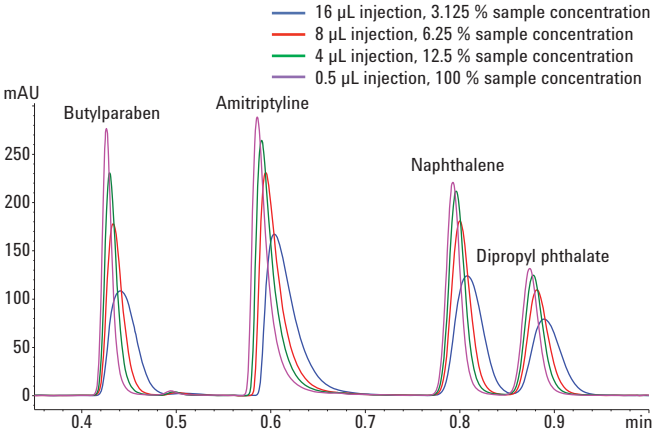

Figure 3A. Injection volumes contribute to overall system volume, and must be kept small to preserve the performance of high-efficiency columns such as a 1.9 µm Agilent InfinityLab Poroshell.

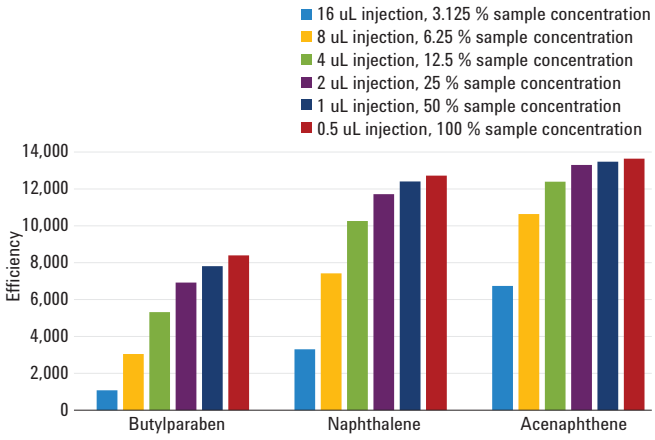

Figure 3B. Injection volumes contribute to overall system volume, and must be kept small to preserve the performance of high-efficiency columns such as a 1.9 µm Agilent InfinityLab Poroshell.

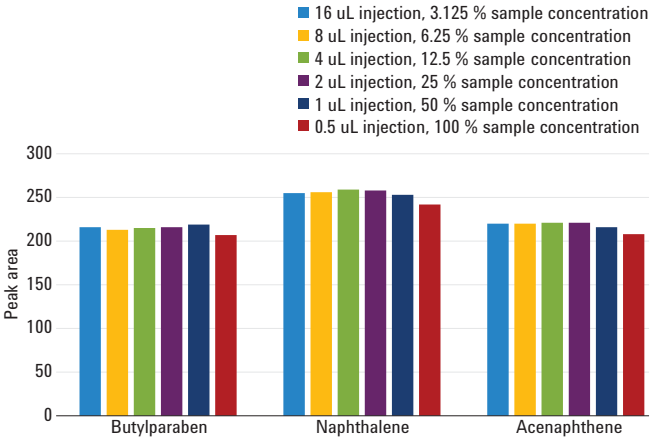

Figure 4. Measuring peak areas shows that the same amount of sample was injected onto the column in this injection volume comparison, reducing the possibility of other variables such as sample loading, and allowing the impact of injection volume to be isolated and studied.

Sample optimizations

In addition to instrument and method optimizations, samples can also be optimized to improve the observed performance of a column. The solvent in which your sample exists can affect chromatographic performance. In Figure 5A, samples were diluted 1:10 in various solvents, then 1.5 µL was injected. Some solvents, such as THF and DMSO, create large baseline disturbances at 254 nm, which could be problematic for very early eluting compounds. The larger issue with sample solvent is the potential for peak shape distortion, as seen with dipropyl phthalate when the sample solvent is THF or IPA. THF also greatly alters the peak shape for the remaining peaks. To prevent peak shape issues related to sample solvent, ensure that your sample is prepared in a solvent of equal or lesser strength than the mobile phase. Figure 5B demonstrates the effect of sample solvent on efficiency. Strong solvents such as THF, DMSO, and IPA greatly reduce efficiency by broadening the chromatographic peaks. The best performance comes from samples made up in mobile phase or water. Interestingly, water sample gave the best results. This is likely due to the sample being focused on the column. One problem with water, however, was that solubility was an issue for some analytes in this sample. The chromatograms in Figure 5A show low peak heights for naphthalene and acenaphthene, as they have poor solubility in water.

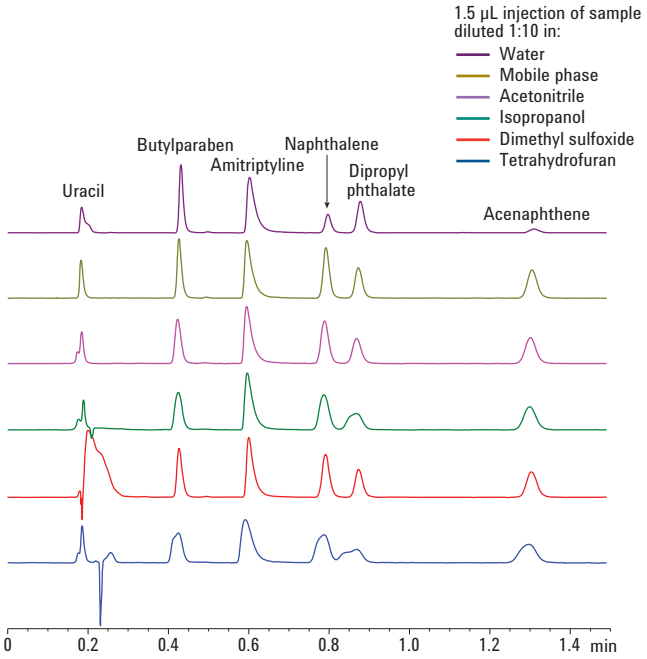

Figure 5A. Sample solvents should be of equal or lesser strength than the mobile phase, otherwise poor peak shape can occur, resulting in poor efficiency. Choosing the correct sample solvent strength becomes more critical as injection volume increases especially for low-volume columns such as an Agilent InfinityLab Poroshell 2.1 × 50 mm, 1.9 µm column.

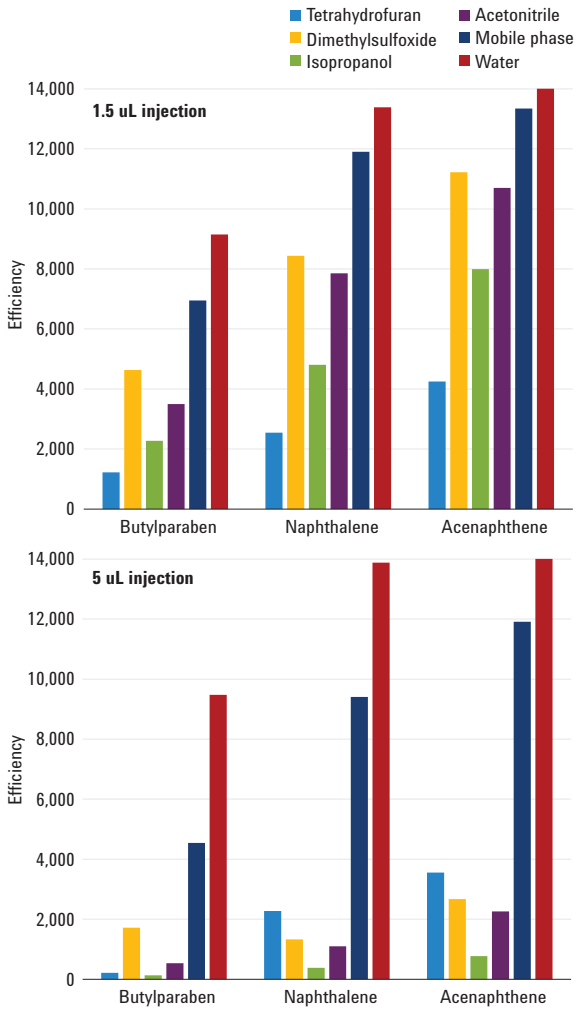

Figure 5B. Sample solvents should be of equal or lesser strength than the mobile phase, otherwise poor peak shape can occur, resulting in poor efficiency. Choosing the correct sample solvent strength becomes more critical as injection volume increases especially for low-volume columns such as an Agilent InfinityLab Poroshell 2.1 × 50 mm, 1.9 µm column.

The concentration of the analytes in the sample can also affect method performance. For the experiment shown in Figure 6, amitriptyline was prepared in mobile phase at various concentrations; all other parameters were held constant. Figure 6A illustrates how peak shape and retention time vary as the sample is loaded onto the column. In this case, performance was measured by efficiency and peak tailing, which are plotted in Figure 6B. There is no clear best concentration of amitriptyline to load onto the column. Ideally, one would choose a concentration at which efficiency was high and tailing low. All analytes will have their own unique behavior for sample loading, so it is important to consider compounds individually.

For the best performance from an InfinityLab Poroshell 2.1 × 50 mm, 1.9 µm column, sample solvent and analyte concentration should be optimized as best as possible for a given analysis. However, some analyses may not have the ability to change sample characteristics such as these.

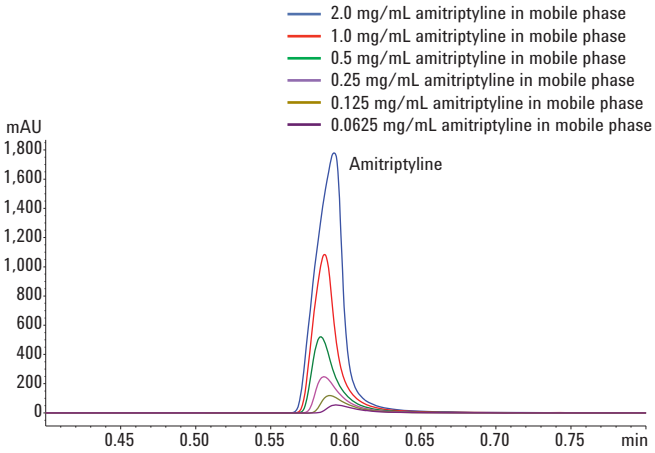

Figure 6A. Method performance can vary by analyte and by how much sample is loaded onto the column.

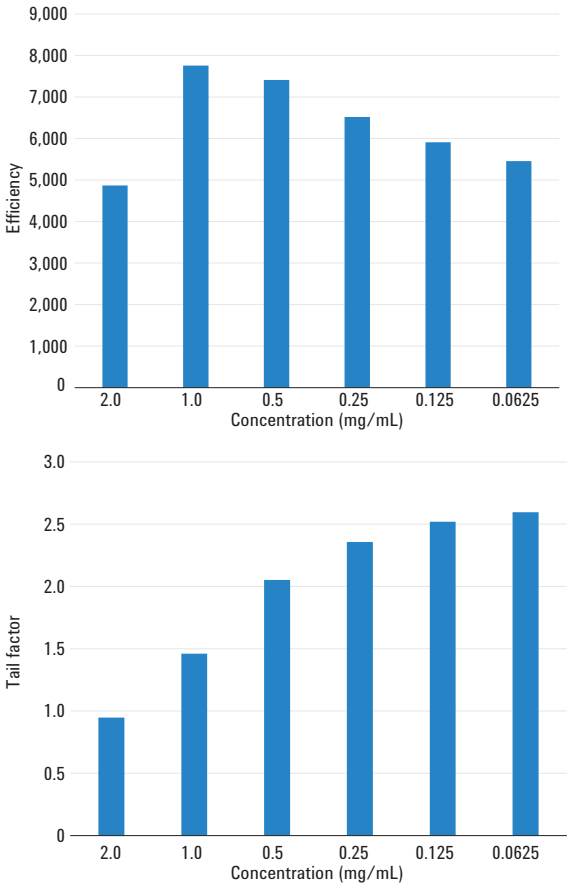

Figure 6B. Method performance can vary by analyte and by how much sample is loaded onto the column.

Conclusions

The highly efficient Agilent InfinityLab Poroshell 1.9 µm column is a power chromatographic separation tool. However, care should be taken to optimize the LC instrument, method, and sample to ensure that the full resolving power is realized. Instruments should be configured with low-volume capillaries and flow cells. Method data collection rates should be set sufficiently high, and injection volumes should be kept as low as feasible. Sample solvents should be of equal or lesser strength compared to the mobile phase, and analyte concentration should be low enough that it does not overload the column and impair peak shape.

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Transfer of Methods between Poroshell 120 EC-C18 and ZORBAX Eclipse Plus C18 Columns

Technical Overview

Introduction

The developement of superficially porous particles has led to the possibility of method transfer from larger 5-µm totally porous particles, as well as from sub-2-µm totally porous particles. One of the benefits of transferring from larger particle columns is significant time savings, as the superficially porous particles are optimally run at a faster flow rate achieving similar resolution with a much shorter column length [1–4]. The high efficiency of superficially porous particles is similar to sub-2-µm totally porous particles because of the short mass transfer distance and substantially narrower particle size distribution. Transferring methods from totally porous sub-2-µm columns may also be desirable. Many development laboratories have chosen to use sub-2-µm columns. However, in some cases the higher operating pressure required of sub-2-µm methods may not be transferable to all HPLC systems. In many cases methods using sub-2-µm columns can be directly transferred to superficially porous particle columns, without adjustment. This is particularly true when columns like the Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 are manufactured to have similar bonding chemistries and use similar retention mechanisms. Additionally, superficially porous particle columns can perform the same analysis as sub-2-µm columns, while generating less backpressure. This allows analysts to increase flow rates for higher throughput, or to increase column length to enhance resolution without exceeding the system pressure limits.

One asset of the Agilent ZORBAX family of HPLC columns is the scalability of methods between particle sizes. This allows a quick and reliable transfer of methods from method development to preparative lab and high throughput analysis.

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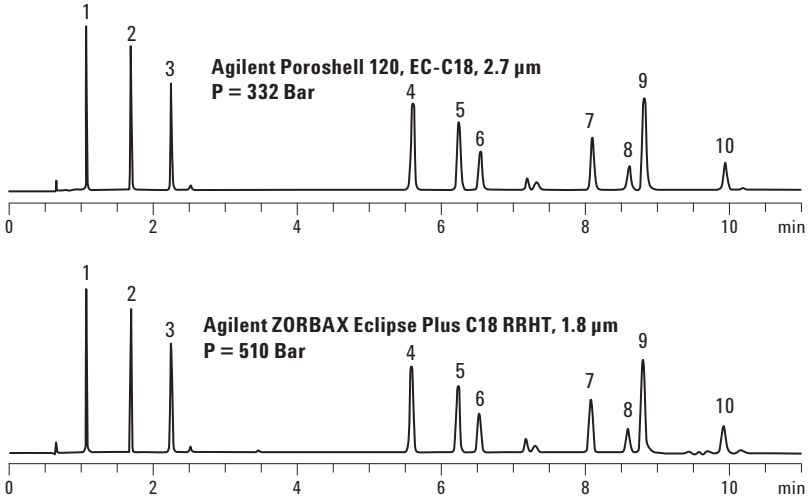
Several recent comparisons of Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 have shown very similar chromatography. Poroshell 120 was designed to deliver 90 % of the efficiency of sub two micron columns such as Eclipse Plus C18 at approximately 60 % of the pressure. Superficially porous particles found in Poroshell 120 have the low pressure benefits of larger particles while achieving the performance of sub two micron particles.

Examples of this chromatographic similarity are shown using environmental phenols in Figure 1 with 0.1 % Formic acid and in Figure 2 in the analysis of soft drink additives using 10 mM ammonium acetate pH 4.8. In both cases, the retention order of the compounds are the same. The similarity of these two examples leads to the larger question, how similar are Poroshell 120 EC-C18 and Eclipse Plus C18, in terms of selectivity over a wider range of operating conditions and with a larger set of compounds including acids bases and neutral materials.



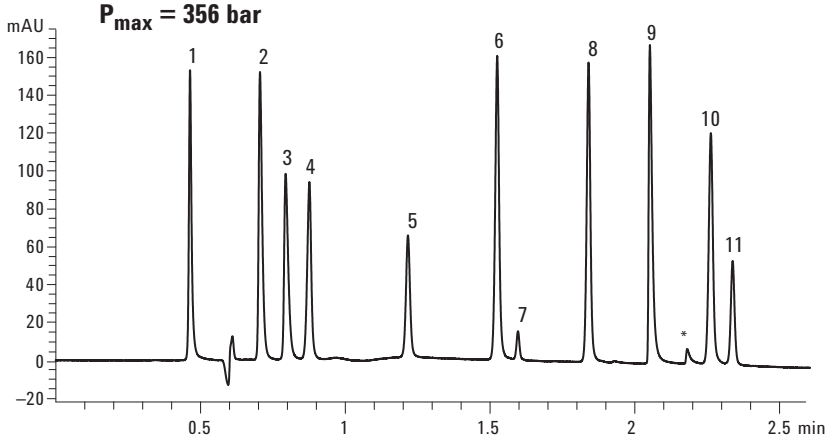
Conditions

Columns	Agilent Poroshell 120 EC-C18, 4.6 mm × 100 mm, 2.7 µm Agilent p/n 689975-902 Agilent ZORBAX Eclipse Plus RRHT C18, 4.6 mm × 100 mm, 1.8 µm Agilent p/n 959964-902
Mobile phase	A: 0.1% Formic acid B: MeCN + 0.1% Formic acid
Temperature	40 °C
Detection	275 nm
Injection volume	10 µL
Flow	2 mL/min
Initial	8% B, 10 min 30% B

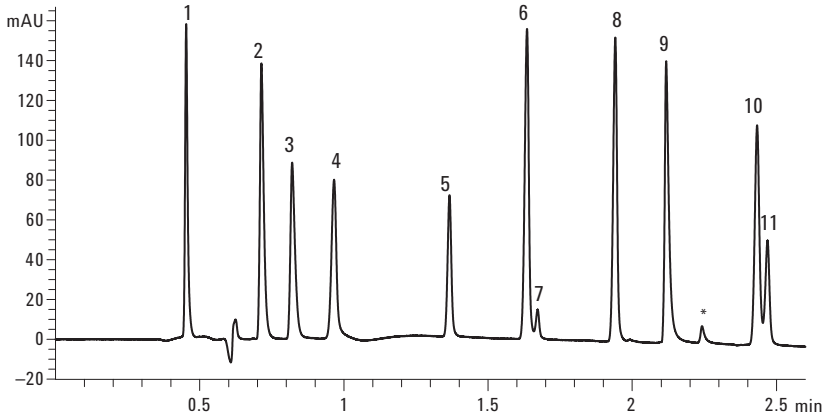
1. Hydroquinone	6. o-cresol
2. Resorcinol	7. 2-Nitrophenol
3. Catechol	8. 2,3 Dimethyl phenol
4. 4-Nitrophenol	9. 2,5 Dimethyl phenol
5. p-cresol	10. 1-Naphtol

Figure 1. Comparison of Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 using acetonitrile and formic acid mobile phase for the analysis of environmental phenols.

Agilent Poroshell 120 EC-C18, 2.7 µm
P_{max} = 356 bar



Agilent ZORBAX Eclipse Plus C18 RRHT, 1.8 µm
P_{max} = 483 bar



Conditions

Columns	Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm, 2.7 µm Agilent p/n 695975-302 Agilent ZORBAX Eclipse Plus C18 RRHT, 3.0 mm × 100 mm, 1.8 µm Agilent p/n 959964-302
Mobile phase	A: 20 mM Ammonium acetate, pH 4.80 B: Acetonitrile
Gradient	14% B at t_0, ramp to 52% B in 2.1 min
Flow rate	0.851 mL/min
Temperature	30 °C

1. Ascorbic Acid
2. Acesulfame K
3. Saccharin
4. p-Hydroxybenzoic Acid
5. Caffeine
6. Benzoic Acid
7. Aspartame
8. Sorbic Acid
9. Quinine
10. Dehydroacetic Acid
11. Methylparaben

* Quinine Impurity

Figure 2. Comparison of Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 using acetonitrile and ammonium acetate mobile phase for the analysis of soft drink addities.

Experimental

Method development is often based upon the use of a generic gradient. Using a short Agilent Poroshell 120 EC-C18, 4.6 x 50 mm column, several different mobile phases can be quickly evaluated. The generic gradient used in this work is run at 2.0 mL/min, starts at 5% organic and increases to 95% organic over 2 min and holds at this concentration for 1 min. Mass spectrometer compatible mobile phases consisting of volatile buffers such as ammonium formate buffer and ammonium acetate buffer are used. These buffers were prepared by dissolving sufficient ammonium formate or ammonium acetate in water to produce 10 mM solutions and titrating the solutions to the desired pH with the appropriate concentrated acid. The pH of these buffers covers a range between 3 and 6.5.

An Agilent 1200 Method Development Solution LC system was used for this work:

- G1312B Binary Pump SL
- G1367D Automatic Liquid Sampler (ALS) SL
- Two G1316C Thermostatted Column Compartments (TCC) SL
- G1315C Diode Array Detector (DAD) SL, using a G1315-60024 micro flow cell (3-mm path, 2-µL volume)
- ChemStation version B.04.01 was used to control the HPLC and to process the data.

Correlation data was calculated and plotted using Microsoft Excel 7.0.

Four Agilent Poroshell 120 EC-C18 columns were used in this work:

- Agilent Poroshell 120 EC-C18, 4.6 mm × 50 mm, 2.7 µm p/n 699975-902
- Agilent Poroshell 120 EC-C18, 3 mm × 100 mm, 2.7 µm p/n 695975-302
- Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 50 mm, 1.8 µm p/n 959943-902
- Agilent ZORBAX Eclipse Plus C18, 3 mm × 100 mm, 1.8 µm p/n 959964-302

Table 1 summarizes the list of compounds studied for this work. These compounds were prepared in water or 50/50 water/acetonitrile and injected individually.

Table 1. Sixty-six Compounds Including Acids, Bases and Neutrals Prepared in 50/50 MeCN/Water and Injected onto 4.6 x 50 mm Columns Individually

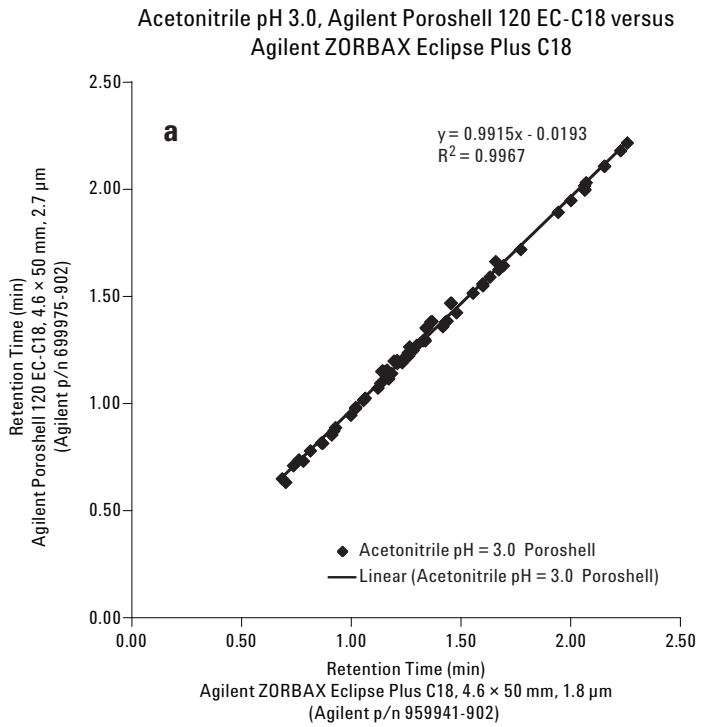
List of tested compounds	
furazolidone	phenacetin
chloramphenicol	acetanilide
impramithue	phenol
norethindrail	resorcial
cortisone acetate	hydroquinone
chloramphenicol	4 nitro phenol
busirone hydrochloride	o cresol
benzocaine	1 napthol
pyrimethamine	imipramine hydrochloride
sulfaquinoxaline	3 4 dihydroxy l phenyl alanine
sulfamonomethoxine	dl phenyalanine
nimopidin	ephedrine hydrochloride
sulfadimethoxine	loperamide
sulfamethoxazole	dibenzofuran
sulfachloropyridazine	procaine hydrochloride
sulfamethoxypyridazine	exonazole nitrate
sulfamethizole	gembigrozil
sulfamerazine	beta estradiol
sulfathiazole	metoprolol
sulfadiazine	protriptyline
benzaldehyde	hydroxy sophthalic
phenanthrene	flufenamic acid
biphenyl	pramoxine hydrochloride
acenaphthene	naproxen
methoxy naphthalene	diphenhydramine
dimethoxy benzene	diflunisal
alpha hydroxyprogesterone	nisoldipin
progesterone	diclofenac
prednisolone	hydrocortisone
deoxycorticosterone	procainamide hydrochloride
chlorphenamine	lidocaine
berberine	terfenaine
chlortetracycline hydrochloride	chlorpheniramine maleate

Discussion

Differences in column performance have been studied by many including Wilson, Nelson, Gilroy, Dolan, Snyder and Carr [5,6]. The United States Pharmacopeia lists many columns [7] and a tool to determine how interchangeable columns may be. Characteristics such as silica chemistry and bonding can change selectivity. Silanol activity affects peak shape dramatically through secondary interactions. It also can affect selectivity through H-bonding or ion-exchange. These effects become more pronounced at higher pH than at lower pH [8]. Both Agilent ZORBAX Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 Columns are made from silica produced by Agilent at the same facility that makes the final columns. Both are intended to be highly inert columns and have been designed to yield excellent peak shape with basic compounds. In addition to the effect of pH, silanol activity can also be affected by differences in solvent. Methanol is an H-bonding solvent that has weaker elution strength than aprotic acetonitrile [10]. By choosing a wide range of conditions, it is more likely that differences in selectivity will be revealed.

Figure 3 shows similar retention of 66 compounds on Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18 columns using a generic gradient analysis with a variety of compounds from different chemical classifications. The high correlation coefficient (R^2) indicates a high degree of similarity between the interactions involved in the separation on the two Agilent C18 columns, while a slope of approximately 1 implies similar interaction strengths [9,10].

Generic Gradients using Acetonitrile, Buffered with 10 mM Ammonium Formate or Ammonium Acetate between pH 3 and 6.5



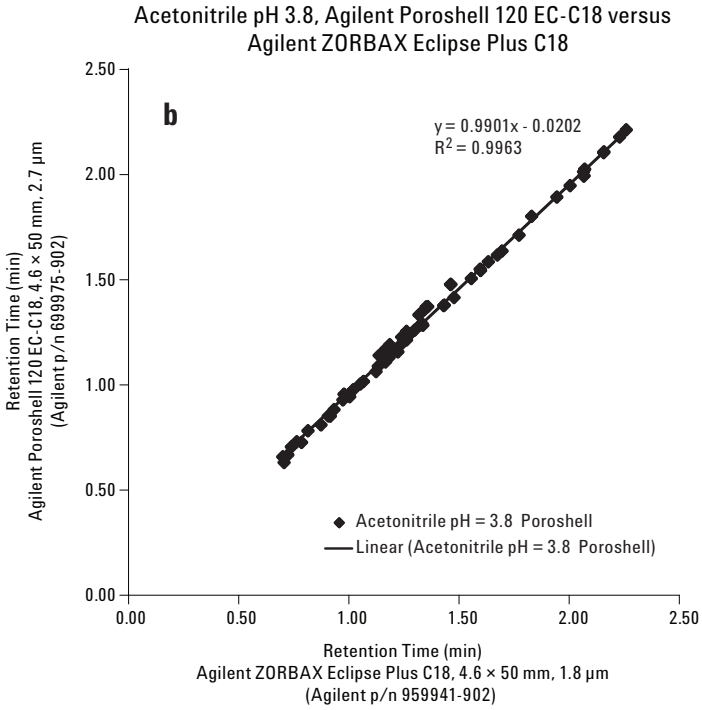
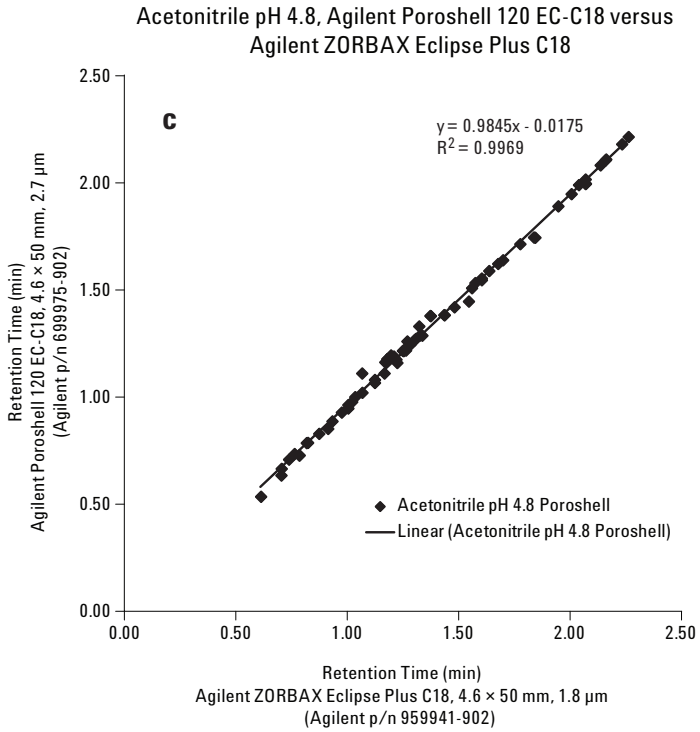
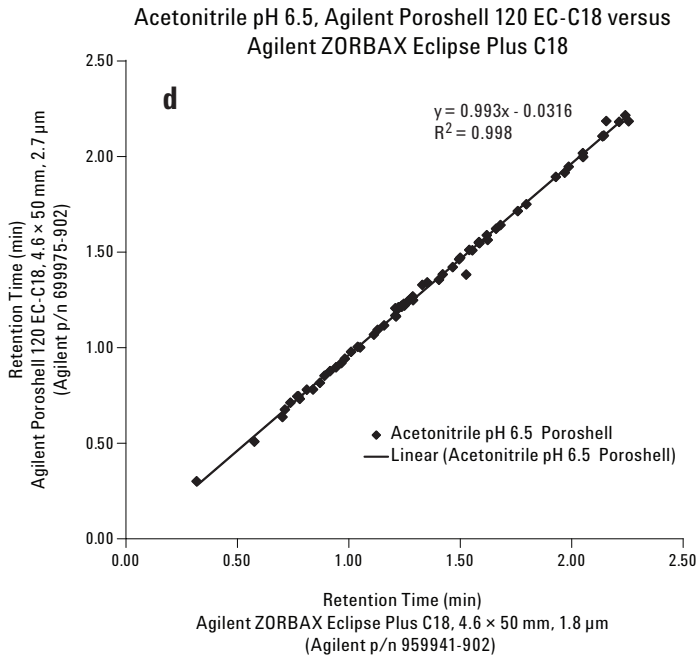


Figure 3. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm. (continued)





Conditions

Mobile phase	A: 10 mM Buffer B: Organic (ACN)
Gradient	5% B at t_0, ramp to 95% B in 2 min, hold 95% B for 1 min
Flow rate	2 mL/min
Sample	1 µL of 1 mg/mL standard in H_2O or H_2O/ACN

Figure 3. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm.

Figure 4 shows scatter plots of the retention times of 66 compounds on Agilent Poroshell 120 EC-C18 versus Agilent ZORBAX Eclipse Plus C18 columns at different pH values between 3 and 6.5 in acetonitrile. Figure 2 shows scatter plots at different pH values between 3 and 6.5 in methanol. The slope and R^2 values for these combinations are summarized in Table 2. As illustrated, the correlation between the two plots is quite good. While retention times sometimes change with the ionic compounds, the changes are proportional on both columns. A slight difference in the slopes of the correlation curves may indicate some difference in H bonding interaction between Agilent ZORBAX Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 when comparing the acetonitrile and methanol data (slope of 0.99 and slope of 1.01), but this is not likely to cause any problems in method transfer and is only measureable given the large number of experiments and compounds studied.

Generic Gradients using Methanol, Buffered with 10 mM Ammonium Formate or Ammonium Acetate between pH 3 and 6.5

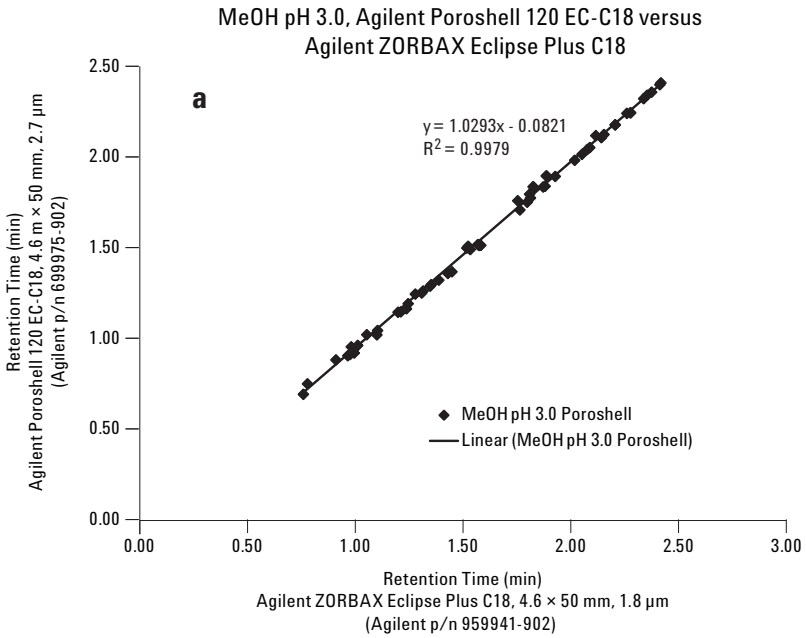
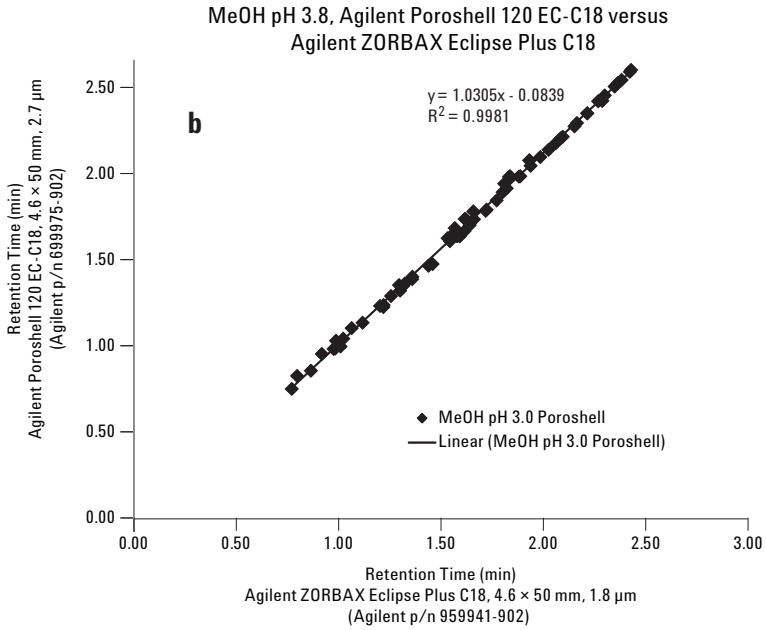


Figure 4. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm. (continued)



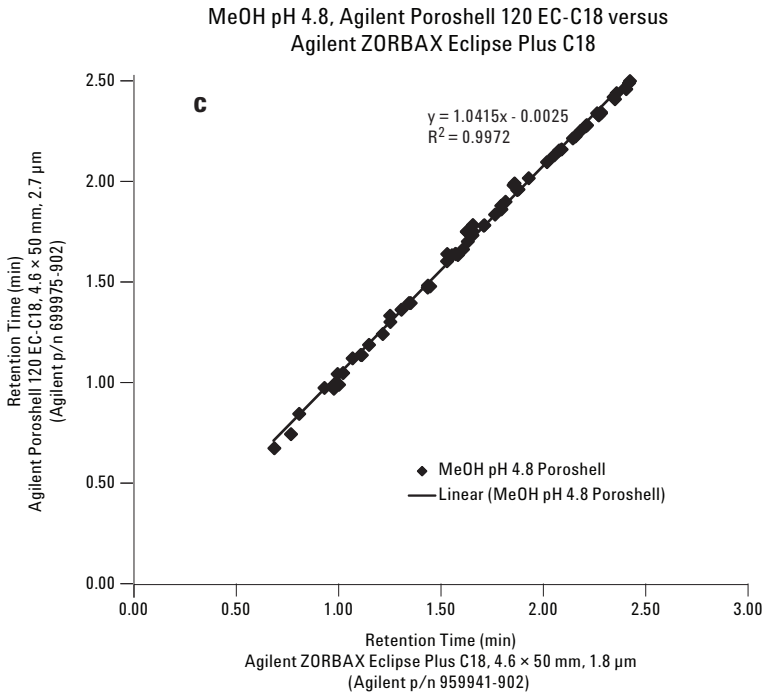
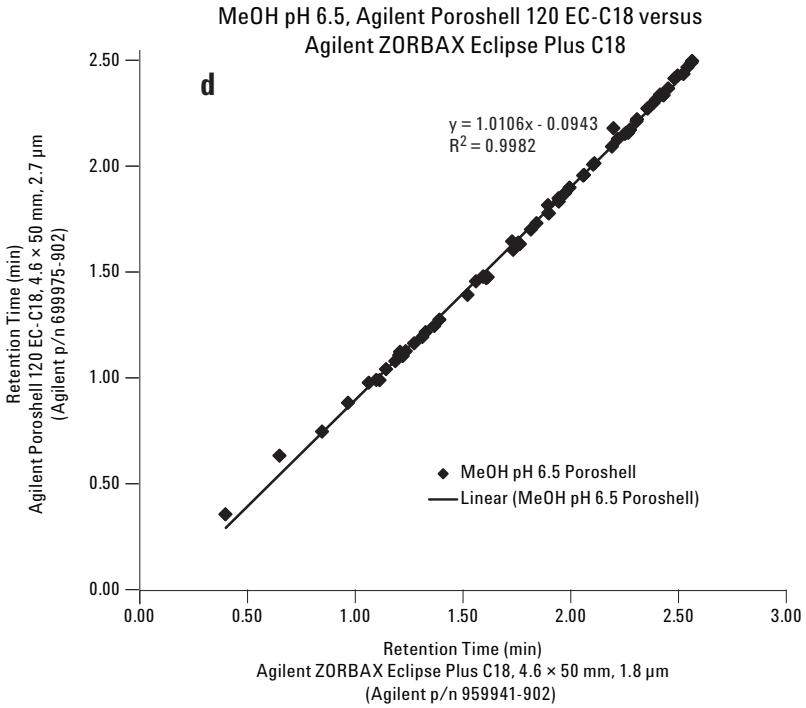


Figure 4. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm. (continued)



Conditions

Mobile phase:	A: 10 mM Buffer
	B: Organic (MeOH)
Gradient:	5% B at t_0, ramp to 95% B in 2 min, hold 95% B for 1 min
Flow rate:	2 mL/min
Sample:	1 µL of 1 mg/mL standard in H_2O or H_2O/ACN

Figure 4. Scatter plot of retention time of 66 compounds on Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm versus Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 1.8 µm.

Table 2. Summary of Correlation Data

Acetonitrile	Methanol
a. pH =3.0 y = 0.9915x -0.0193 R² = 0.9967	a. pH =3.0 y = 1.0293x -0.0821 R² = 0.9979
b. pH =3.8 y = 0.9901x -0.0202 R² = 0.9963	b. pH =3.8 y = 1.0305x -0.0839 R² = 0.9981
c. pH =4.8 y = 0.9845x -0.0175 R² = 0.9969	c. pH =4.8 y = 1.0415x -0.002 R² = 0.9972
d. pH =6.5 y = 0.993x -0.0316 R² = 0.998	d. pH =6.5 y = 1.0106x -0.0943 R² = 0.9982

Another benefit of the Agilent Poroshell 120 columns over sub-2-µm columns is lower operating pressure. The pressure is related to the particle size of the column; larger particles naturally yield lower pressure than smaller particles. In addition to the particle size, the pressure generated inside a column is dependent upon several other factors including solvent linear velocity, and solvent viscosity at a given composition and temperature. While this is a gradient study, the most viscous solvent composition in this study occurs between 40/60 and 50/50 methanol/water. At 25 °C the viscosity of this solvent is 1.62 cP. The most viscous acetonitrile composition is 10/90 acetonitrile/water. At 25 °C the viscosity of this solvent is 1.01 cP [11]. As indicated in the references the viscosity of the solutions is inversely dependent on the temperature. The pressure verses linear velocity graphs for Agilent Poroshell 120 EC-C18 columns and Agilent ZORBAX Eclipse Plus C18 1.8 µm columns are shown for both solvent pairs as Figures 5 and 6. In this case 100 mm columns are used. As stated earlier, this benefit can allow the use of longer columns achieving the same pressure (and larger injection volumes), or higher flow rates.

Differences in selectivity are more likely to occur in cases where the pore size difference becomes more important, typically for compounds between 1500 and 2500 mw. Compounds such as PAHs that involve shape selectivity may also be problematic.

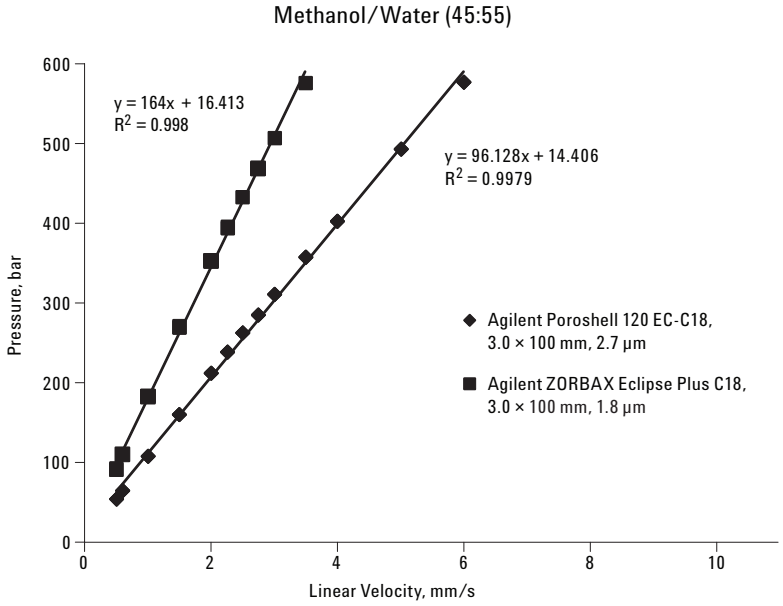


Figure 5. Pressure measured at varied linear velocities indicates lower operating pressure for Agilent Poroshell 120 than an a 1.8 µm column of similar length.

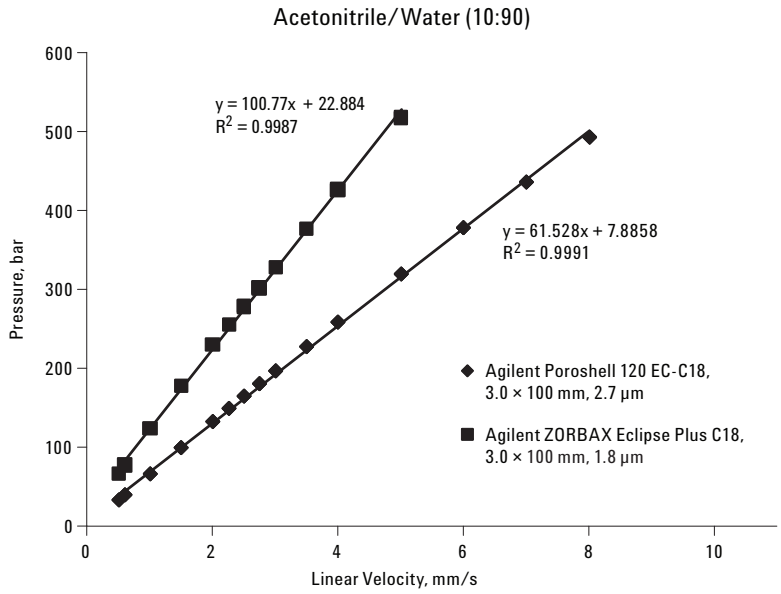


Figure 6. Pressure measured at varied linear velocities indicates lower operating pressure for Agilent Poroshell 120 than an a 1.8 µm column of similar length.

Conclusions

This work has demonstrated the equivalence of selectivity between Agilent ZORBAX Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 columns across a wide range of pH and mobile phase conditions. Both column chemistries are manufactured using similar materials with similar proprietary bonding chemistries. Both columns were designed to achieve excellent peak shapes for bases without sacrificing low pH peak shape and performance for other compounds. The benefit of using Agilent Poroshell 120 EC-C18 columns is high efficiency at a lower backpressure. Based on this work, it is expected that if the need arises methods developed on Agilent ZORBAX Eclipse Plus C18 columns can be reliably transferred to Agilent Poroshell 120 EC-C18 columns and conversely with low risk.

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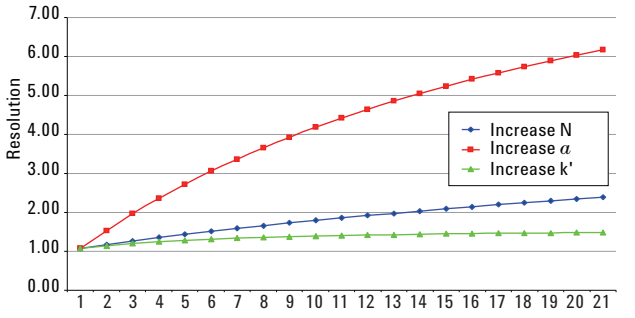
Agilent Technologies

Use of Agilent Poroshell HPH-C18 Columns at Elevated pH as a Tool for Method Development

Technical Overview

Introduction

HPLC method development for chemical and pharmaceutical analysis is a challenging task. It involves screening a range of chromatographic parameters to generate sufficient resolution and robust separations. While there are many approaches to method development, such as one factor at a time, and quality by design (QbD), the goals and factors used for optimizing separations are the same. Several factors affect chromatographic resolution (RS), efficiency (N - controlled by particle size, particle morphology, and column length), retention factor (k - controlled by solvent strength), and selectivity (α - controlled by bonded phase choice and mobile phase)(Figure 1). Selectivity or α is the most powerful of these factors.



Plates:	5,000	10,000	15,000	20,000	25,000
Alpha:	1.10	1.35	1.60	1.85	2.1
k':	2.0	4.5	7.0	9.5	12.0

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$$

Figure 1. Typical method development parameters; effects of selectivity, efficiency, and retention on resolution.

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Method development involves the separation of simple and complex mixtures. Selectivity can be controlled though several factors, including the choice of stationary phase, the type of organic modifier, gradient slope, flow rate, and temperature. For ionizable compounds, the pH of the buffer is also a powerful parameter. Optimizing separation of ionizable compounds in order to find robust conditions has become an important part of method development in liquid chromatography [1]. Most pharmaceutical and biological compounds contain ionizable functions such as carboxylic or amino groups. Using pH is a very powerful selectivity tool for reversed-phase liquid chromatography (RPLC) separations. Low-pH separations involve protonated acids and bases, but these acids and bases are deprotonated at high-pH. Because retention in reversed-phase chromatography is strongly dependent upon the analyte charge, pH can be used to make large changes in selectivity. At acidic pH, acids have their maximum retention because they are neutral, but bases have their minimum retention because they are fully charged. At basic pH (above the pKa of the compound), bases have their maximum retention because they are neutral, and acids are fully ionized and have their minimum retention. For the best peak shape, retention and sample loading of basic analytes in RPLC, the mobile phase pH should be two units higher than the pKa of the compound of interest. The retention of neutral compounds is unaffected by pH. In this work, adjustment of pH was used to control selectivity using an Agilent Poroshell HPH-C18 column that is designed to be stable in high pH mobile phases.

Materials and Methods

An Agilent 1260 Infinity LC was used for this work.

- Agilent 1260 Infinity Binary Pump G1312B
- Agilent Automatic Liquid Sampler (ALS) G1367C
- Agilent 1260 Infinity Thermostatted Column Compartment (TCC) SL G1316C
- Agilent 1290 Infinity Diode Array Detector (DAD) G4220A (10-mm path, 1-µL volume)
- OpenLab version C.01.05 was used to control the HPLC and to process the data.
- Agilent Poroshell HPH-C18, 2.1 × 50 mm, 2.7 µm (p/n 699775-702) or Poroshell HPH-C18, 4.6 × 50 mm, 2.7 µm (p/n 699975-702)

In some experiments, an Agilent 6140D Single Quadrupole LC/MS was also employed.

Table 1. Compounds used in retention correlation.

Sample name
1,2-Dimethoxybenzene
1,2-Dinitrobenzene
1,2,3-Trimethoxybenzene
1,2,4-Trimethoxybenzene
1,2,5-Trimethoxybenzene
1,3-Dimethoxybenzene
1,3-Dinitrobenzene
1,4-Dinitrobenzene
2,3-Dimethylphenol
2,4-Dichlorophenol
2,4-Dimethyl benzoic acid
2,5-Dihydroxyl benzoic acid
2,5-Dimethyl phenol
2-Hydroxyhippuric acid
2-Napthalene sulfonic acid
3,4-Dimethoxybenzoic acid
3-Nitrophenol
4-Hydrobenzaldehyde
4-Hydroxybenzoic acid
4-Nitrophenol
5-hydroxy-isophthalic acid
8-Hydroxyquinoline
Acebutolol
Acetylsalicylic acid
Alprenolol
Amitriptyline
Andro
Antipyrin
APAP
Atenolol
Atorvastatin
Beta estradiol
Beclomethasone
Benzocaine
Benzoic acid
Benzophenone
Benzyl alchohol
Betamethasone
Biphenyl (DMSO)
Butacaine
Butyl benzene
Butyl paraben
Butylated hydroxy anisole
Butylated hydroxy toluene
Butyrophenone
Caffeine
Catechol
Chlorammphenicol
Corticosterone
Desimpramine
Dexametasone
Diclofenac
Diethyl phthalate
Diflunisal
Diisopropyl phthalate
Dioctyl phthalate
Dipropyl phthalate
Doxepim
Esterone
Ethinylestradiol
Ethyl-4-hydroxybenzoate
Fenprofen
Fluoxetine
Furazolidone
Hesperidin
Hydrocortisone
Irganox 1330
Ketoprofen
Labetalol
m-Nitrophenol
Mefamic acid
Naldolol
Naproxen
Nargingenin
Nisoldipin
Norethindrone acetate
Nortryptyline
p-Cresol
p-Nitrophenol
Pentachlorophenol
Phenacetin
Phenantranene
Pindolol
Piperdine
Piroxicam
Pravastatin
Prednisone
Procaine
Progesterone
Promazine
Propranolol
Protriptyline
Pyrimethamine
Quinine
Resorcinol
Salicytic acid
Salycilic acid
Sulfachloropyridazine
Sulfadiazine
Sulfadimethoxine
Sulfamerazine
Sulfamethiazine
Sulfamethiazole
Sulfamethoxazole
Sulfamethoxypyridazine
Sulfamonomethoxine
Sulfaquinoxaline
Sulfathiazole
Sulindac
Testosterone
Tetracaine
Tolemetin
Triamcinalone
Trimipramine
Ultranox 276
Uracil
Valerophenone

Mobile phases compatible with mass spectrometry, consisting of volatile buffers such as ammonium formate buffer, ammonium acetate, and ammonium bicarbonate buffer, were used. These buffers were prepared by dissolving sufficient ammonium formate or ammonium bicarbonate in water to produce 10 mM solutions, and adjusting the solutions to the desired pH with the appropriate concentrated acid (formic acid or acetic acid) or concentrated base (ammonium hydroxide). The mixture evaluated included acids (acetyl salicylic acid, and diflunisal), bases (procainamide, dipyrimadole, and diltiazem), and neutral compounds (hexanophenone and impurity (valerophenone)). Caffeine does not ionize and was also included.

Use of pH to affect selectivity

Figure 2 depicts how the elution order of a mixture consisting of acidic, basic, and neutral compounds changed as pH of the mobile phase was changed. In this work, a generic gradient was used with the organic modifier (acetonitrile) concentration changing from 10 to 90% over 4 minutes. Chromatograms at pH 3 (ammonium formate), pH 4.8 (ammonium acetate), and pH 10 (ammonium bicarbonate) are shown using buffers compatible with mass spectrometry. The flow rate was 2 mL/min.

As shown, the three chromatograms use the same gradient and column. The neutral (hexanophenone) and nonionized compounds (caffeine) remained at the same elution time. They were not affected by the change in pH. As the mobile phase pH was increased from pH 4.8 to pH 10, the acidic compounds became charged and their retention time decreased. This is depicted by the red arrows in Figure 2. As the pH is increased, the retention time of the bases increased as shown with the blue arrows. The peak elution order changed dramatically as did the spacing. In all three chromatograms, the peak shape was excellent. In this case, the spacing of the compounds was greater using the pH 10 buffer than either of the other buffers. In addition to longer retention of bases, better peak shape was also found when using high pH mobile phases as compared to low pH mobile phase.

Another way to look at selectivity is by plotting retention time using two different conditions for a group of acids, bases, and neutral compounds. A list of the compounds used in this study is found in Table 1. In this case, 117 compounds were run using the Poroshell 120 HPH-C18 column with identical gradients and two organic modifiers (methanol and acetonitrile) and at two pHs (pH 3 and pH 10). The generic gradient used here was 0.42 mL/min, starting at 5% organic and increasing to 95% organic over 4 minutes, and held at this concentration for 2 minutes.

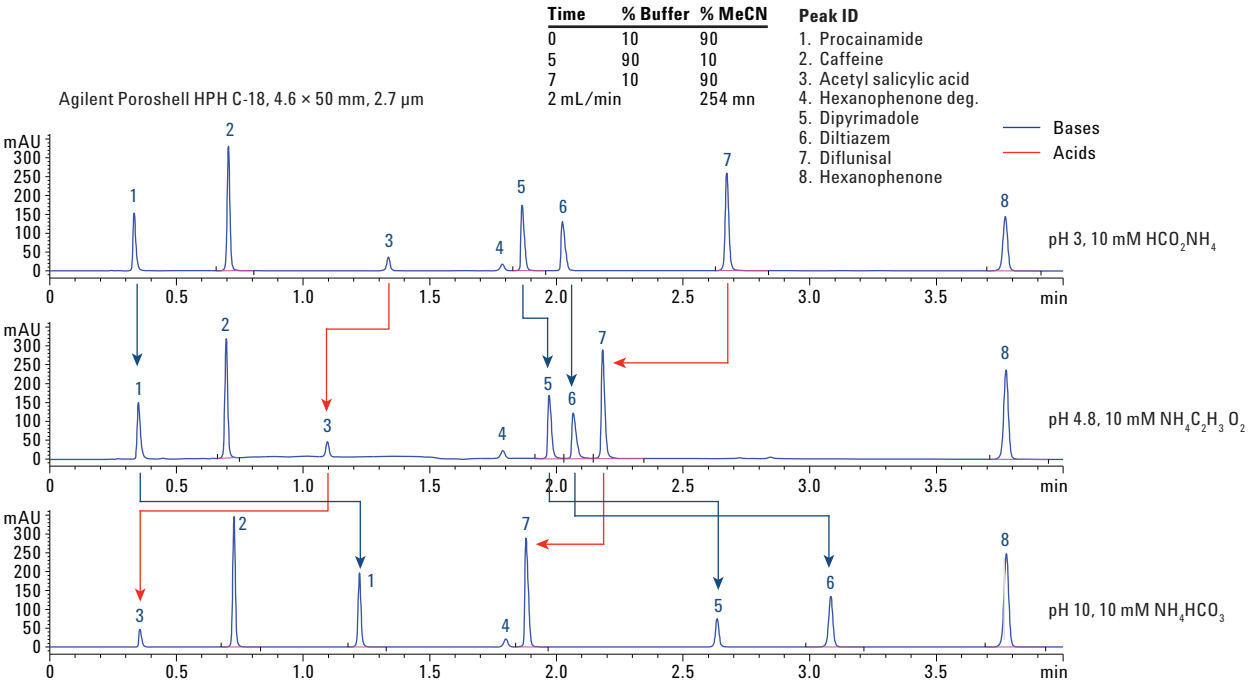

Figure 2. Selectivity control by altering pH with an Agilent Poroshell HPH-C18, 4.6 × 50 mm, 2.7 µm LC column at pH 3, 4.8, and 10.

As shown in Figure 3A, a subgroup of analytes lined up perfectly with a slope of 1. These compounds were neutral or nonionizable with methanol as the organic modifier. They include substituted benzenes, steroids, phenols, and phenones. The retention time of these materials was not affected by the pH of the mobile phase, as expected. This method was applied and discussed in previous work where two highly similar columns (Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18) were compared under similar chromatographic conditions [2]. Analytes that appear above the line are bases. At pH 3, these compounds were charged, and as they became uncharged when the pH increased to 10, the retention time increased. The correlation coefficient of retention times is a measure of the difference of the separation under two different pH conditions. A highly correlated plot would have a value close to 1. This would indicate that the chromatographic separations were very similar. Conversely, a very low correlation value (close to 0.5 or lower) indicates a more orthogonal or dissimilar separation. A second comparison is also shown in Figure 3B, where a comparison of low and high pH gradients was made using acetonitrile as the organic modifier. In this case, the correlation coefficient was smaller, than when using methanol [2,3,4].

Improved LC-MS sensitivity for basic compounds at high pH

In a third experiment, LC/MS of several bases was compared at high and low pH using a generic gradient in positive mode electrospray. Normally one expects that the ionization state of analyte molecules is dependent on the pH of the mobile phase, and that the ionization efficiency in LC/MS with electrospray in positive ion mode will be drastically lowered in high pH mobile phases since the compounds become neutral. However, many researchers investigating different types of samples (including proteins, peptides, and amino acids) have observed either an insensitivity to change of mobile phase pH or even increases.

Successful detection of basic compounds in ESI+ when using high pH buffers in the mobile phase has been reported [5-10]. High pH mobile phases do not suppress the ionization of basic compounds in ESI+; positive ions are formed abundantly, and analyte responses are often better in high pH compared to acidic mobile phases. This finding is significant as it extends the applicability of generic elution methods to the analysis of polar basic compounds previously difficult to retain.

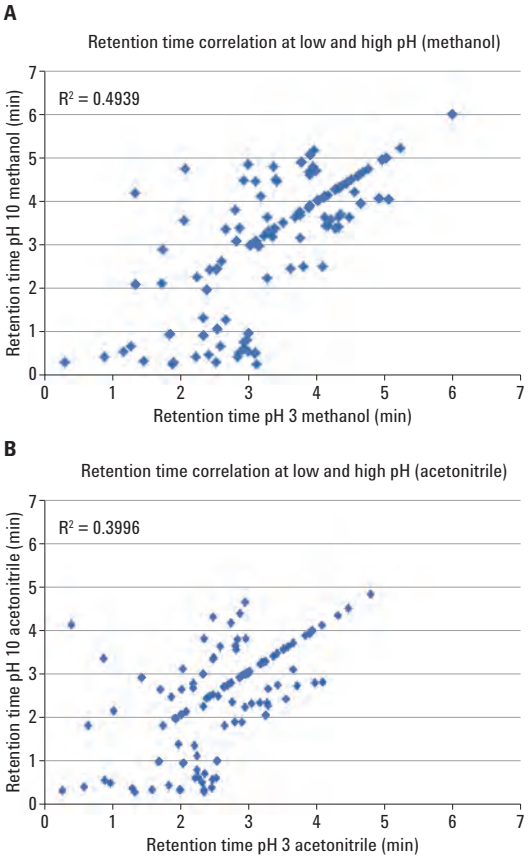

Figure 3. Retention time correlation with an Agilent Poroshell HPH-C18, pH 3 versus pH 10. A) methanol, and B) acetonitrile.

In this experiemnt, a gradient was run using acetonitrile as the organic modifier. The aqueous solvent contained 0.1% formic acid, the low pH mobile phase modifier or 10 mM pH 10 ammonium bicarbonate. In the example shown above, lidocaine was prepared in water at 0.01 mg/mL. A 1 µL injection was made. As shown, the sample was injected on a Poroshell HPH-C18 column, the lower trace shows the sample analyzed at low pH, the analyte is retained only slightly and the peak tailed. In the upper trace, the better retained analyte peak was well shaped and twice as tall. Due to the elution in a mobile phase having a higher organic content, which is beneficial for LC/MS detection, the peak area was also significantly larger. In general, ionization in the more volatile organic phase was more efficient leading to higher signal intensity.

Procainamide and diltiazem were also analyzed. The signal intensity increase of these compounds was not as dramatic as for lidocaine. Solvent evaporation rate during droplet formation is a function of the mobile phase vapor pressure. Higher volatility of a greater proportioned acetonitrile:water mobile phase favors ESI ionization.

The results in Figures 4A to 4C show that the use of high pH mobile phases for the analysis of basic compounds offered a good alternative to using low pH mobile phases in ESI+ LC/MS.

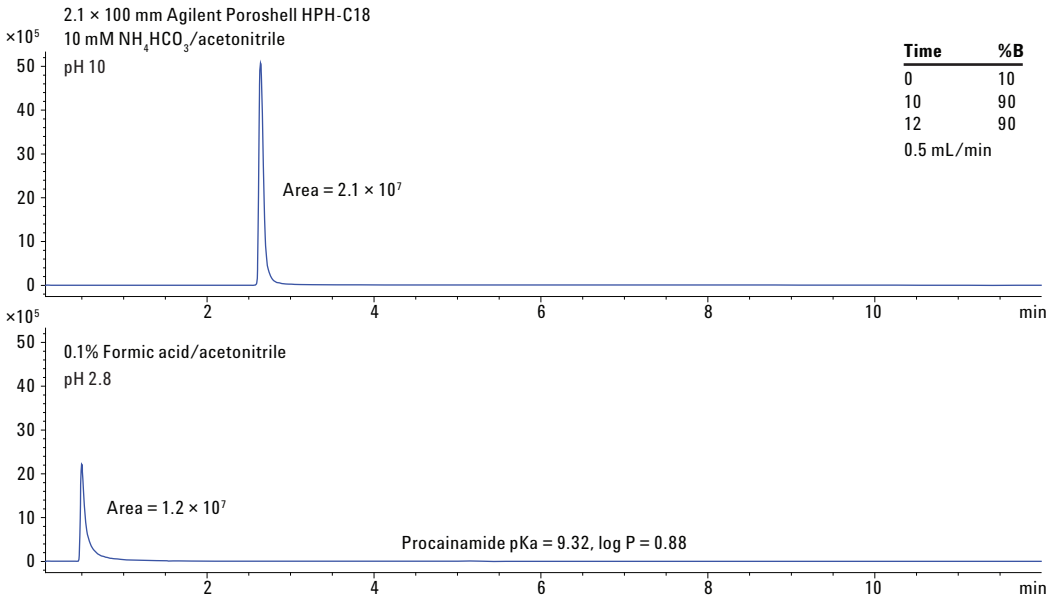

Figure 4A. Comparison of LC/MS of bases (procainamide, pKa 9.32, logP 0.88) in positive ion electrospray mode at high and low pH. Agilent Poroshell HPH-C18, 2.1 × 100 mm.

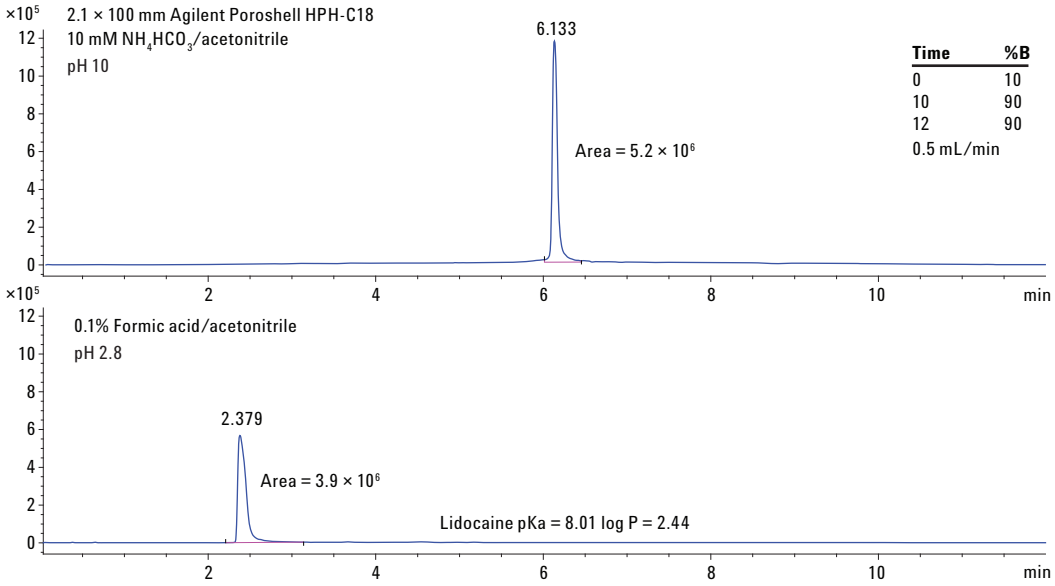

Figure 4B. Comparison of LC/MS of bases (lidocaine, pKa 8.01, logP 2.44) in positive ion electrospray mode at high and low pH. Agilent Poroshell HPH-C18, 2.1 × 100 mm.

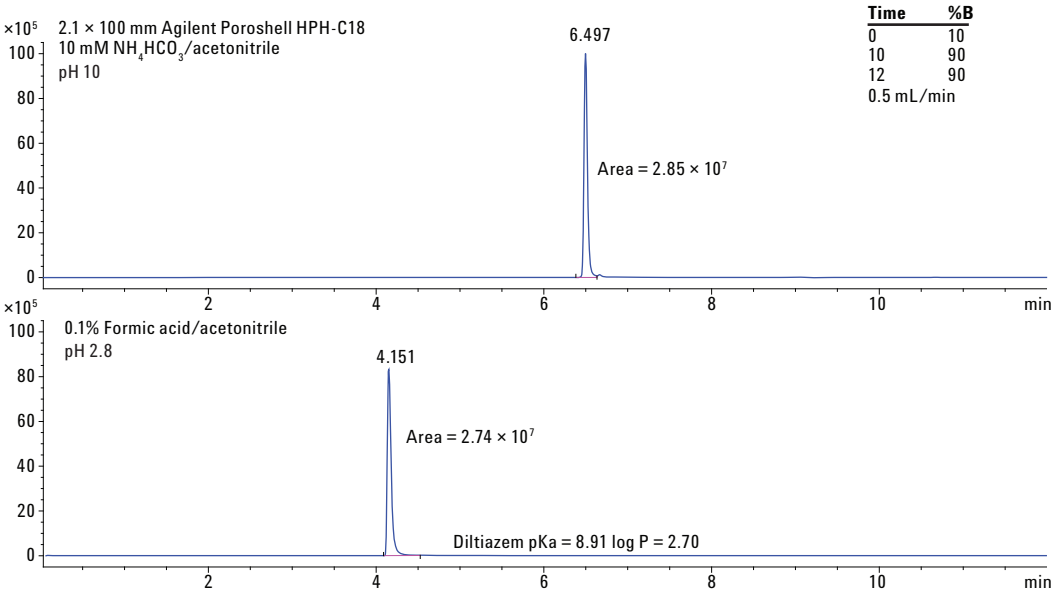

Figure 4C. Comparison of LC/MS of bases (diltiazem, pKa 8.91, logP 2.70) in positive ion electrospray mode at high and low pH. Agilent Poroshell HPH-C18, 2.1 × 100 mm.

Stability of Poroshell HPH-C18 at high pH

HPLC column stability is one of the critical factors affecting method performance and has been widely studied. Column stability can be affected by temperature, type of aqueous buffer and its concentration, choice of organic solvents, additives, and mobile phase pH. Prescreening of compounds and columns should enable scientists to arrive at successful separations more quickly. HPLC column stability is one of the critical factors affecting method performance. A robust HPLC method using a durable column leads to successful support of new clinical and manufacturing projects. A column that is not stable during method development leads to inaccurate results and frustration.

Column degradation is caused by silica dissolution, bonded-phase removal, or through the exposure of silanols by the removal of end capping (hydrolysis). Both dissolution and hydrolysis of silica columns are known to be related to pH and temperature (increased degradation rate at higher pH/temperatures). Other causes of column degradation include poor sample preparation (dirty samples) and column bed instability.

A good criterion for column stability under a given pH is 500 injections. This allows development, adjustment, and use for a column under an established method. In this section of the work, a Poroshell HPH-C18 column was evaluated in a gradient using ammonium bicarbonate and acetonitrile at pH 10. Acidic, neutral, and basic compounds were used. To evaluate columns from a variety of manufacturers, a common stress gradient was used while changing the analytes to accommodate differences in selectivity. In all cases, at least two acid, base, and neutral compounds were employed.

The protocol discussed here evaluated the impact of mobile phase modifier on column stability [11]. The impact of sample solution was minimal, as typically only a few µg of sample were loaded. The test mixture was chosen to assess column performance, not to assess the impact of the test probes themselves on column stability. A low flow rate was used to minimize column bed stability problems during development. As shown in Figure 5A, the retention time of all compounds remained stable throughout the 2,000 injections with the exception of nortryptyline. This compound, with a pKa very close to the pH of the mobile phase, moved slowly to give longer retention times.

A second column from another brand was subjected to the same experimental conditions. Most of the analytes remained at the same retention time throughout the 2,000 injections. Nortryptyline moved rapidly to later elution times. Within 500 injections, nortryptyline began to coelute with the next compound, neutral hexanophenone. The peak continued to migrate through this peak, totally coeluting by injection 2,000. This experiment indicated greater degradation of the non-Agilent column compared to the Poroshell HPH-C18 column. Differences in peak height occurred as the sample changed.

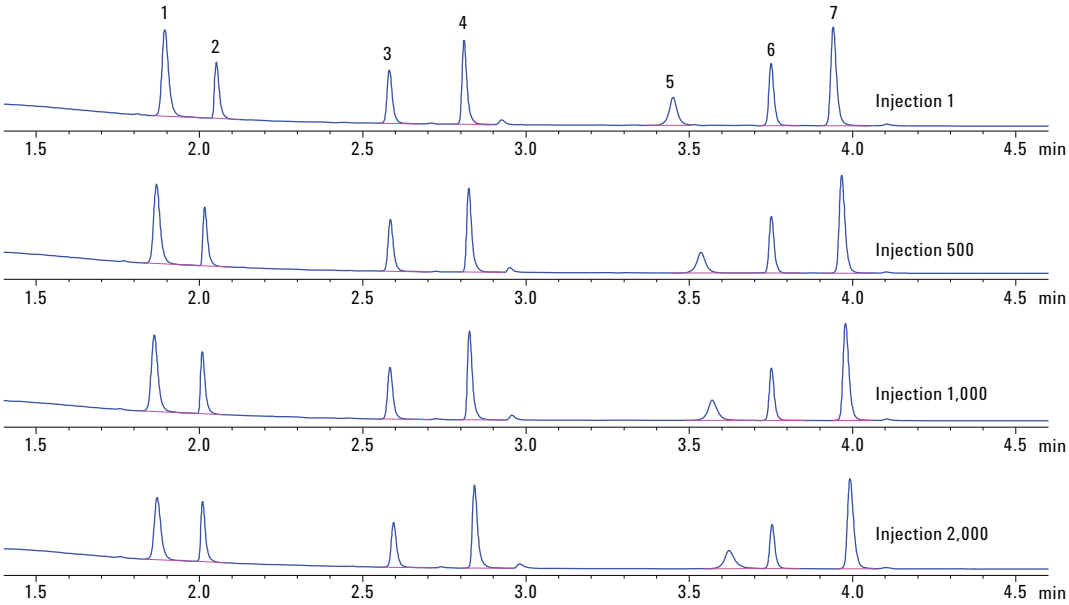

Figure 5A. Excellent retention on the Agilent Poroshell HPH-C18, 2.1 × 50 mm, 2.7 µm column even under high pH bicarbonate conditions, (total method run time = 7 minutes, flow rate 0.4 mL/min).

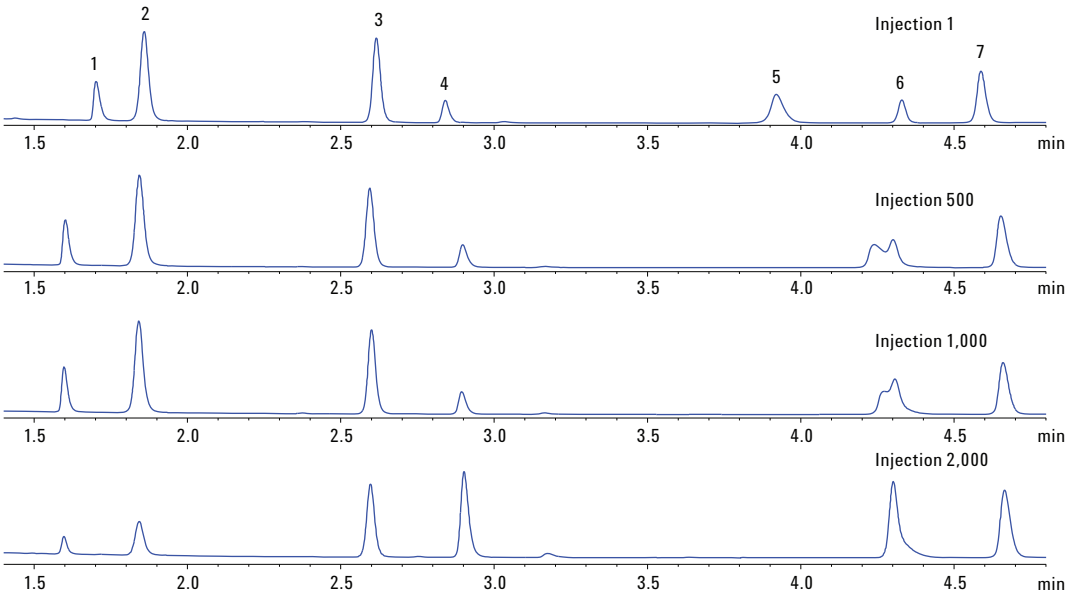

Figure 5B. A competitor 3 µm column suffered greater degradation under high pH bicarbonate.

Conclusion

Using an Agilent Poroshell HPH C18, pH can be used to adjust selectivity without sacrificing column lifetime at elevated pH. By keeping a gradient constant and altering pH, the elution order of a group of eight acid, base, and neutral compounds could be dramatically changed, and hence chromatographic resolution. In a second experiment, the correlation coefficient of the retention times was determined using a generic gradient plotted for pH 3 and pH 10. Using R^2 as a measure of orthogonality, we found that the two conditions offered different selectivity. Using pH as a method development tool was very effective, especially when the sample contained acidic or basic compounds. We also investigated positive ion electrospray mass spectrometry of several basic compounds using gradients HPLC at high and low pH. In this case, we showed that the peak shape of basic compounds improved and retention time increased. We also observed a signal increase as measured by the peak area. This effect was not the same in all cases and was likely to be compound-dependent. In no case was a signal decrease observed for bases at elevated pH. Finally, we determined that a Poroshell HPH C18 could be used for extended periods (over 2,000 injections) at pH 10 in ammonium bicarbonate at 25 °C. By using pH as a method development tool with a Poroshell HPH-C18, chromatographers can maximize flexibility in their method development and analyses, while still benefiting from the rugged and long lifetime of the Agilent Poroshell 120 family.

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Easy Method Transfer and Improved Performance with Agilent Poroshell 120 4 µm Columns

Application Note

Food Testing and Agriculture

Author

William Long
Agilent Technologies, Inc.

Abstract

A method for separating nine phenol compounds originally developed on a 4.6 × 100 mm, 5 µm column was transferred to Agilent Poroshell 120 EC-C18, 4.6 × 100 mm columns with 4 µm and 2.7 µm particles. The performance of these columns was compared to the performance of a similarly sized column with 1.8 µm totally porous particles. Gradient and flow rate were scaled, maintaining a constant retention index to determine the optimal flow rate for each column. By switching to a Poroshell 120 EC-C18 4 µm column and optimizing the gradient, the peak capacity increased from 50 to 67. Simple guidelines for transferring a method are provided. The pressure of the 4 µm method is below 200 bar and can be easily transferred to any HPLC system.

Agilent Technologies

Introduction

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or pathogens. In the last decade, there has been much interest in polyphenolss as dietary plants [1-3].

In food, polyphenols can contribute to bitterness, astringency, color, flavor, odor, and oxidative stability. All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid [4].

Phenolic-flavonoids found in plants include ellagic acid, catechol, gallic acid, quercetin, resorcinol, tannic acid, vanillin, salicylic acid, acetyl salicylic acid, and benzoic acid.

Environmental phenols are generated by industrial processes. These phenols include antioxidants used in plastics, pesticides, combustion of coal, petroleum and wood, and the manufacture of phenolic resins. In many cases, phenols are generated by natural processes. However, when these compounds are discharged to ground water, they can devastate many aquatic organisms. The analytical determination of phenolic compounds is, therefore, important because of the toxicity of these compounds and their widespread use [5,6].

For many years, the 5 µm HPLC column has been the technology standard. Many investigators were willing to sacrifice good efficiency and robustness for high pressure, unfamiliar instrumentation, and possible column clogging. Agilent Poroshell 120 4 µm columns can improve separating power over methods using similarly sized 5 µm columns. Poroshell 120 4 µm columns generate only slightly more pressure than 5 µm columns, and can be easily used on the same instrument. In addition, they use the same 2 µm frits found on 5 µm columns, making them robust against column clogging, and requiring no additional sample preparation.

In this work, a gradient method was transferred from a 4.6 × 100 mm, 5 µm column to a Poroshell 120, 4.6 × 100 mm, 4 µm column, or a Poroshell 120 2.7 µm column. Gradients were scaled to determine the flow rate at optimum peak capacity. Finally, the pressures of the experiments were compared, showing how Poroshell 120 columns can be easily used on any LC.

Experimental

An Agilent 1260 Infinity LC was used throughout this study. A G1312B Binary Pump SL was set up with mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in acetonitrile). The gradient is shown in Table 1, with time segments proportionally scaled relative to the flow rate for a constant retention index throughout the experiment. The pump was configured with the pulse damper and mixing column removed.

Table 1. Gradient program used with 4.6 × 100 mm columns.

% B	Time (min)						
5	4	2	1.33	1	0.8	0.67	0.34
40	34	17	11.33	8.5	6.8	5.67	2.84
40	40	20	13.33	10	8	6.67	3.34
5	42	21	14	10.5	8.4	7	3.5
5	50	25	16.67	12.5	10	8.34	4.17
Flow rate (mL/min)	0.5	1	1.5	2	2.5	3	3.5

A G1367C Automatic Liquid Sampler was used, with 20 µL injection volumes. The G1316B Thermostatted Column Compartment was set to 35 °C. A G4212A Diode Array Detector was set to 270,4 nm with a reference wavelength of 360,100 nm, incorporating a G4212-60008 flow cell, with 10 mm path and 1 µL capacity. Agilent Open Lab software version 1.05C was used to control the HPLC and process the data.

Columns

- Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 4 µm (p/n 695975-902)
- Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-902)
- Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 1.8 µm (p/n 959964-902)
- Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 5 µm (p/n 959996-902)

The compounds of interest are shown in Figure 1, with their respective structures. Compounds were dissolved in water at 1 mg/mL. Equal aliquots were combined to produce a mixed sample, which was diluted 1/10 in water. Thiourea was used as a void volume marker in all samples to determine t_0.

Thiourea, hydroquinone, resorcinol, phenol, 4-nitrophenol, *p*-cresol, *o*-cresol, 2,3 dimethylphenol, 2,5 dimethylphenol, 1-naphthol, and formic acid were purchased from Sigma-Aldrich, Corp. Acetonitrile was purchased from Honeywell. Water was 18 MΩ Milli-Q, made on site.

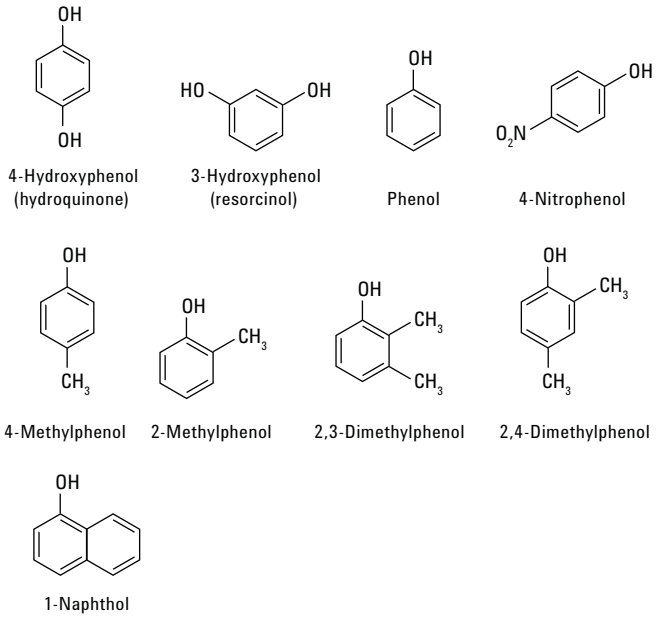

Figure 1. Compounds of interest.

Results and Discussion

The work by Coman and Moldovan [6] showed an excellent separation and quantitation scheme for phenols commonly identified in drinking and surface water. The objective in our work was to chromatographically improve the method, either by increasing peak capacity of the analysis, or by substantially shortening the chromatographic run time. The use of formic acid instead of acetic acid lowers the mobile phase pH. In addition, including formic acid in both the aqueous and organic mobile phases results in more level baselines. However, the k* value must be maintained when varying these column conditions so as not to change selectivity while gaining peak capacity. As shown in a previous note [6], the initial gradient was scaled, keeping column volumes constant and preserving method selectivity. In this case, the flow rate was varied between 0.5 and 3.5 mL/min at 0.5 mL/min intervals. Using Equation 1 as a guideline, the conditions listed in Table 1 were developed. These conditions were calculated manually and were the basis of the chromatographic programs used for the 100 mm columns. As can be seen, all steps in the program were proportionately shortened as the flow rate increased.

$$k^* = (t_g F)/(d/2)2L(\Delta\%B) \qquad \textbf{Equation 1}$$

Where:
t_g is the gradient time
F is the flow rate
L is the column length
d is the column diameter
Δ%B is the change in organic content across the gradient segment

A sample chromatogram is shown in Figure 2, including 1.8 µm and 5 µm totally porous ZORBAX Eclipse Plus C18, and 4 µm and 2.7 µm Poroshell 120 EC-C18. As can be seen, the elution order and relative spacing was the same for all four columns. However, the retention for the two superficially porous columns (4 and 2.7 µm) was slightly less than for the two totally porous columns (5 and 1.8 µm). This method was easily transferred between these columns.

Figure 3 demonstrates the relationship between pressure and particle size of the four columns. Larger particles generated lower pressure. Both the 5 µm column and the 4 µm Poroshell 120 column were under 200 bar using the conditions shown in Figure 2 (1.5 mL/min). Most methods with the 4 µm Poroshell 120 EC-C18 column will be run at this flow rate.

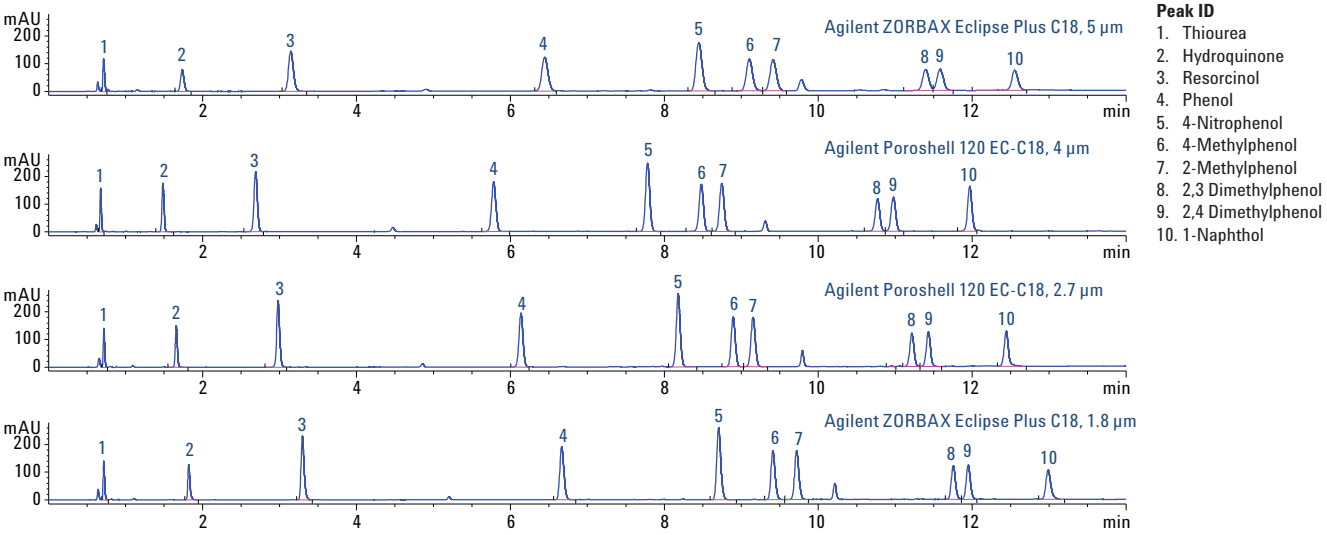

Figure 2. Overlay of chromatograms at 1.5 mL/min.

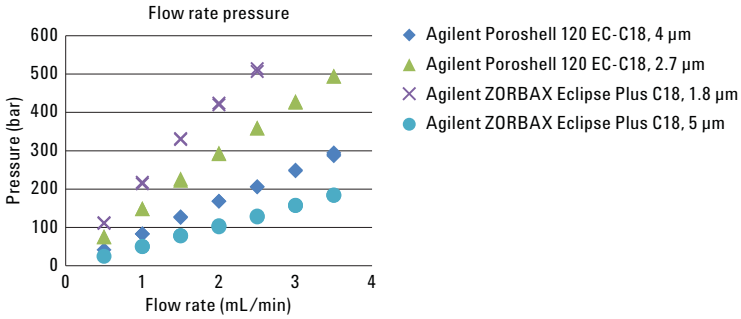

Figure 3. Pressure versus flow rate for different columns.

Conditional peak capacity nc = $(t_{R,n} - t_{R,1})/w$ **Equation 2**

Where:
$t_{R,n}$ and $t_{R,1}$ are retention times of the last and first eluting peaks
w is the 4σ peak width, = (W½ /2.35) × 4
W½ is peak width at half height

Peak capacity for each of the chromatograms is shown in Figure 4. The highest peak capacity was found for the 1.8 µm ZORBAX Eclipse Plus C18 column at 2.5 mL/min. It is possible that the peak capacity would have been higher at faster flow rates. However, this would have exceeded the system pressure limit of 600 bar. The 100 mm Poroshell 120 EC-C18, 2.7 µm column generated the next highest peak capacity, between 2 and 3 mL/min.

As can be seen in Figure 4, at lower flow rates the 2.7 µm Poroshell 120 and the 1.8 µm ZORBAX Eclipse Plus C18 had nearly identical peak capacities. The Poroshell 120 EC-C18, 100 mm, 4 µm column generated the next highest peak capacity, between 1 and 2 mL/min, a flow rate that is within the starting range of most chromatographers using 4.6 mm id columns. The 5 µm column has an optimal peak capacity between 1 and 1.5 mL/min. In general, with totally porous columns of the same dimensions, larger particle columns yield lower peak capacities at lower optimal flow rates.

Figures 5A and 5B show how resolution of two peak pairs changed on each column. Peak pairs 6/7 are 4-methyl phenol and 2-methyl phenol, Peak pairs 8/9 are 2,3-dimethylphenol and 2,4-dimethylphenol. These peak pairs of highly related compounds represent the type of problem most method development chemists face daily. As shown in Figure 5B, optimal peak resolutions were 2.4 at 2.5 mL/min for the 2.7 µm Poroshell 120, 2 at 2.5 mL/min for the 1.8 µm column, 1.9 at 1.5 mL/min for the 4 µm Poroshell 120, and 1.2 at 1.5 mL/min for the 5 µm column. As with peak capacity, larger particle columns yield lower optimal peak resolution. In addition, the optimal peak resolution of larger particles is found at lower flow rates.

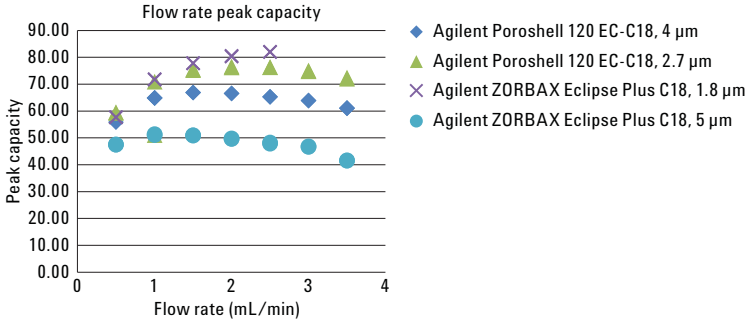

Figure 4. Optimization of peak capacity between 0.5 and 3.5 mL/min.

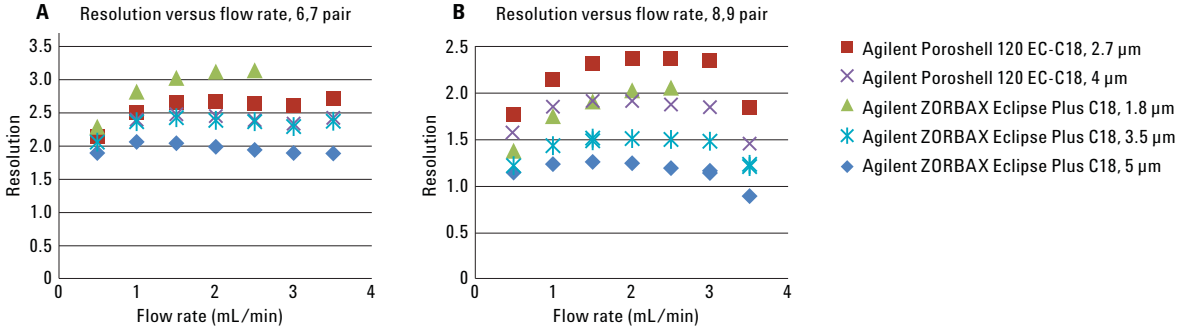

Figure 5. Resolution optimization between 0.5 and 3.5 mL/min.

Conclusions

HPLC columns packed with superficially porous particles offer many advantages over columns packed with conventional, fully porous particles. The Agilent Poroshell 120 EC-C18, 4 µm column offers a substantial increase of efficiency and peak capacity compared to 5 µm totally porous columns.

The superficially porous 2.7 µm Poroshell 120 EC-C18 offers similar efficiency and selectivity to the 1.8 µm Agilent ZORBAX Eclipse Plus C18, without the high backpressure. Due to the similar selectivity between Poroshell 120 EC-C18 and ZORBAX Eclipse Plus C18 columns, methods can be easily transferred to decrease run time, improve throughput, and increase peak capacity.

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

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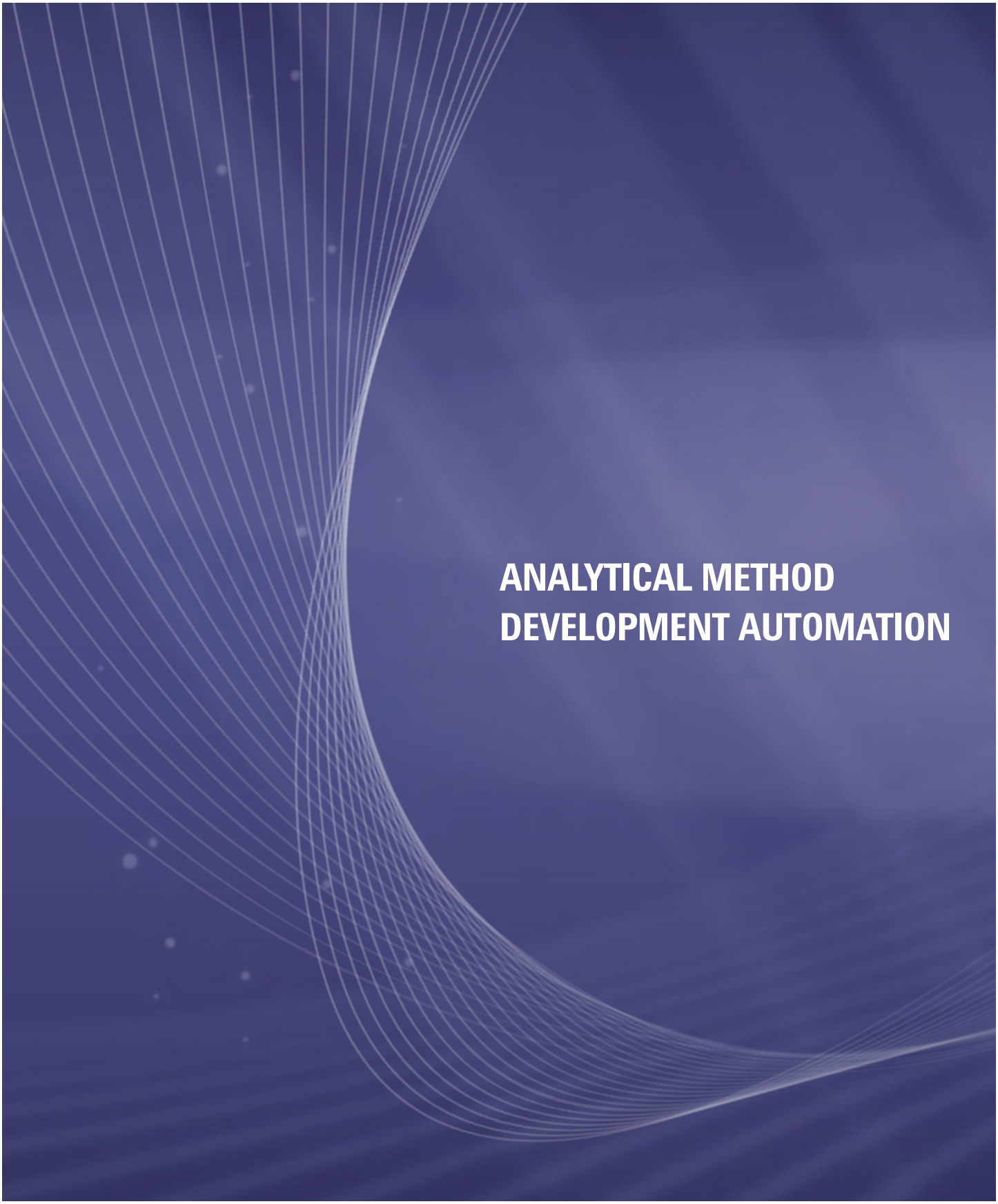

ANALYTICAL METHOD
DEVELOPMENT AUTOMATION

Universal Analytical Method Development for Various HPLC Systems Using the Agilent 1290 Infinity II Method Development Solution

On-The-Fly Target System Emulation Using Intelligent System Emulation Technology – ISET

Application Note

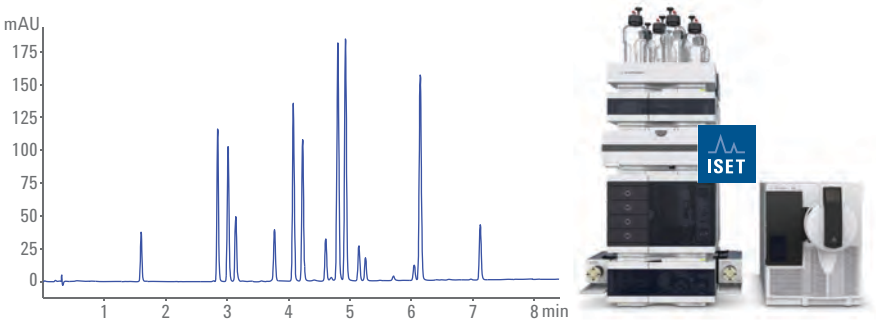
Small Molecule Pharmaceuticals

Authors

Edgar Naegele and Andreas Borowiak
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Application Note describes a combined approach to analytical method development using the Agilent ChemStation Method Scouting Wizard, and method transfer using Agilent Intelligent System Emulation Technology (ISET) for direct emulation of target HPLC systems. Based on the demonstrated success for highly dissimilar target systems such as the Agilent 1100 Series LC and Waters Acquity UPLC H-Class, the proposed workflow presents a general approach to develop analytical methods with the need for only one parent analytical method development system.



Agilent Technologies

Introduction

Today, analytical method development facilities are facing the challenge to develop LC methods for a high diversity of target systems used across different departments, or even within one analytical laboratory, because systems differ in manufacturer or LC generation. To overcome the need for a large number of method development systems, this Application Note presents a workflow that combines analytical method development with on-the-fly target system emulation using Agilent Intelligent System Emulation Technology (ISET). Ideally, the Agilent 1290 Infinity II Method Development Solution is used as a parent system, which develops analytical methods for different target systems without the need for manual system changes or dedicated analytical method development systems that can only address a limited number of target LCs.

In a previously published workflow, a UHPLC method was developed by mobile phase and column screening with subsequent transfer to standard HPLC conditions and ISET emulation of the target LC system[1]. In contrast, the workflow described in this Application Note directly develops the target system's analytical method using the 1290 Infinity II Method Development Solution and ISET emulation of the target LC system. Figure 1 shows a schematic overview of the workflow. First, columns, solvents, and temperatures are screened for suitable methods under ISET conditions. The initial screening is followed by a refinement campaign, which further optimizes the methods that showed the best separation regarding resolution and run time. After identification of a suitable separation analytical method, the method is transferred to the target system, and method robustness is tested over multiple injections.

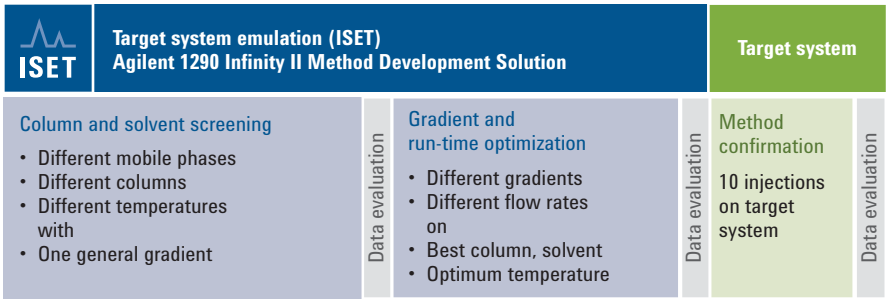


Figure 1. General workflow for the development of a chromatographic method directly towards a chosen target system by a combination of the Agilent ChemStation Method Scouting Wizard and Agilent Intelligent System Emulation Technology (ISET).

This Application Note demonstrates a workflow that combines the Agilent ChemStation Method Scouting Wizard and ISET for direct analytical method development towards a chosen target system. It shows that analytical method development for highly different target systems such as the Agilent 1100 Series LC and Waters Acquity UPLC H-Class is possible with one hardware setup. To test the proposed workflow, a complex sample comprising 15 compounds were used, and the resulting methods were compared for equivalency on the Agilent 1290 Infinity II Method Development Solution and the chosen target system.

Experimental

Instrumentation

The Agilent 1290 Infinity II Method Development Solution comprised the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A) with ISET enabled
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B)
- Agilent 6140 Single Quadrupole LC/MS (G6140B)

In addition, the following parts are required to run the 1290 Infinity II Method Development Solution for automated method development:

- Agilent InfinityLab Quick Change 8-column selection valve (G4239C)
- Agilent 1290 Infinity Valve Drive (G1170A) with InfinityLab Quick Change 12-position/13-port valve (G4235A)
- Low dispersion capillary kit, 0.12 mm id, p/n 5067-4248

Instrumental setup

The 1290 Infinity II Flexible Pump was clustered with an InfinityLab Quick Change 12-position/13-port valve for solvent selection in the Agilent OpenLAB CDS ChemStation Edition instrument configuration. The solvents were defined in the ChemStation pump setup dialog. The Agilent 1290 Infinity II Multicolumn Thermostat (MCT) was equipped with the InfinityLab Quick Change 8-column selection valve, and clustered in the ChemStation instrument configuration. All columns were used with column ID tags (p/n 5067-5917) for automated column recognition in ChemStation and assigned in the ChemStation MCT dialog. Methods necessary for column and gradient screening as well as instrument flushing and column equilibration were automatically created using of the Method Scouting Wizard. The emulation of the target systems was done using ISET.

The Agilent 1100 Series LC comprised the following modules:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Degasser (G1379A)
- Agilent 1100 Series Standard Autosampler (G1329A)
- Agilent 1100 Series Thermostatted Column Compartment (G1316A)
- Agilent 1100 Series Diode Array Detector (G1315B)

The Waters Acquity UPLC H-Class comprised the following modules:

- Acquity UPLC H-Class bio-Quaternary Solvent Manager
- Acquity UPLC bio-Sample Manager FTN
- Acquity UPLC Column Manager
- Acquity UPLC TUV Detector

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, version C.01.07 with Agilent ChemStation Method Scouting Wizard, version A02.06
- Agilent OpenLab CDS version 2.1 for control of Waters Acquity H-Class

Columns

For Agilent 1100 Series LC as target system:

- Agilent InfinityLab Poroshell EC-C18 USP L1, 4.6 × 150 mm, 2.7 µm, p/n 683975-902
- Agilent InfinityLab Poroshell EC-C8 USP L7, 4.6 × 150 mm, 2.7 µm, p/n 683975-906
- Agilent InfinityLab Poroshell Bonus-RP USP L60, 4.6 × 150 mm, 2.7 µm, p/n 693968-901
- Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 3.5 µm, p/n 959963-902

For Waters Acquity UPLC H-Class as target system:

- Agilent InfinityLab Poroshell EC C18, 2.1 × 100 mm, 1.9 µm, p/n 695675-902
- Agilent InfinityLab Poroshell EC PFP, 2.1 × 100 mm, 1.9 µm, p/n 695675-408
- Agilent InfinityLab Poroshell EC Phenyl-Hexyl, 2.1 × 100 mm, 1.9 µm, p/n 695675-912
- Agilent InfinityLab Poroshell EC C8, 2.1 × 100 mm, 1.9 µm, p/n 695675-906

Final methods

System	Agilent 1100 Series LC	Waters Acquity UPLC H-Class
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 3.5 µm	Agilent InfinityLab Poroshell EC PFP, 2.1 × 100 mm, 1.9 µm,
Temperature	40 °C	40 °C
Solvent	A) Water, 0.1 % (v:v) formic acid B) Acetonitrile, 0.1 % (v:v) formic acid	A) Water, 0.1 % (v:v) formic acid B) Acetonitrile, 0.1 % (v:v) formic acid
Flow Rate	1.7 mL/min	0.85 mL/min
Gradient	10 %B at 0 minutes 49 %B at 11.5 minutes 55 % B at 17 minutes	10 %B at 0 minutes 47 %B at 7.5 minutes 10 %B at 7.6 minutes
Stop time	17 minutes	9.5 minutes
Post time	3 minutes	None
UV Detection	254/10 nm, reference 360/100 nm, data rate 20 Hz	

Sample

As a test sample, a complex mixture of 15 pesticides and pharmaceuticals was used. The individual compounds were dissolved in acetonitrile (1 mg/mL) and finally mixed in equal amount. Table 1 outlines the compounds, their formulae, and masses.

Chemicals

All solvents were HPLC grade, and purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). All Chemicals were purchased from Sigma-Aldrich (Germany).

Result and Discussion

Target system: Agilent 1100 Series LC

The initial method development campaign for the separation of a complex sample on the Agilent 1100 Series LC as the target system was done as a column, solvent, and temperature screening on the Agilent 1290 Infinity II Method Development Solution using the Method Scouting Wizard and ISET. In this screening campaign, four standard HPLC columns (see Experimental section), two solvents (methanol and acetonitrile), and three temperatures (30, 40, and 50 °C) were used. The initial generic gradient went from 5 to 70 % organic solvent in 30 minutes. Figure 2 shows the best possible separation of the complex test sample, obtained after the initial Method Scouting Wizard screening campaign.

Table 1. Composition of the test sample (mixture of 1 mg/mL solutions of each compound in acetonitrile).

Name	Chemical formula	*m/z* [M+H⁺]
Atrazine-desethyl	$C_6H_{10}ClN_5$	188.06
Metoxuron	$C_{10}H_{13}ClN_2O_2$	229.07
Hexazinone	$C_{12}H_{20}N_4O_2$	253.16
Terbuthylazine-desethyl	$C_7H_{12}ClN_5$	202.08
Methabenzthiazuron	$C_{10}H_{11}N_3OS$	222.06
Chlorotoluron	$C_{10}H_{13}ClN_2O$	213.08
Atrazine	$C_8H_{14}ClN_5$	216.10
Diuron	$C_9H_{10}Cl_2N_2O$	233.02
Metobromuron	$C_9H_{11}BrN_2O_2$	259.00
Metazachlor	$C_{14}H_{16}ClN_3O$	278.10
Nifedipine	$C_{17}H_{18}N_2O_6$	347.10
Sebuthylazine	$C_9H_{16}ClN_5$	230.11
Terbuthylazine	$C_9H_{16}ClN_5$	230.11
Linuron	$C_9H_{10}Cl_2N_2O_2$	249.02
Nimodipine	$C_{21}H_{26}N_2O_7$	419.18

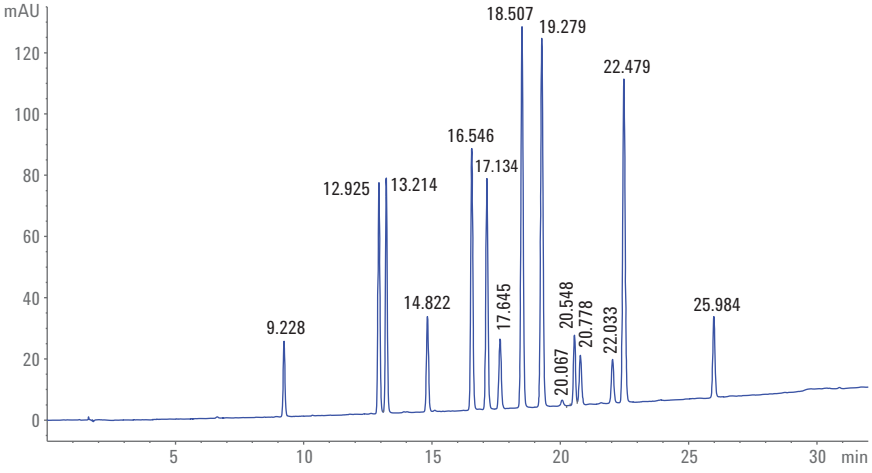


Figure 2. Best possible separation of the complex test sample, which was obtained after the initial Agilent ChemStation Method Scouting Wizard screening campaign. This screening campaign was run under ISET conditions set to the chosen target LC system, the Agilent 1100 Series LC.

To optimize this method, the initial percentage of organic solvent was set to 10 %, and the stop time and composition was set to 30 seconds after the last eluting compound. This method was optimized by a second campaign using gradients with increasing steepness and flow rates in fixed rates of 10 %, respective to flow rate and gradient time. To optimize the resolution of the critical pair of compounds, which eluted at 12.037 and 12.156 minutes in the final chromatogram, the slope of the gradient was decreased between 11.5 minutes and the end of the run at 17 minutes. In the final method, a compromise between speed and resolution of the critical pair was accepted (Figure 3).

To identify the compounds during the process of method development and optimization, their masses were tracked by the single quadrupole mass spectrometer. The method achieved after the final optimization was transferred directly to the target system, the 1100 Series LC, and 10 replicate injections of the sample were run (Figure 4).

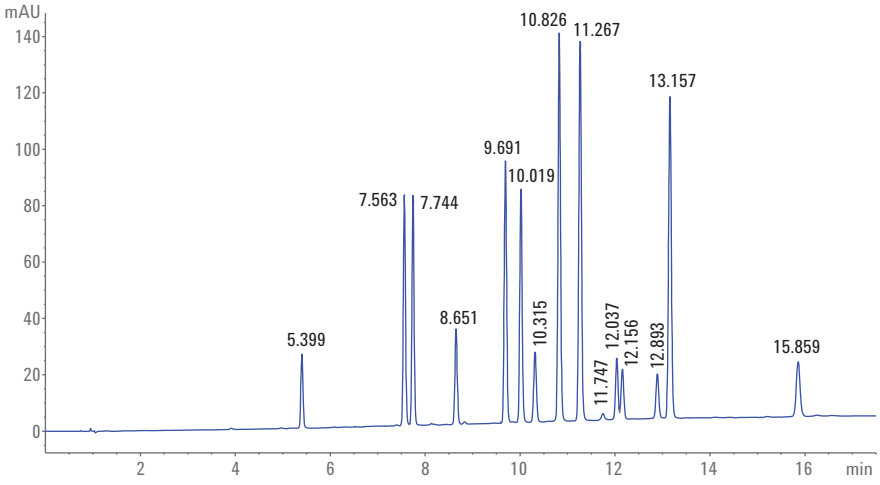


Figure 3. Best possible separation of the complex test sample, which was obtained after the refinement Agilent ChemStation Method Scouting Wizard screening campaign. This screening campaign was run under ISET conditions of the chosen target LC system, the Agilent 1100 Series LC.

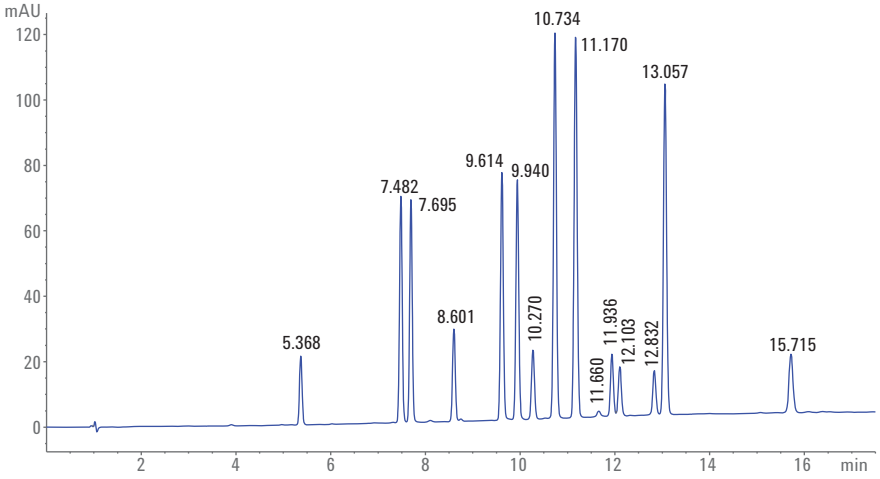


Figure 4. Final separation obtained on the target system, the Agilent 1100 Series LC.

After evaluation of the replicate runs on the target system, typical standard deviations of the retention times at or below 0.003 minutes could be found. The corresponding RSD values were typically below 0.03 %. The differences in retention time between the method development system and the target system were typically below 1 % (Table 2 and Figure 5). The complete method development time took approximately 35 hours for the first large screening campaign, approximately 8 hours for the optimization, and approximately 5 hours for the evaluation on the target system, which amounted to approximately 48 hours.

Table 2. Comparison of retention time, standard deviation and RSD values obtained on the Agilent 1290 Infinity II Method Development Solution and the target system, the Agilent 1100 Series LC (tr = retention time, $\bar{x}$ = average, σ = standard deviation, RSD = relative standard deviation).

No.	Compound	Agilent 1100 Series LC $\bar{x}$(tr) (min)	Agilent 1100 Series LC σ(tr) (min)	Agilent 1100 Series LC RSD (%)	Agilent 1290 Infinity II Method Development Solution $\bar{x}$(tr) (min)	Agilent 1290 Infinity II Method Development Solution σ(tr) (min)	Agilent 1290 Infinity II Method Development Solution RSD (%)	Δtr (%)
1	Atrazine-desethyl	5.365	0.002	0.035	5.401	0.002	0.037	0.7
2	Metoxuron	7.479	0.003	0.037	7.565	0.002	0.027	1.1
3	Hexazinone	7.692	0.003	0.037	7.745	0.001	0.016	0.7
4	Terbuthylazine-desethyl	8.599	0.002	0.027	8.653	0.002	0.022	0.6
5	Methabenzthiazuron	9.613	0.002	0.023	9.692	0.002	0.020	0.8
6	Chlorotoluron	9.939	0.002	0.023	10.021	0.002	0.019	0.8
7	Atrazine	10.269	0.002	0.020	10.316	0.002	0.016	0.5
8	Diuron	10.734	0.002	0.017	10.827	0.002	0.023	0.9
9	Metobromuron	11.170	0.002	0.020	11.269	0.003	0.024	0.9
10	Metazachlor	11.658	0.003	0.023	11.748	0.002	0.016	0.8
11	Nifedipine	11.935	0.003	0.025	12.039	0.002	0.017	0.9
12	Sebuthylazine	12.103	0.002	0.019	12.158	0.002	0.016	0.5
13	Terbuthylazine	12.833	0.002	0.019	12.895	0.002	0.016	0.5
14	Linuron	13.058	0.003	0.020	13.159	0.003	0.020	0.8
15	Nimodipine	15.718	0.004	0.027	15.861	0.003	0.019	0.9

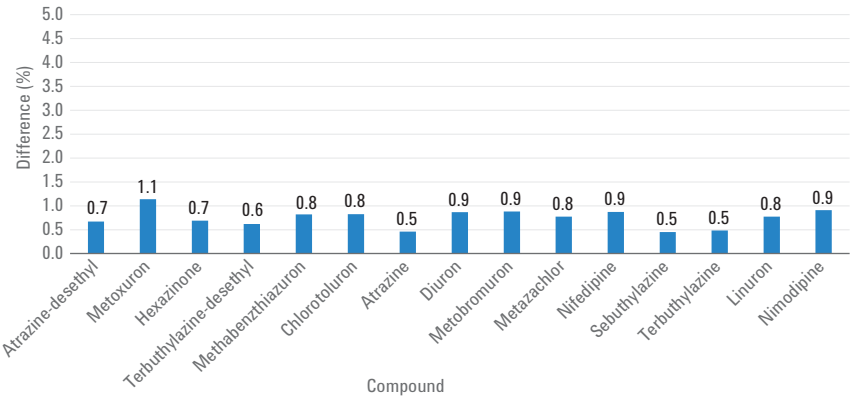


Figure 5. Retention time differences of the individual compounds in the comparison of the target system, the Agilent 1100 Series LC, with the Agilent 1290 Infinity II Method Development Solution.

Target system: Waters Acquity UPLC H-Class

For the development of a separation method suitable for the Waters H-Class as a target system, Agilent InfinityLab Poroshell columns with smaller particles (1.9 µm) were used. As a starting point, C8, C18, phenyl-hexyl, and pentafluoro-phenyl (PFP) phases were used. These columns had more typical dimensions used for UHPLC instruments (2.1 × 100 mm). The initial campaign was run with methanol and acetonitrile as organic solvents, and three different temperatures were tested. The initial generic gradient had a length of 20 minutes, and the organic solvent increased from 5 to 70 %. As expected, the columns with the C8 and C18 material showed a similar separation behavior as already obtained for the conventional LC method (data not shown). Surprisingly, the PFP stationary phase showed a dramatically earlier elution with slightly different selectivity compared to the C8 and C18 phases (Figure 6).

Because all the compounds were already separated, and the last peak eluted at a retention time of 12 minutes, this separation was taken for optimization. In a second campaign, different flow rates and gradients were tested to separate the pesticide sample on the PFP column in a shorter run time and with optimum resolution. Finally, the separation could be achieved in only 7.4 minutes applying a gradient from 10 to 47 % acetonitrile at a flow rate of 0.85 mL/min (Figure 7).

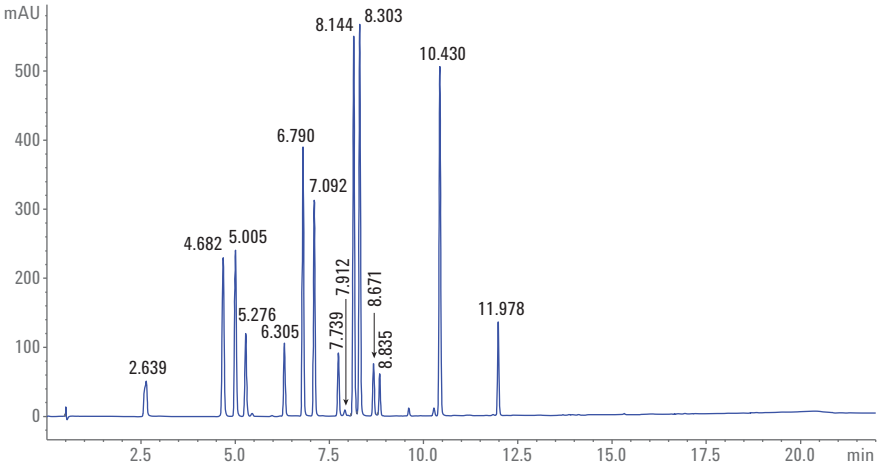


Figure 6. Separation of the pesticide sample on a PFP column with a gradient starting at 5 % acetonitrile and increasing to 70 % in 20 minutes at 40 °C. This screening campaign was run under ISET conditions set to the chosen target LC system, the Water H-Class.

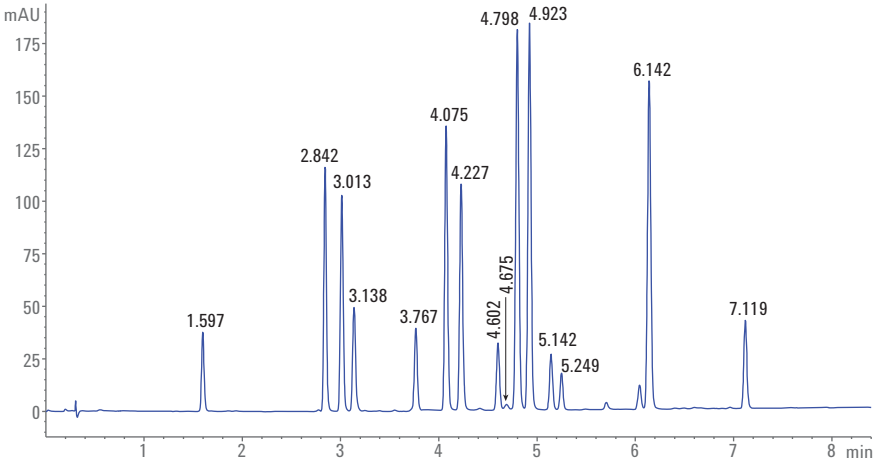


Figure 7. Final optimized separation of the pesticide sample on a PFP column with a gradient starting at 10 % acetonitrile and increasing to 47 % in 7.4 minutes at 40 °C. This screening campaign was run under ISET conditions set to the chosen target LC system, the Water H-Class.

This method was directly transferred to the target system, the Waters H-Class system, for evaluation. The identity of the retention times could be seen in the comparison of the chromatogram obtained on the 1290 Infinity II Method development system (Figure 7) and the Waters H-Class system (Figure 8).

For statistical evaluation, 10 replicate runs were done. As a result of this evaluation, typical standard deviations of the retention times below 0.01 minutes could be found. According to the short retention times, corresponding RSD values were typically below 0.2 %. The differences in retention time between the development system and the target system were typically below 2 % (Table 3 and Figure 9). The complete method development took approximately 37 hours.

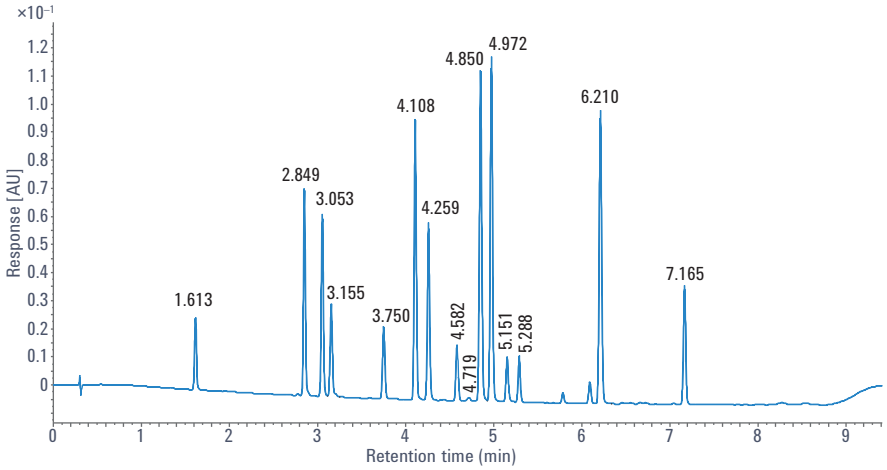


Figure 8. Final optimized separation of the pesticide sample on the Waters H-Class system using a PFP column and applying a gradient starting at 10 % acetonitrile and increasing to 47 % in 7.4 minutes with a flow rate of 0.85 mL/min, and a column temperature of 40 °C.

Table 2. Comparison of retention time, standard deviation and RSD values obtained on the Agilent 1290 Infinity II Method Development Solution and the target system, the Agilent 1100 Series LC (tr = retention time, $\bar{x}$ = average, σ = standard deviation, RSD = relative standard deviation).

No.	Compound	Waters H-Class $\bar{x}$ (tr) (min)	Waters H-Class σ(tr) (min)	Waters H-Class RSD (%)	Agilent 1290 Infinity II Method Development Solution $\bar{x}$ (tr) (min)	Agilent 1290 Infinity II Method Development Solution σ(tr) (min)	Agilent 1290 Infinity II Method Development Solution RSD (%)	Δtr (%)
1	Atrazine-desethyl	1.616	0.004	0.220	1.587	0.003	0.164	-1.8
2	Hexazinone	2.852	0.003	0.105	2.833	0.004	0.159	-0.6
3	Metoxuron	3.056	0.003	0.112	2.997	0.005	0.161	-1.9
4	Terbuthylazine-desethyl	3.157	0.003	0.101	3.126	0.005	0.168	-1.0
5	Atrazine	3.753	0.003	0.070	3.764	0.005	0.132	0.3
6	Methabenzthiazuron	4.110	0.003	0.0.62	4.062	0.005	0.113	-1.2
7	Chlorotoluron	4.261	0.002	0.055	4.213	0.005	0.114	-1.1
8	Sebuthylazine	4.585	0.002	0.046	4.601	0.005	0.104	0.3
9	Metazachlor	4.734	0.041	0.871	4.676	0.005	0.104	-1.2
10	Metobromuron	4.864	0.038	0.788	4.781	0.006	0.122	-1.7
11	Diuron	5.992	0.055	1.101	4.908	0.005	0.097	-1.7
12	Terbuthylazine	5.166	0.043	0.824	5.137	0.005	0.100	-0.6
13	Nifedipine	5.340	0.150	2.806	5.240	0.005	0.089	-1.9
14	Linuron	6.212	0.001	0.024	6.121	0.006	0.105	-1.5
15	Nimodipine	7.167	0.001	0.016	7.108	0.005	0.066	-0.8

Conclusion

This Application Note demonstrates the use of the Agilent 1290 Infinity II Method Development Solution with the Agilent ChemStation Method Scouting Wizard for the direct development of analytical separation methods under ISET control for a chosen target system. The analytical method development for the separation of a complex sample was done for an Agilent 1100 Series LC and a Waters Acquity UPLC H-Class as target systems. Both instruments showed excellent correlation between the method, which was developed on the Agilent 1290 Infinity II Method Development Solution, and the target systems. The retention time deviation was typically below 2 %. The time needed for the development of the method on the 1290 Infinity II Method Development Solution and their evaluation on the target system typically took two days or less.

Reference

1. Huesgen, A. G. Fast screening of mobile and stationary phases with the Agilent 1290 Infinity LC and seamless method transfer to an Agilent 1200 Series LC using ISET, *Agilent Technologies Application Note*, publication number 5991-0989EN, **2012**.

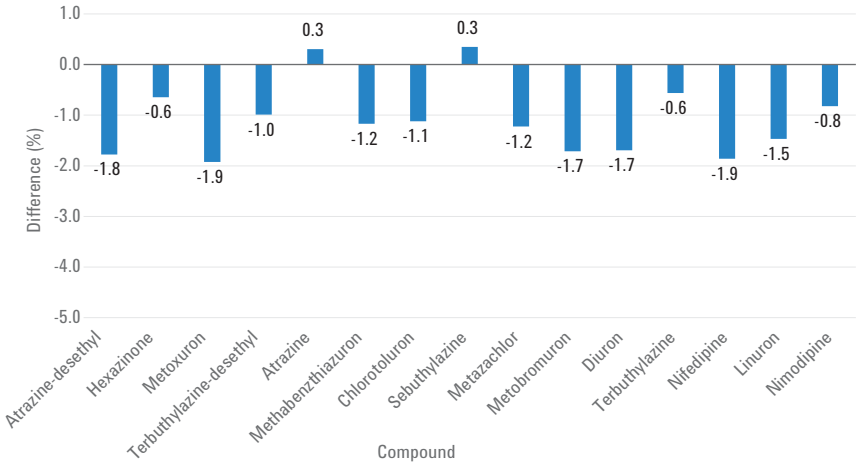


Figure 9. Retention time differences of the individual compounds in the comparison of the target system, the Waters H-Class, to the Agilent 1290 Infinity II Method Development Solution.

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Agilent Technologies

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

Authors

William J. Long
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Margaret Antler, Andrey Vazhentsev
Advanced Chemistry Development, Inc.
110 Yonge Street, 14th Floor
Toronto, Ontario M5C 1T4
Canada

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.

Agilent Technologies

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

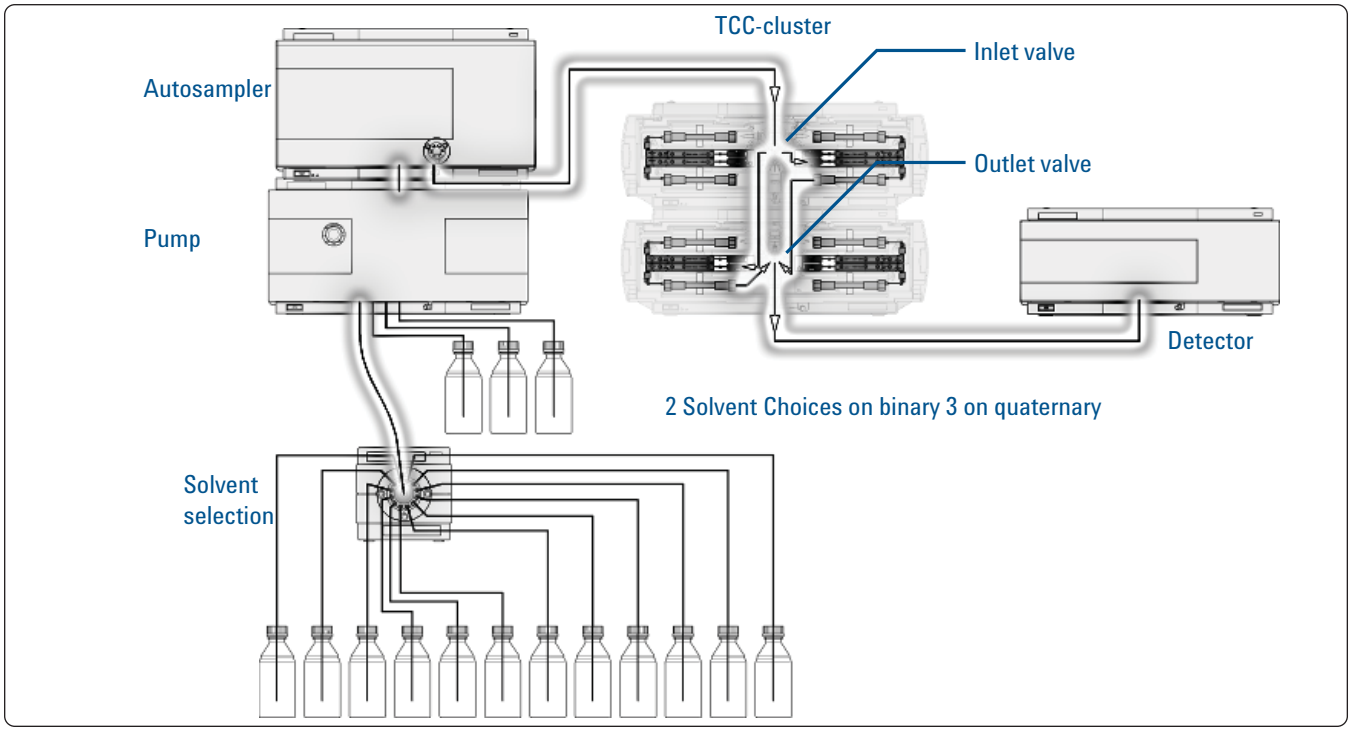

Figure 1. Instrument diagram.

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

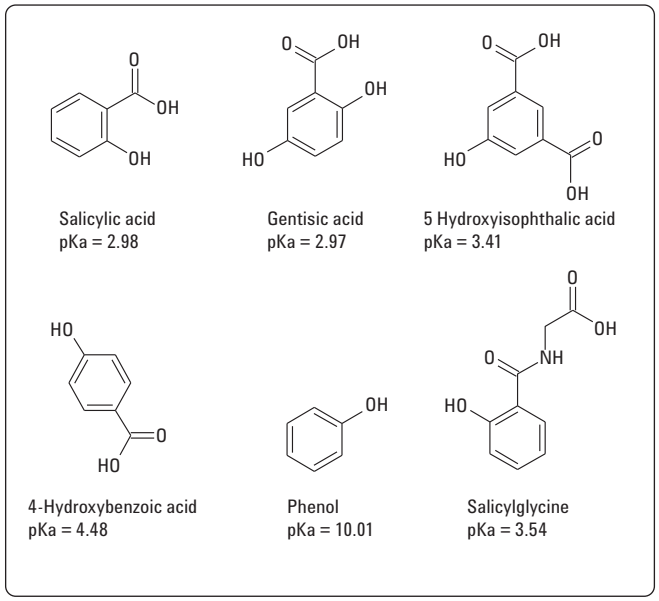

Figure 2. Structures and pKa values of the mixture components.

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.

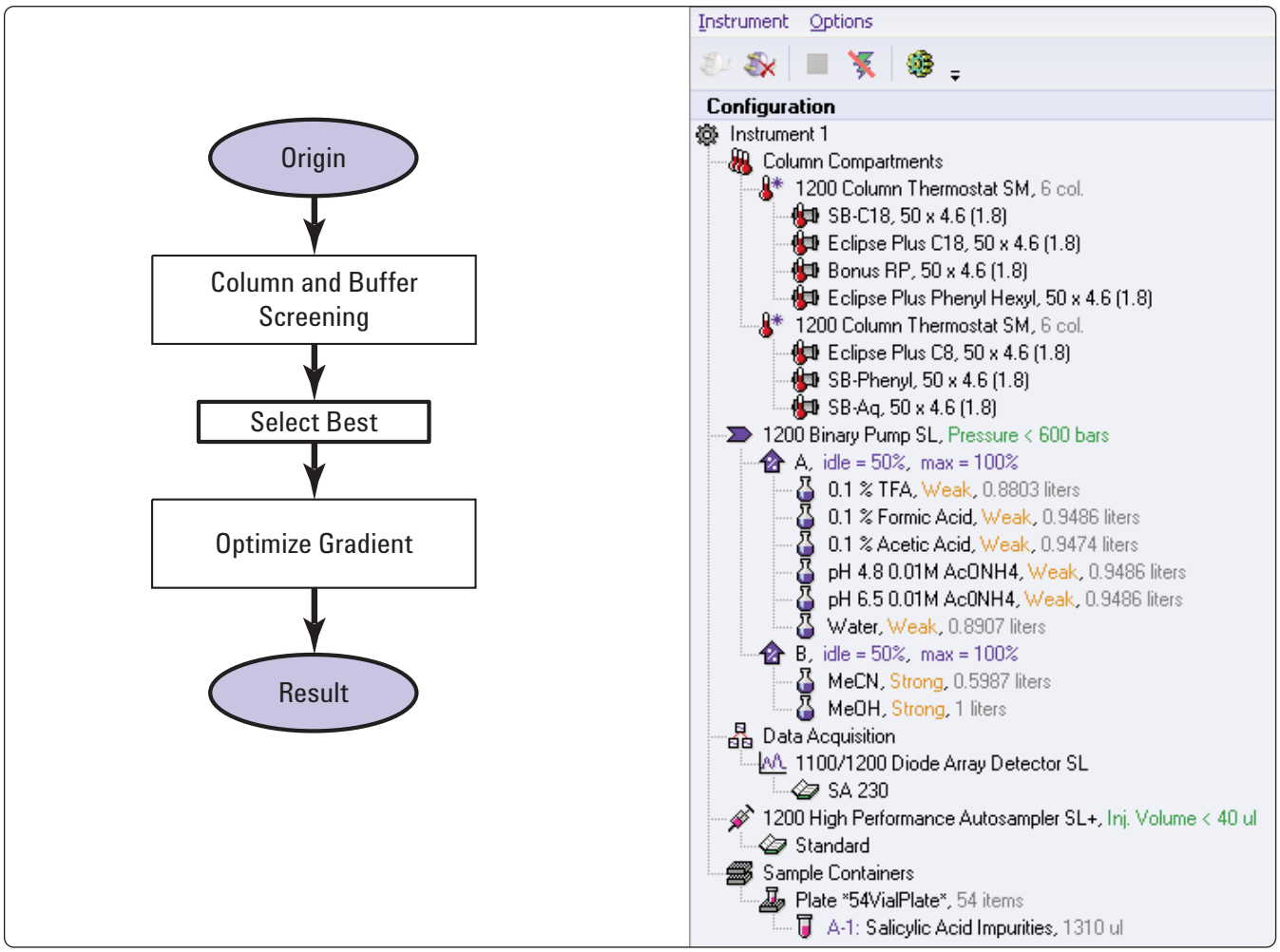

Figure 3. Autochrom strategy screenshot.

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

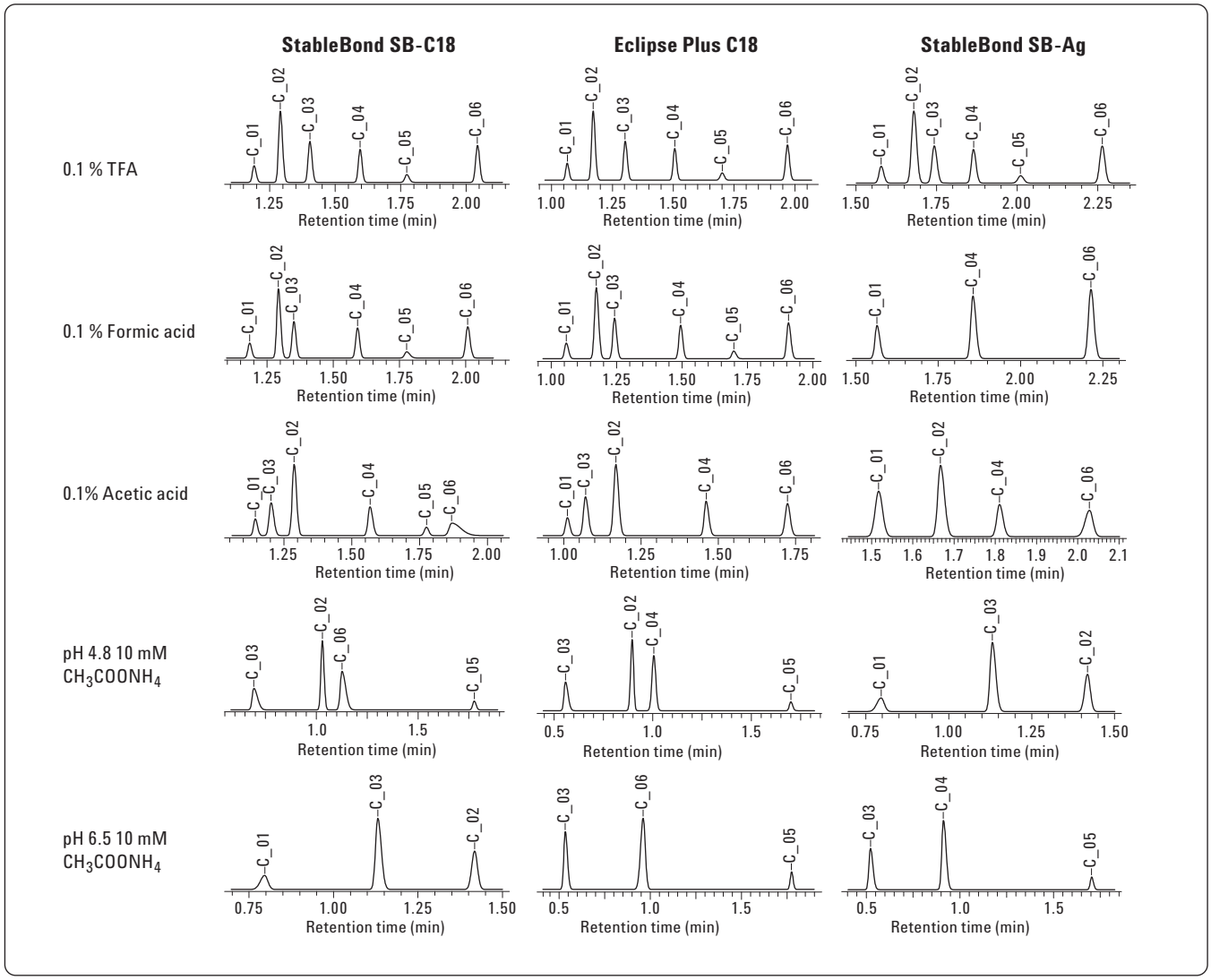

Figure 4. Fifteen Chromatograms from wave 1.

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

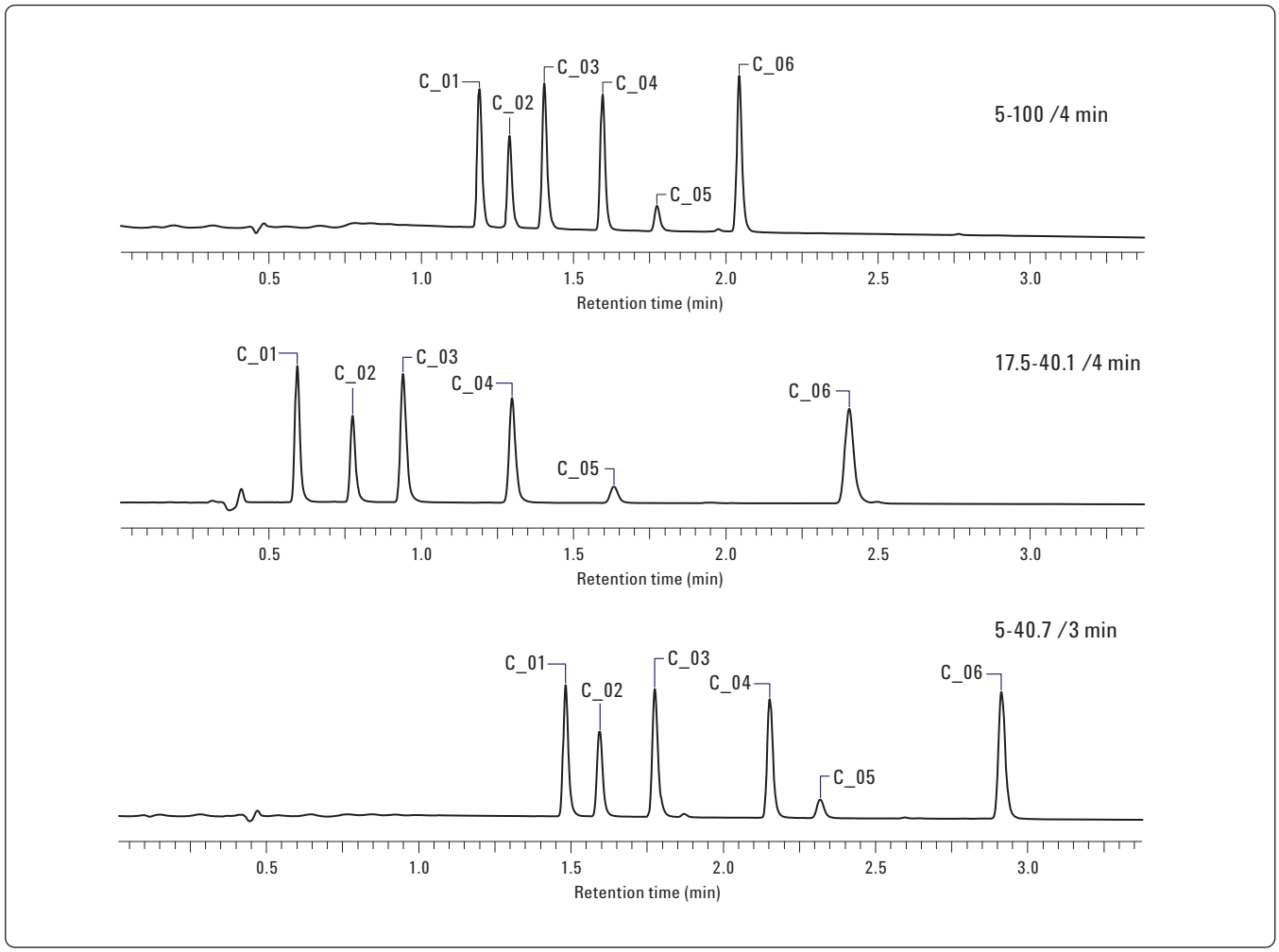

Figure 5. Experimental chromatograms from gradient optimization experiments.

	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

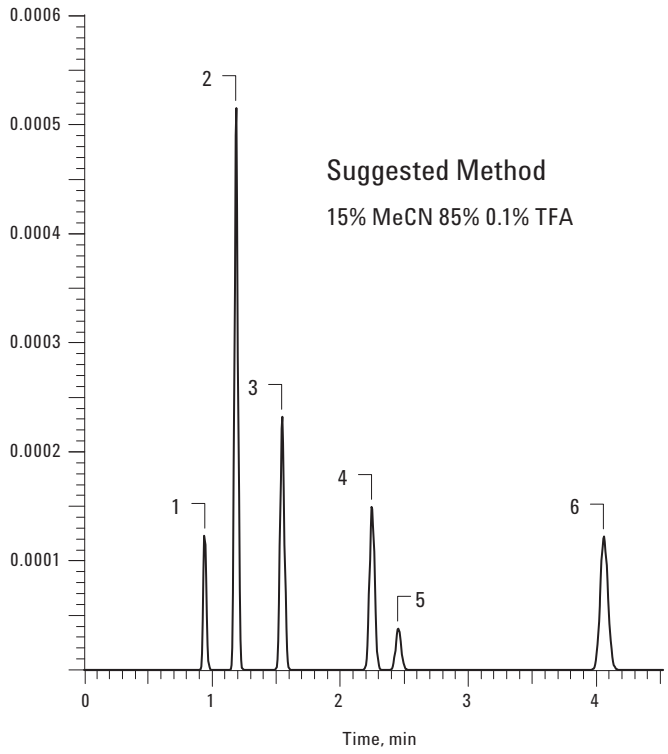

Figure 6. First three chromatograms and system suggested analysis (no model built, more data needed).

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.

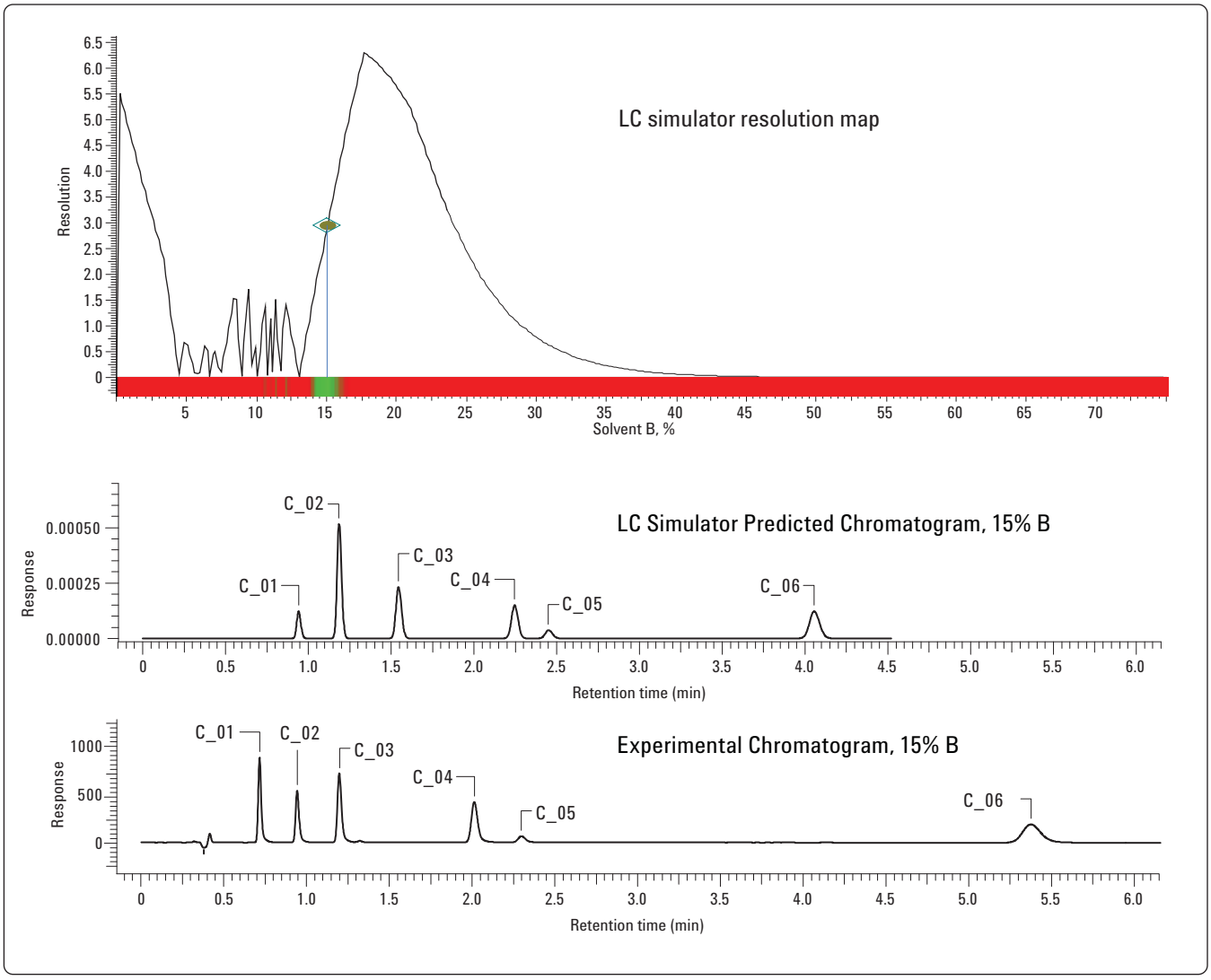

Figure 7. LC simulator resolution map and predicted chromatogram, compared with experimental chromatogram from ChemStation.

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.

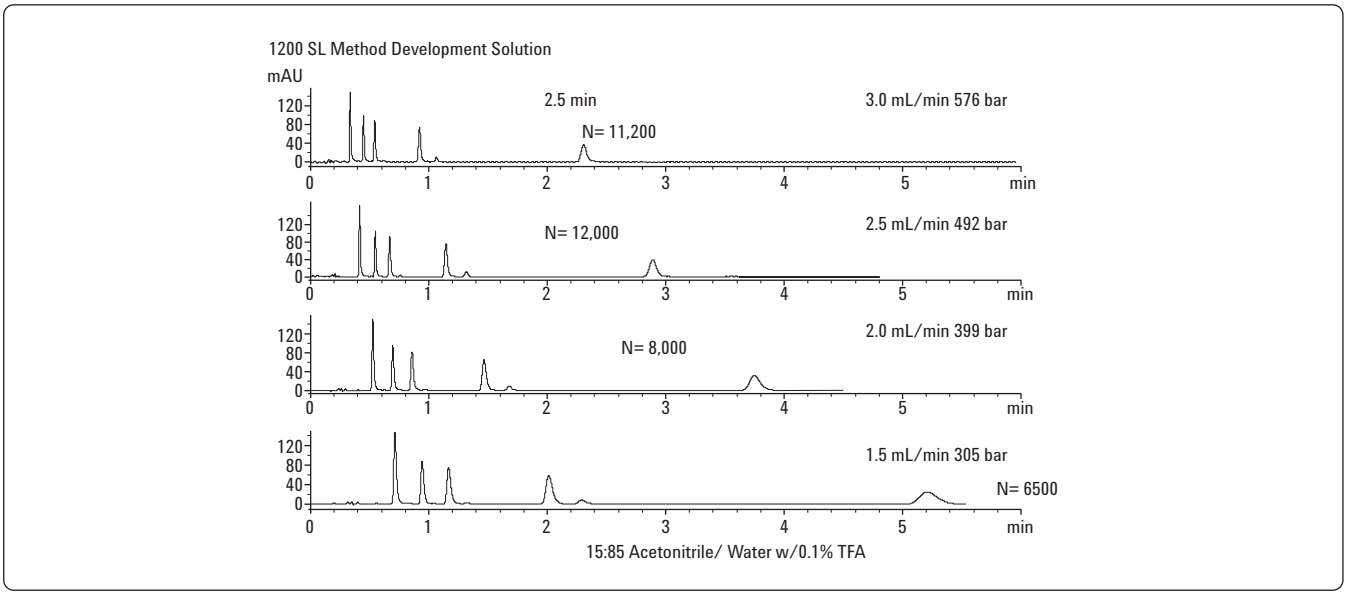

Figure 8. Four chromatograms run at different flow rates between 1.5 ml/min and 3 ml/min.

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Quality-by-Design-Based Method Development Using an Agilent 1290 Infinity II LC

An Efficient Method Development Workflow Combined with ISET-mediated Method Transfer Under Waters Empower 3 CDS Control

Application Note

Pharmaceutical Developments and QA/QC

Authors

Vinayak A.K and Andreas Tei

Agilent Technologies, Inc

Abstract

This Application Note demonstrates an UHPLC method for the separation of Amlodipine and its known EP impurities based on Quality by Design (QbD) principles. This method was translated and transferred in a second step for use on HPLC systems. Agilent Instrument Control Framework (ICF) was used as an interface to control the Agilent 1290 Infinity II LC by Waters Empower 3 chromatography data system (CDS). Fusion QbD (S-Matrix Corp, Eureka, CA) software was integrated to realize a QbD-based method development process.

The method, developed on a sub-2 µm column under UHPLC conditions, was translated using a freeware method translator tool into routine QA/QC workflows where HPLC systems are in use. For further optimization and evaluation processes, the performance characteristics of the target HPLC system was emulated using Agilent Intelligent System Emulation Technology (ISET) on an Agilent 1290 Infinity II method development system. After the transfer to the target system, all Critical Method Attributes (CMAs) were met, and the reproducibility was verified.

Agilent Technologies

Introduction

Quality by Design (QbD) based method development and method validation aligned with the ICH Q8 (R2) and ICH Q2 (R2) guidance is getting more attention in the pharmaceutical Analytical R&D community[2]. During the screening phase of different column chemistries, efficiency can dramatically be increased when using UHPLC methods on short, sub-2 µm columns. However, the final method may need to be transferred to QA/QC departments where most of the LC systems are conventional HPLC systems. Transferring a method from UHPLC to HPLC without compromising the critical method attributes (CMAs) is a challenging process[2]. A method developed on a UHPLC system, even when done using conventional HPLC columns, may not provide the same performance when transferred to an HPLC system due to differences in system delay volumes and gradient mixing precision. To overcome these issues, Agilent Intelligent System Emulation Technology (ISET) has been developed to emulate the properties of commonly used target systems[3].

This Application Note demonstrates the use of the Agilent 1290 Infinity II LC as a versatile UHPLC solution for robust QbD-based method development processes as well as the use of a third-party QbD software (Fusion QbD) with the 1290 Infinity II LC under Waters Empower 3 CDS. Finally, it demonstrates how Agilent ISET can be used to emulate the performance characteristics of different target LC systems that are frequently used in QA/QC environments under third-party software control.

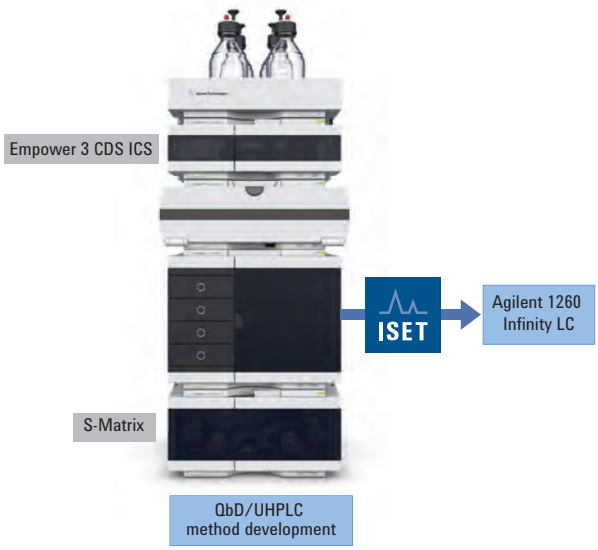

Figure 1. Agilent Intelligent System Emulation Technology-mediated method transfer under Waters Empower 3 CDS control.

Experimental

Instrumentation

An Agilent 1290 Infinity II LC method development system was used for method development. The individual modules and components of the 1290 Infinity II method development solution were:

- Agilent 1290 Infinity valve drive (G1170A) and 12 position/13-port solvent selection valve (G4235A)
- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) maintained at 4 °C
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with 8 pos/18 port column selector valve (5067–4233)
- Agilent 1290 Infinity II Diode Array Detector (G7117B)

Minimum firmware requirements for all Agilent 1290 Infinity II modules are: B, C, and D.06.70

An Agilent 1260 Infinity LC was used to verify the reproducibility of transferred method. The individual modules of the 1260 Infinity LC were:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity Autosampler (G1367E)
- Agilent 1260 Infinity Thermostated Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector(G4212B)

Software

- Fusion QbD Automated LC Method Development Software (S-Matrix Corp, Eureka, CA) (Version: 9.7.1, Build 458)
- Waters Empower Software (Version 3 build 3471) - with system suitability package.
- Waters Instrument Control Software (ICS) 2.1 HF1 includes Agilent ICF and driver package (A.02.03 DU1 HF2)
- ISET 4 (Driver Version A.02.11)

Reagents and samples

All solvents were HPLC grade (RCI Labscan Ltd, Thailand). Standards of Amlodipine Besylate (API) and the known EP impurities A, B, D, E, F, and G were obtained from Anant Pharmaceuticals Pvt Ltd, India. European pharmacopeia (EP) sample preparation protocol for Amlodipine Besylate was followed for the entire experiment, in which the API is spiked with known impurities[4].

Workflow

The method development workflow began with a screening process to determine the best chromatographic separation conditions using the Amlodipine Besylate standard and impurities on seven short sub-2 µm columns combined with two organic solvents and seven different pH levels (aqueous solvents) as liquid phases. This column chemistry screening experiment was performed using a 1290 Infinity II LC method development system and Fusion QbD Software under Empower 3 control (Figure 2). The chromatographic conditions found to be best (meeting the Analytical Target Profile (ATP) requirement of the screening phase) after the initial screening phase were further optimized by multivariate statistic experiments creating a design space according to QbD principles, creating a robust UHPLC method (satisfying the ATP requirement of the optimization phase).

The UHPLC method was transferred in a second step to two HPLC columns having different particle sizes. To mimic the performance characteristics of the target system, the Agilent 1290 Infinity II UHPLC was operated in emulation mode after activating the ISET tool. The gradient mixing behavior and autosampler delay volume of the Agilent 1260 Infinity system were emulated.

The performance results of the Agilent 1290 Infinity II LC in emulation mode were compared with the results from the target system. The reproducibility of RT, area, and resolution of system suitability impurities (impurities B and G) and API were determined.

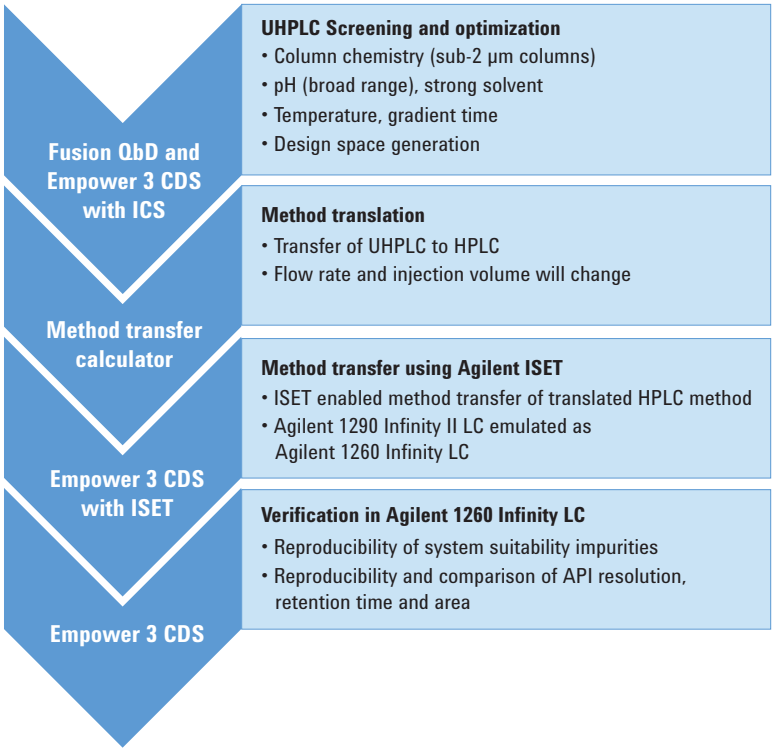

Figure 2. Overall workflow used for the study. The software packages used are shown on the left side of the flowchart, while detailed steps of the workflow are shown on the right side.

Results and Discussion

UHPLC Screening and optimization

The ATP of the screening phase was to develop a fast UHPLC method that meets the system suitability criteria of the EP method (resolution between Amlodipine impurities B and G should be greater than 2.0). Table 1 shows that, to achieve this, various sub-2 µm column chemistries, a broad range of pH, and organic solvents (ACN and MeOH) were screened. Table 2 shows the column chemistry screening experiment that provided the best overall chromatographic conditions. The chromatographic performance at this condition was found to be satisfactory, and all ATP criteria were met (Figure 3).

Table 1. The critical method parameters (CMPs) used in the screening phase experiments. Seven pH buffers, seven columns, three different flow rates, and two strong organic solvents were screened.

CMP	Range/Level(s)
Strong solvent type	Methanol, acetonitrile
Pump flow rate (mL/min)	0.8, 1.0, 1.2
pH	2.00 – 10 mM Trifluoroacetic acid 3.00 – 20 mM Formic acid 4.00 – 5 mM Formic acid + 10 mM ammonium formate 5.00 – 5 mM Acetic acid + 10 Mm ammonium acetate 7.00 – 10 mM Ammonium acetate 8.00 – 10 mM Ammonium hydrogen carbonate 9.00 – 10 mM Ammonium acetate+ 5 mM ammonia
Column type (3.0 × 50 mm, 1.8 µm)	Agilent ZORBAX Eclipse plus c18 Agilent ZORBAX Eclipse plus c8 Agilent ZORBAX SB Aq Agilent ZORBAX Eclipse Plus phenyl hexyl Agilent ZORBAX SB CN Agilent ZORBAX SB C18 Agilent ZORBAX Bonus RP*

*Column diameter used for Bonus RP column was 2.1 mm

Table 2. The best conditions for CMPs in screening phase experiments.

CMPs	Level setting
Strong solvent type	Acetonitrile
Pump flow rate (mL/min)	1.200
pH	2.00
Column type	Agilent ZORBAX Eclipse plus C8

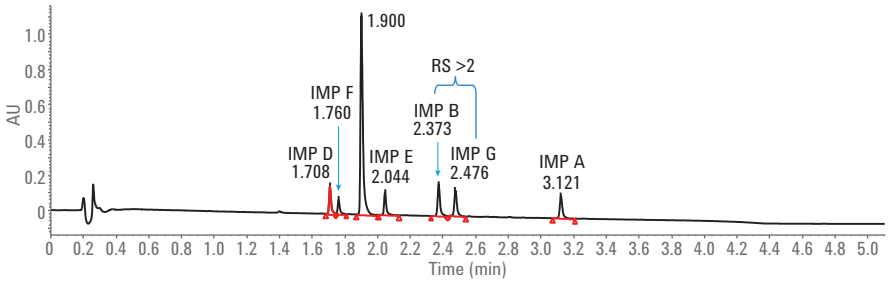

Figure 3. Resolution between impurities B and G was greater than 2, which met the ATP criteria of the screening phase.

The ATP for the optimization phase was to reduce the run time of the best condition of the screening phase, without compromising the system suitability criteria of the EP method and the resolution of API and other impurities. Critical method parameters (CMPs) such as pump flow rate, gradient time, and oven temperature were varied, as mentioned in Table 3. Data analysis of these experiments leads to a robust design space (Figure 4), which meets the previously established ATP criteria. The proven acceptable region (PAR), aligned with the ATP goal, was drawn in the design space. The resolution values of system suitability impurities were plotted for the five different conditions in the PAR (Table 4) and the respective chromatograms (Figure 5). The point prediction tool of Fusion QbD predicted the values of critical method attributes (CMAs), and compared and verified the experimental values (Table 5). The reproducibility of the final UHPLC method after optimization was verified, and an overlay of six replicates was plotted (Figure 6).

Table 3. CMPs varied in optimization phase.

CMP	Range/Level(s)
Pump flow rate (mL/min)	1.200–1.500
Gradient time (min)	1.0 ≤ Gradient time ≤ 4.0
Final hold time (min)	0.5 ≤ Final hold time ≤1.5
Oven temperature (°C)	25.0, 30.0, 35.0

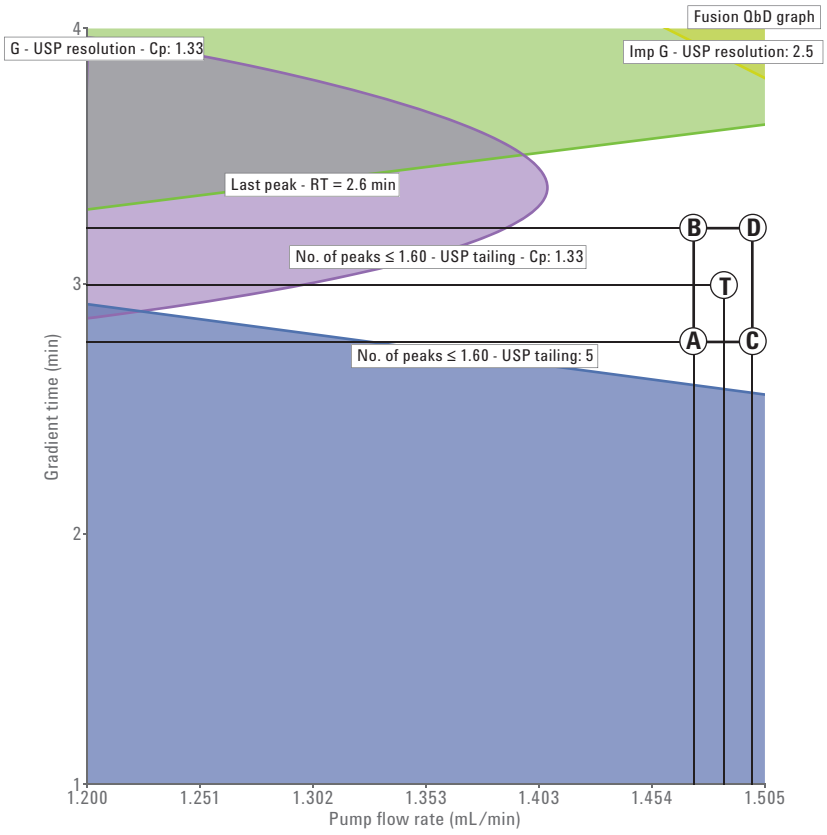

Figure 4. Final design space showing the PAR.

Table 4. CMPs and resolution of system suitability impurities reflecting five points of the PAR.

Conditions	Flow rate	Grad time	Final hold time	Oven temperature	Resolution b/w impurities B and G
A	1.47	2.76	0.5	30	3.28
B	1.47	3.08	0.5	30	3.16
T – Center point	**1.48**	**2.92**	**0.5**	**30**	**3.20**
C	1.50	3.08	0.5	30	3.22
D	1.50	2.92	0.5	30	3.10

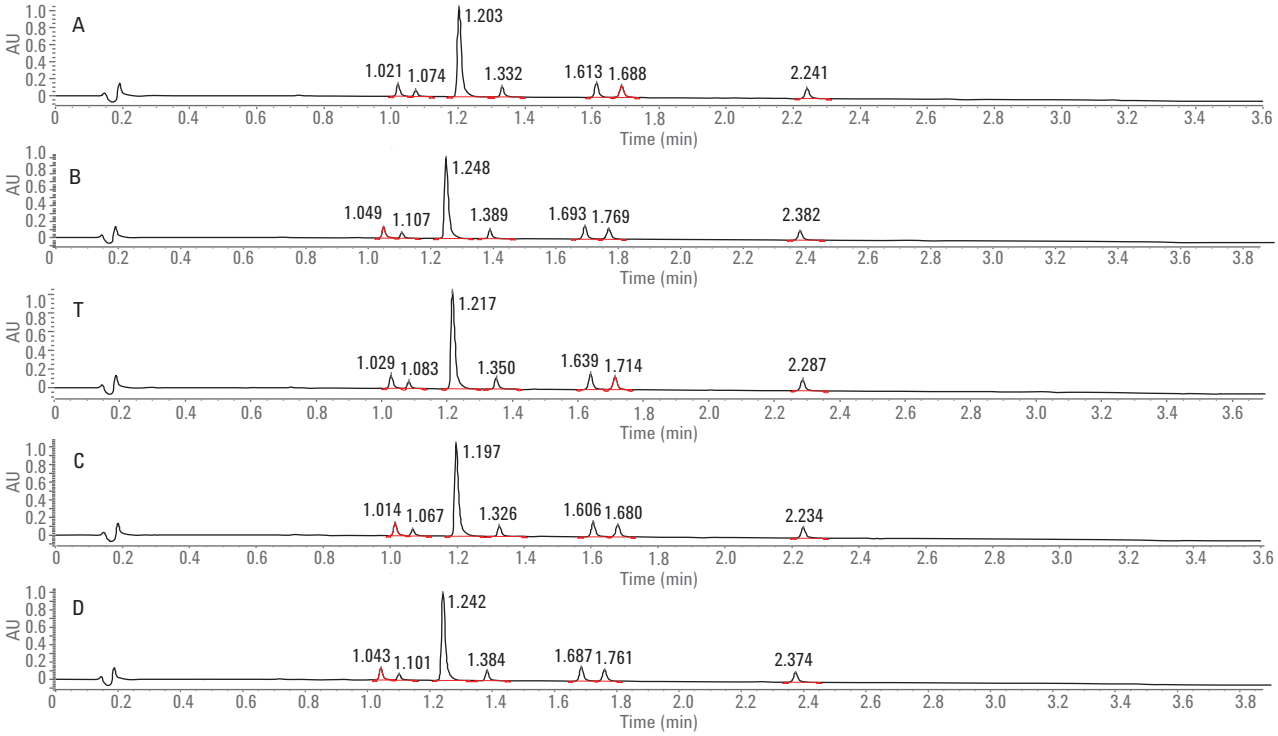

Figure 5. Chromatograms reflecting the conditions of five points (A, B, T, C, D) in PAR.

Table 5. Fusion QbD software-predicted response CMA values from the center point (T) of the PAR. The experimental results were compared with predicted values, and found to be within the Sigma confidence limit.

CMA	Predicted	Experimental	–2 Sigma confidence limit	+2 Sigma confidence limit
No. of peaks ≥2.00 – USP resolution	6.14	6.00	5.68	6.60
No. of peaks ≤1.60 – USP tailing	5.80	6.00	5.06	6.65
Last peak - retention time	2.28	2.28	2.28	2.29
USP Resolution b/w impurities B and G	2.97	3.20	2.90	3.20

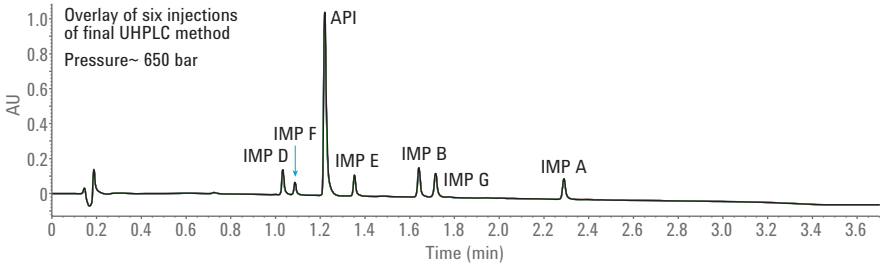

Figure 6. Reproducibility of final UHPLC method, using an overlay of six chromatograms.

Method translation and transfer

The UHPLC method developed on sub-2 µm columns was translated into three different HPLC methods using conventional particle sizes. The Microsoft excel-based method translation calculator from the University of Geneva was used for this purpose[5]. Initially, the UHPLC method was translated to the HPLC method (Agilent ZORBAX Eclipse Plus, 4.6 × 150 mm, 3.5 µm as column 1) with a reasonable run time of 27 minutes (Table 6). This method was evaluated on the 1290 Infinity II system using the ISET emulation mode of the target system (an Agilent 1260 Infinity LC), and later verified with the results of a 1260 Infinity LC system (Figure 7). The emulated solvent delivery module and autosampler were G1312B v1.0 and G1367E-100 µL syringe v1.0. The method transfer to column 1 (HPLC method 1) was achieved without compromising the ATP criteria, with a reasonable run time, however, the observed pressure range was approximately 300 bar (70 % of the pressure limit of conventional HPLC pumps). This might be a point of concern for users of legacy HPLC systems having pressure limitations. As a result, the UHPLC method was translated into HPLC method 2 using a column with larger particle sizes (column 2 = ZORBAX Eclipse Plus, 4.6 × 150 mm, 5 µm), reducing the backpressure (Table 6). The method translation calculator suggested a lower flow rate of 1.2 mL/min and a longer run time of 37 minutes. In HPLC method 3, the runtime was reduced by increasing the flow rate to 1.8 mL/min, without compromising the resolution. The results of HPLC method 3 were also verified with the results on a 1260 Infinity LC system (Figure 8), and could be used with systems having pressure limitations. Table 7 and Table 8 summarize the RT and resolution deviations of emulated and actual systems for the respective methods. Six replicates of HPLC methods 1 and 3 were performed to check the reproducibility of the respective methods and RSD values of resolution, RT, and area of API, and system suitability impurities were found to be ≤1.1 % (Table 9).

Table 6. The method parameters of UHPLC and all other translated HPLC methods.

Parameter	UHPLC method	HPLC method 1	HPLC method 2	HPLC method 3
Column	Agilent ZORBAX Eclipse Plus 3.0 × 50 mm, 1.8 µm	Agilent ZORBAX Eclipse Plus 4.6 × 150 mm, 3.5 µm	Agilent ZORBAX Eclipse Plus 4.6 × 150 mm, 5 µm	Agilent ZORBAX Eclipse Plus 4.6 × 150 mm, 5 µm
Flow rate (mL/min)	1.5	1.8	1.2	1.8
Injection volume (µL)	2	14	14	14
Gradient	Time %B 0.00 25 0.30 25 3.20 95 3.70 95 3.80 25 4.30 25	Time %B 0.00 25 2.87 25 19.79 95 22.71 95 23.29 25 26.21 25	Time %B 0.00 25 4.10 25 28.27 95 32.44 95 33.27 25 37.44 25	Time %B 0.00 25 2.87 25 19.78 95 22.70 95 23.28 25 26.20 25
Pressure (bar)	~650	~300	~130	~180

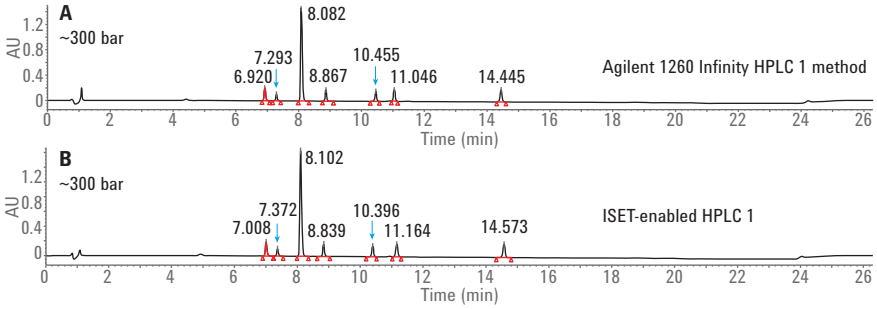

Figure 7. Overlaid chromatograms showing the similarity of the ISET-emulated method on the Agilent 1290 Infinity II system and the Agilent 1260 Infinity system for the HPLC method 1.

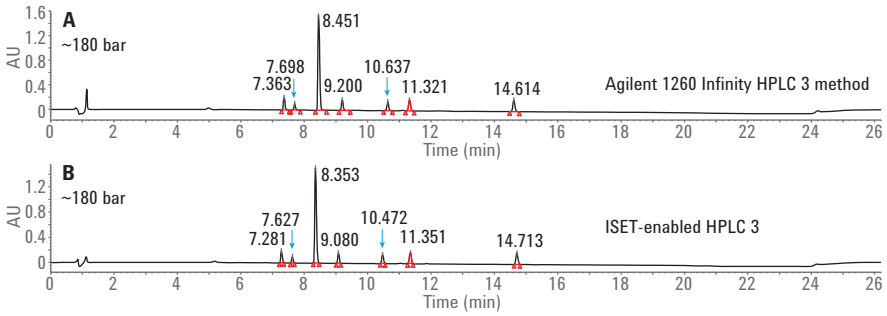

Figure 8. Overlaid chromatograms showing the similarity of ISET-emulated and an Agilent 1260 Infinity LC system for HPLC method 3.

Table 7. Calculated percentage deviations for HPLC method 1. All deviations were found to be within the allowed limit of acceptance criteria (resolution –5 % and retention time ±5 %).

	API Resolution	API RT (min)	Impurity B RT (min)	Impurity G RT (min)	Impurity G resolution
Agilent 1260 Infinity HPLC 1	8.01	8.08	10.45	11.05	5.63
ISET enabled HPLC 1	8.46	8.06	10.38	11.07	6.52
Percentage deviation (%)	+4.3	–0.24	–0.6	+0.1	+13.6

Table 8. Calculated percentage deviations for HPLC method 3. All deviations were found to be within the allowed limit of the acceptance criteria (resolution –5 % and retention time ±5 %).

	API Resolution	API RT (min)	Impurity B RT (min)	Impurity G RT (min)	Impurity G resolution
Agilent 1260 Infinity HPLC 3	7.63	8.45	10.63	11.35	5.9
ISET enabled HPLC 3	7.32	8.35	10.47	11.32	7.3
Percentage deviation (%)	–4.2	–0.24	–0.6	+0.1	+13.6

Table 9. RSD values showing the reproducibility of HPLC methods 1 and 3.

	Impurity G Rs	Impurity G RT	API RT	API Rs	Impurity G area	API Area
HPLC Method 1						
Average (min)	5.46	11.03	8.07	8.05	748,439	5,778,226
SD	0.05	0.008	0.008	0.01	2,951.619	5,852.5
RSD	0.96	0.07	0.10	0.14	0.39	0.10
HPLC Method 3						
Average (min)	5.52	11.04	8.07	8.07	752,041.8	5,784,162
SD	0.06	0.005	0.004	0.01	3,421.39	4,937.24
RSD	1.13	0.04	0.05	0.16	0.45	0.08

Conclusion

Agilent Instrument Control Framework (ICF) software was used as an interface to control the Agilent 1290 Infinity II LC by Waters Empower 3 chromatography data system, Waters ICS, and Fusion QbD software was used to develop a fast and robust UHPLC method. According to the QbD principles, a design space was generated after optimization. The five points of the design space were checked with the acceptance criteria, and found that all criteria were met. The predicted values of CMAs were found to match the experimental values. System suitability requirements (resolution >2 for impurities B and G) were met, and all peaks were baseline-separated in a gradient time of 3.7 minutes. The final UHPLC method was reproducible (API and impurity G area RSD <0.5) and robust.

A seamless method transfer from an Agilent 1290 Infinity II UHPLC system to an Agilent 1260 Infinity system was achieved using Agilent ISET technology. The method was adapted to the pressure limits of the target system. The results in emulation mode and the results of the target system were compared. Thus, it was shown that method development, QbD principles, and method transfer can be achieved seamlessly by combining Agilent 1290 Infinity II LC, ISET, ICF, and third-party CDS and Fusion QbD software.

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1. Metzlaff, M. Agilent 1290 Infinity II LC with ISET– Emulation of the Agilent 1100 Series LC Through Waters Empower Software. *Agilent Technologies, Application Note*, publication number 5991-6541EN, **2016**.

2. Vinayak, A. K. QbD Based Method Development on an Agilent 1290 Infinity UHPLC System Combined with a Seamless Method Transfer to HPLC Using Intelligent System Emulation Technology. *Agilent Technologies Application Note*, publication number 5991-5701EN, **2015**.

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4. European pharmacopeia 8.7, 04/2016:1491, pg: 5703.

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Automated Scouting of Stationary and Mobile Phases Using the Agilent 1290 Infinity II Method Development Solution

Technical Overview

Authors

Edgar Naegele and Sonja Schneider
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Technical Overview demonstrates the use of the Agilent 1290 Infinity II Method Development Solution for automated scouting of stationary and mobile phases. The solution is equipped with an Agilent 1290 Infinity II Multicolumn Thermostat that enables automated switching between up to eight columns. The Agilent 1290 Infinity II Flexible Pump is clustered with two solvent-selection valves for the connection of up to 26 different solvents. The Agilent Method Scouting Wizard of the Agilent OpenLAB CDS ChemStation Edition Software facilitates easy setup of methods for different combinations of columns, mobile phases, and temperatures in a single sequence.

Agilent Technologies

Introduction

In today's pharmaceutical, food, and environmental analysis laboratories, many chemically different compounds are analyzed by UHPLC methods. For these analyses, many different stationary phases, solvents, and modifiers are used. As a consequence, the development of corresponding methods to solve these analytical problems can be time-consuming. To overcome this challenge, the Agilent 1290 Infinity II Method Development Solution can be used for automated scouting of stationary and mobile phases. The Agilent 1290 Infinity II Multicolumn Thermostat can handle up to eight different columns, and enables precise column-temperature control over a broad temperature range. Heat transfer is done by heat exchangers with lowest internal volume and highest efficiency to achieve excellent retention time stability for highly reproducible retention times[1]. The Agilent 1290 Infinity II Flexible Pump is equipped with two solvent-selection valves, providing up to 26 channels for different solvents and buffers. This capability enables more than 1,000 possible analytical conditions. To set up the corresponding methods, the Agilent Method Scouting Wizard is available as a plug-in tool for Agilent OpenLAB CDS ChemStation Edition Software. This wizard enables the scouting of columns, solvents, gradients, and temperatures in an easy setup scheme. At the end of the setup process, a sequence comprising methods for all possible combinations is created. This sequence also contains the necessary flushing methods, column equilibration methods, and column storage conditions.

This Technical Overview demonstrates the typical setup of the 1290 Infinity II Method Development Solution. In particular, the setup of the Agilent Infinity II Multicolumn Thermostat and the 1290 Infinity II Flexible Pump is demonstrated from a hardware perspective. The setup of the experiments in the Method Scouting Wizard for column, solvent, and temperature scouting with the final creation of the scouting sequence is described from a software perspective.

Experimental

Instrumentation

The Agilent 1290 Infinity II Method Development Solution comprised the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) including valve drive (option #058) equipped with Agilent Quick-Change column selection valve (G4239C, 1,300 bar) including capillary kit (option #005) for installation of up to eight columns
- Agilent 1290 Infinity II Diode Array Detector (G7117B)
- Agilent 1290 Infinity valve drives (2x G1170A) equipped with Agilent Quick-Change 12-position/13-port solvent selection valves (2x G4235A)

Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, revision C.01.07 (minimum driver requirement A.02.11 SP1) with Method Scouting Wizard, revision A.02.05

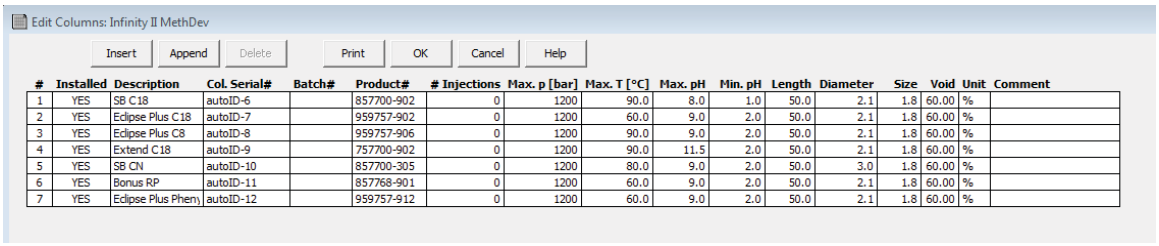
Columns

- Agilent ZORBAX RRHD SB-C18, 2.1 × 50 mm, 1.8 µm (p/n 857700-902)
- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)
- Agilent ZORBAX RRHD Eclipse Plus C8, 2.1 × 50 mm, 1.8 µm (p/n 959757-906)
- Agilent ZORBAX RRHD Extend C18, 2.1 × 50 mm, 1.8 µm (p/n 757700-902)
- Agilent ZORBAX RRHD SB-CN, 2.1 × 50 mm, 1.8 µm (p/n 857700-305)
- Agilent ZORBAX RRHD Bonus RP, 2.1 × 50 mm, 1.8 µm (p/n 857768-901)
- Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl, 2.1 × 50 mm, 1.8 µm (p/n 959757-912)

Instrument setup

The columns used for all applications were defined in the ChemStation columns list (Figure 1). For each column, a description, geometric data, particle size, and limitations such as pH, pressure, and temperature can also be given. Installed columns should be marked with YES in the first column of the table (Figure 1) to ensure that the column description is assigned to the respective results.

The column list is connected directly to the column assignment in the 1290 Infinity II Multicolumn Thermostat (Figure 2). In this assignment, the position of the column at the right or left side of the 1290 Infinity II Multicolumn Thermostat can be chosen, and associated with a color code.



#	Installed	Description	Col. Serial#	Batch#	Product#	# Injections	Max. p [bar]	Max. T [°C]	Max. pH	Min. pH	Length	Diameter	Size	Void	Unit	Comment
1	YES	SB C18	autoID-6		857700-902	0	1200	90.0	8.0	1.0	50.0	2.1	1.8	60.00	%	
2	YES	Eclipse Plus C18	autoID-7		959757-902	0	1200	60.0	9.0	2.0	50.0	2.1	1.8	60.00	%	
3	YES	Eclipse Plus C8	autoID-8		959757-906	0	1200	90.0	9.0	2.0	50.0	2.1	1.8	60.00	%	
4	YES	Extend C18	autoID-9		757700-902	0	1200	90.0	11.5	2.0	50.0	2.1	1.8	60.00	%	
5	YES	SB CN	autoID-10		857700-305	0	1200	80.0	9.0	2.0	50.0	3.0	1.8	60.00	%	
6	YES	Bonus RP	autoID-11		857768-901	0	1200	60.0	9.0	2.0	50.0	2.1	1.8	60.00	%	
7	YES	Eclipse Plus Phen	autoID-12		959757-912	0	1200	60.0	9.0	2.0	50.0	2.1	1.8	60.00	%	

Figure 1. An Agilent ChemStation column list. It provides an overview on all columns available.

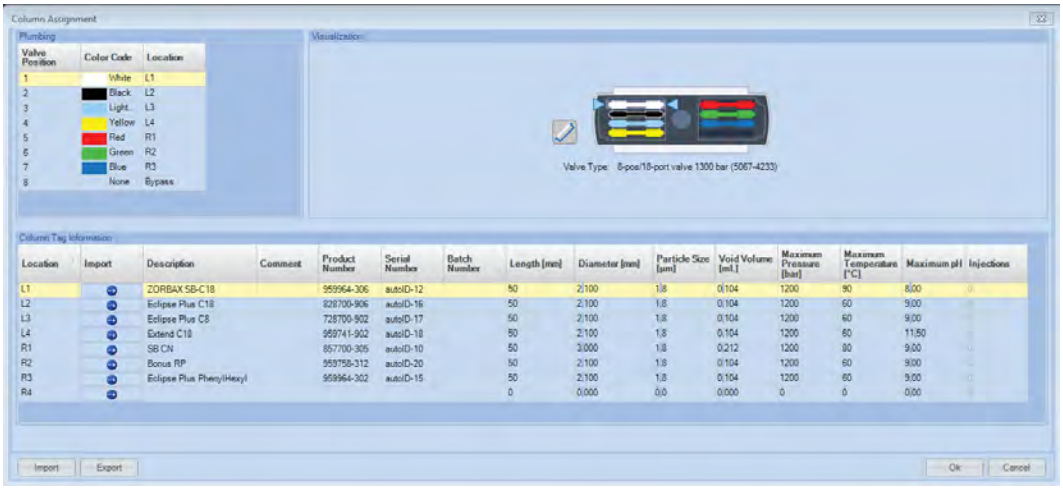


Figure 2. Column assignment in the Agilent 1290 Infinity II Multicolumn Thermostat. Each column is assigned to an unambiguous location.

The column used for the individual method can be selected in the method tab of the 1290 Infinity II Multicolumn Thermostat (Figure 3). In contrast to previous versions, columns are assigned to respective positions in the 1290 Infinity II Multicolumn Thermostat, and then selected accordingly. The appropriate column can be selected either by the drop-down menu, which shows all assigned columns, or by just clicking the column with the correct color code in the image of the 1290 Infinity II Multicolumn Thermostat. The current valve position, which connects automatically to the chosen column, is shown. For quick information, the valve position, the color code of the chosen column, and its product number is shown in Agilent ChemStation (Figure 4).

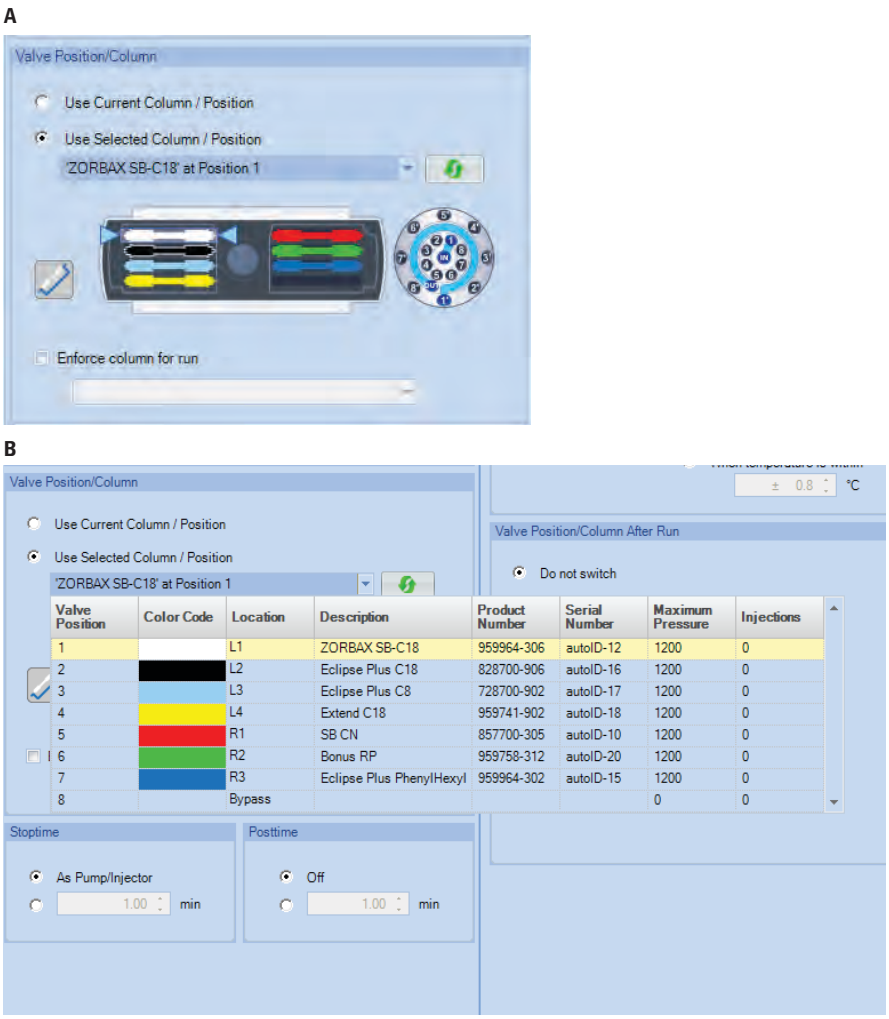


Figure 3. Column selection in the method tab of the 1290 Infinity II Multicolumn Thermostat using the drop-down menu.

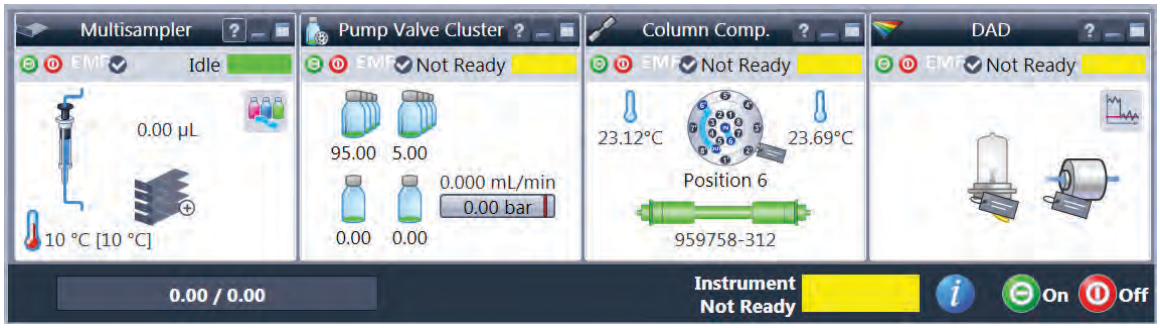


Figure 4. Instrument panel in Agilent ChemStation, highlighting the currently active column.

The assignment of the solvents can be done in a similar way. The solvent selection valves are assigned to the pump channels in the instrument configuration (not shown). In the Pump Valve Cluster Configuration tab, the positions of the solvent selection valve can be named by the connected solvent (Figure 5). The compressibility calibration can be chosen by taking the correct solvent or type of solvent from the drop-down menu, and pH and molarity values can be added. In the Pump Valve Cluster Method tab, the appropriate solvents for channels A and B can be chosen, and the valve will automatically be switched to the correct positions (Figure 6). The accessible multiple solvents are sketched out in the pump section of the ChemStation instrument panel (Figure 4).

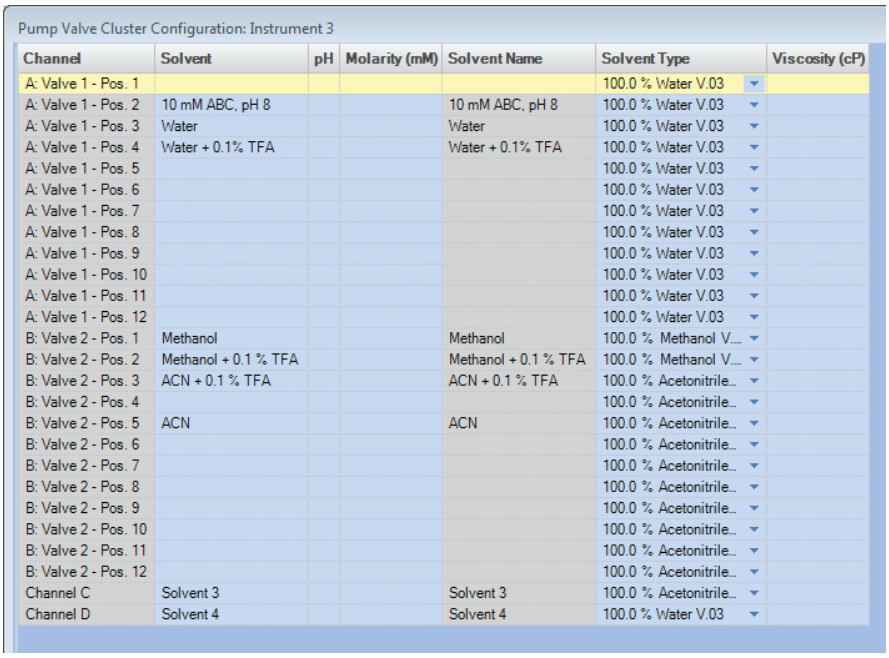


Figure 5. Pump Valve Cluster Configuration tab in Agilent ChemStation.

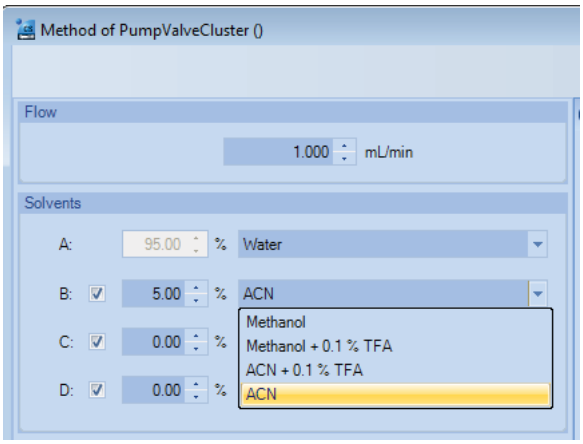
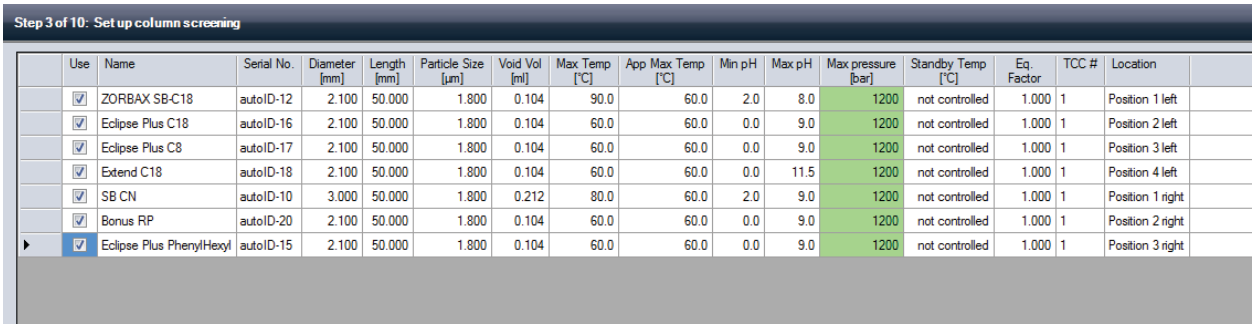


Figure 6. Pump Valve Cluster Method tab in Agilent ChemStation.

Method Scouting Wizard

The Method Scouting Wizard enables the easy setup of many sequences to scout different LC conditions including different columns, solvents, gradients, and temperatures. Finally, the Method Scouting Wizard automatically creates a sequence, which also includes solvent exchanges, re-equilibrations, and column storages when changing columns and solvents. The user is able to choose options of the method scouting campaign by selecting method scouting for columns, gradients, temperature, and solvents. In the dialog box for column scouting, the columns necessary for a study can be checked (Figure 7). If the columns differ by inside diameter and length, the method will be transferred to the current column geometry to make the results comparable.

The different solvents that have to be used can be set in the dialog box for solvent scouting (Figure 8). The Agilent 1290 Infinity II Flexible Pump, which is a quaternary pump, can be used to generate binary, ternary, or quaternary gradients. The current study was set up with the binary pump configuration, in which two solvent channels are connected to deliver the solvent for a binary gradient.



Use	Name	Serial No.	Diameter [mm]	Length [mm]	Particle Size [µm]	Void Vol [ml]	Max Temp [°C]	App Max Temp [°C]	Min pH	Max pH	Max pressure [bar]	Standby Temp [°C]	Eq. Factor	TCC #	Location
☑	ZORBAX SB-C18	autoID-12	2.100	50.000	1.800	0.104	90.0	60.0	2.0	8.0	1200	not controlled	1.000	1	Position 1 left
☑	Eclipse Plus C18	autoID-16	2.100	50.000	1.800	0.104	60.0	60.0	0.0	9.0	1200	not controlled	1.000	1	Position 2 left
☑	Eclipse Plus C8	autoID-17	2.100	50.000	1.800	0.104	60.0	60.0	0.0	9.0	1200	not controlled	1.000	1	Position 3 left
☑	Extend C18	autoID-18	2.100	50.000	1.800	0.104	60.0	60.0	0.0	11.5	1200	not controlled	1.000	1	Position 4 left
☑	SB CN	autoID-10	3.000	50.000	1.800	0.212	80.0	60.0	2.0	9.0	1200	not controlled	1.000	1	Position 1 right
☑	Bonus RP	autoID-20	2.100	50.000	1.800	0.104	60.0	60.0	0.0	9.0	1200	not controlled	1.000	1	Position 2 right
☑	Eclipse Plus PhenylHexyl	autoID-15	2.100	50.000	1.800	0.104	60.0	60.0	0.0	9.0	1200	not controlled	1.000	1	Position 3 right

Figure 7. The Agilent Method Scouting Wizard dialog box for column screening. From the columns installed in the Agilent 1290 Infinity II Multicolumn Thermostat, those necessary for the current study can be checked. Conditions such as flow rate are adjusted automatically to different column geometries.

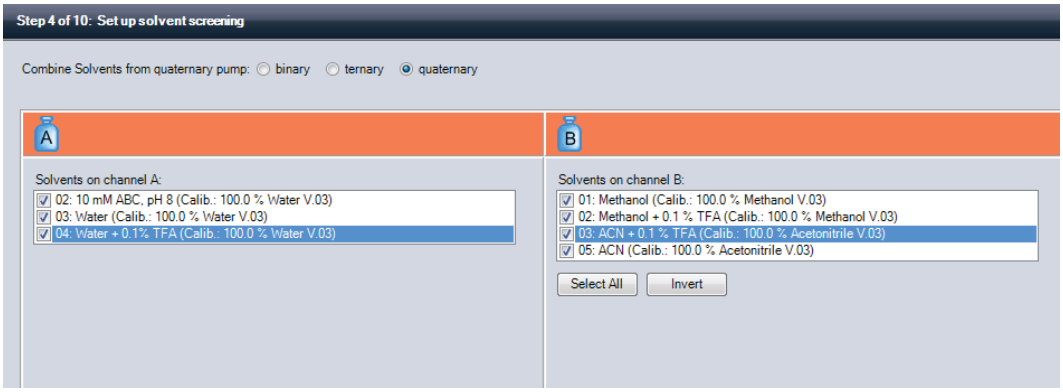


Figure 8. The Agilent Method Scouting Wizard dialog box for solvent screening. The Agilent 1290 Infinity II Flexible Pump is used in a binary mixing setup.

The final sequence was created, and included all necessary solvent exchanges (blue), column equilibration (green), and column storage methods (yellow) as separate runs without injection (Figure 9).

The solvent volumes that are going to be used during the campaign are calculated by the Method Scouting Wizard and shown in the Solvent Usage tab of the Step 10 of 10: Summary (Figure 10).

Step 10 of 10: Summary

You have set up method screening campaign "rest" as summarized:

Description | Sequence | Solvent Usage

Solvent	Estimated Volume [l]	Bottle Fill [l]	Bottle Capacity [l]
A02: 10 mM ABC, pH 8 (Calib.: 100.0 % Water V.03)	0.976	0.000	0.000
B01: Methanol (Calib.: 100.0 % Methanol V.03)	0.200	0.000	0.000
C: Solvent 3 (Calib.: 100.0 % Acetonitrile V.03)	0.000	0.000	0.000
D: Solvent 4 (Calib.: 100.0 % Water V.03)	0.000	0.000	0.000
B02: Methanol + 0.1 % TFA (Calib.: 100.0 % Methanol V.03)	0.203	0.000	0.000
B03: ACN + 0.1 % TFA (Calib.: 100.0 % Acetonitrile V.03)	0.203	0.000	0.000
B05: ACN (Calib.: 100.0 % Acetonitrile V.03)	0.203	0.000	0.000
A03: Water (Calib.: 100.0 % Water V.03)	0.976	0.000	0.000
A04: Water + 0.1% TFA (Calib.: 100.0 % Water V.03)	0.978	0.000	0.000
Sum of solvents to be disposed in waste	3.740	20.485	0.000

Figure 10. The Agilent Method Scouting Wizard calculates solvent usage for the complete campaign setup.

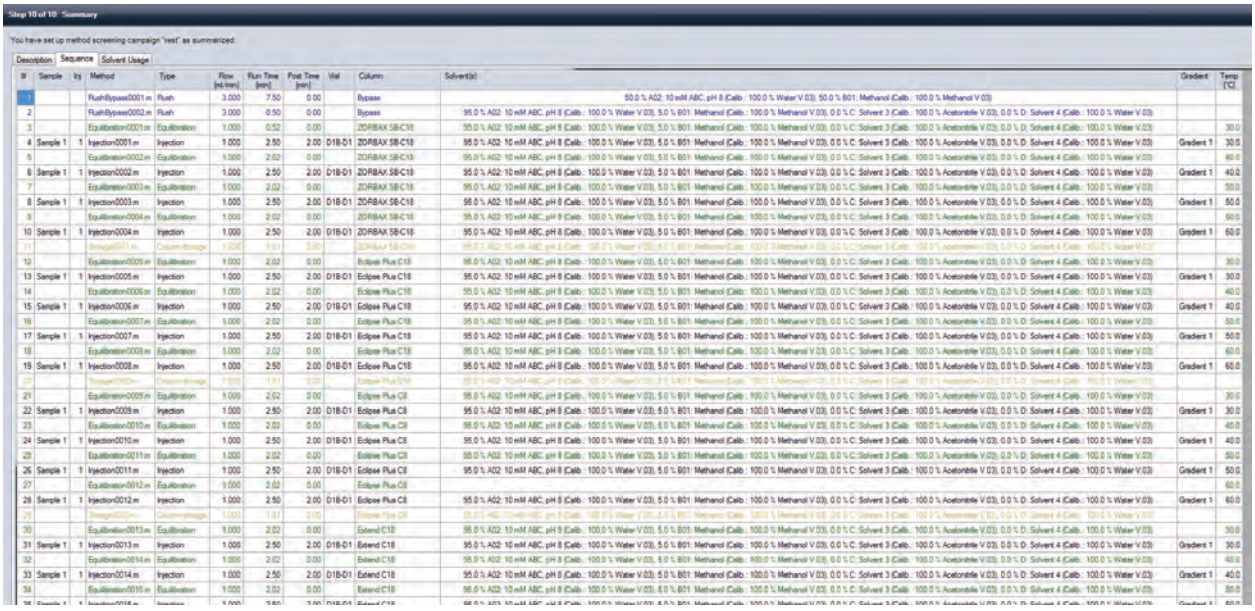


Figure 9. The Agilent Method Scouting Wizard dialog box for sequence setup includes the solvent exchange method, equilibration methods, and column storage methods.

Initial method for the Agilent Method Scouting Wizard

Parameter	Value
Flow	1 mL/min
Gradient	5 %B at 0 minutes, 75 %B at 1.5 minutes, 95 %B at 2 minutes
Stop time	2.5 minutes
Post time	2 minutes
Injection volume	1 µL
Detection	254/10 nm, reference 360/100 nm, data rate 80 Hz
Temperature scouting using 30, 40, 50, and 60 °C	

Solvent scouting using solvents

Mobile phase A

Water, ammonium bicarbonate buffer (ABC), pH 8, water + 0.1 % TFA

Mobile phase B

Methanol (MeOH), MeOH + 0.09 % TFA, acetonitrile (ACN), ACN + 0.09 % TFA

Samples

HPLC standard mixture

Chemicals

All chemicals were purchased from Sigma-Aldrich, Germany. All solvents were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

Results and Discussion

For the method scouting campaign, seven columns were used in combination with three aqueous and four organic solvents. The eighth position in the multicolumn thermostat was used as a bypass to flush the system quickly when a solvent exchange was necessary. In addition, temperature scouting was performed at 30, 40, 50, and 60 °C. A generic gradient was used during the complete campaign. The necessary sequence was created by the Method Scouting Wizard, resulting in a total of 166 sample injections as well as corresponding flush, equilibration, and storage runs.

Figure 11 shows the column scouting of the separation of the standard sample mix at 40 °C using water and acetonitrile as solvents. Seven chromatograms representing the seven used columns are compared. Using this temperature and solvent combination, the Agilent ZORBAX SB-C18 and Agilent ZORBAX Eclipse Plus C8 columns showed the best separation, followed by the Agilent ZORBAX Eclipse Plus C18, Agilent ZORBAX Extend C18, and Agilent ZORBAX Bonus RP columns. The separations obtained on the Agilent ZORBAX SB-CN and the Agilent ZORBAX Eclipse Plus Phenyl-Hexyl columns were not promising for further optimization.

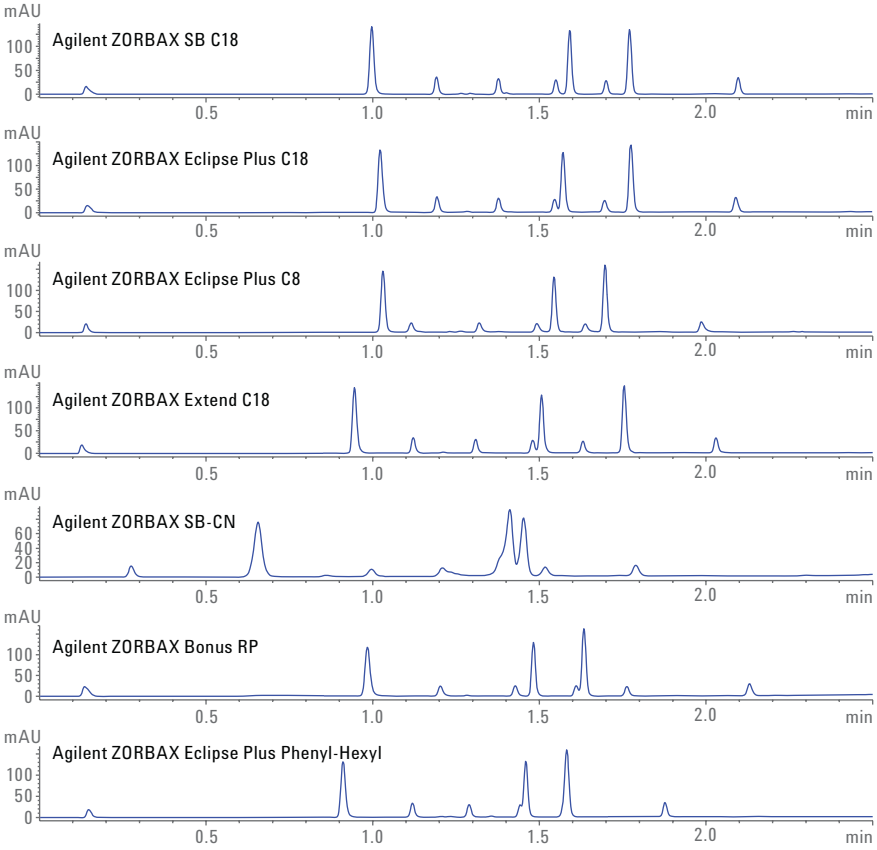


Figure 11. Column scouting using seven different columns, using water (A) and acetonitrile (B) at 40 °C.

Figure 12 shows the solvent scouting on a ZORBAX Eclipse Plus C18 column at 40 °C. For this column and temperature combination, a 10 mM ammonium bicarbonate buffer and acetonitrile delivered the best resolution.

Figure 13 shows the temperature scouting on a ZORBAX Eclipse Plus C18 column with water and acetonitrile. For this column and solvent combination, 60 °C delivered the best resolution.

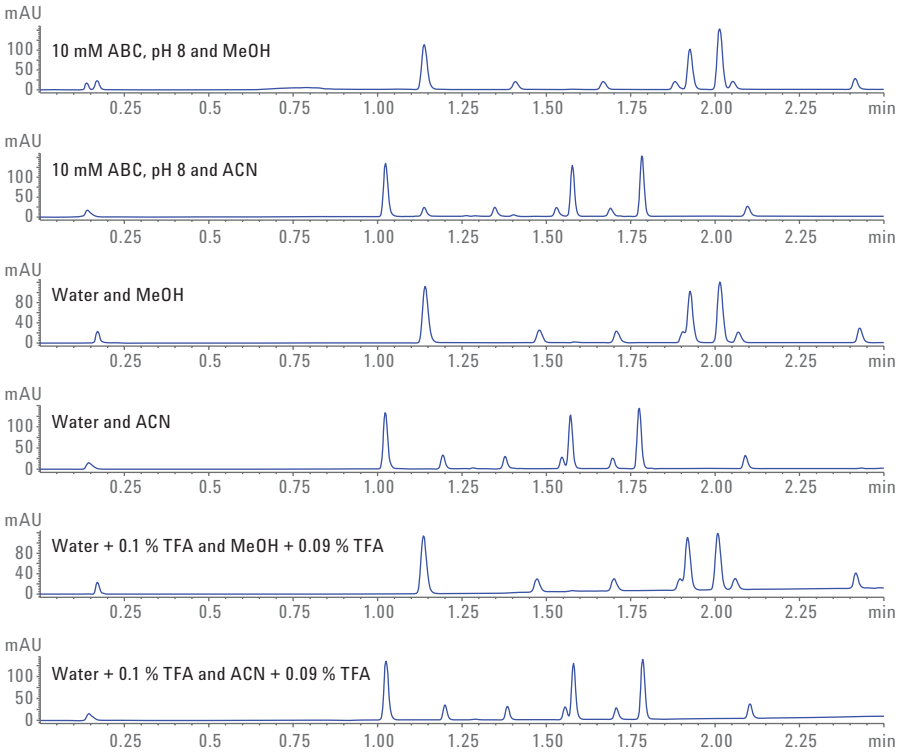


Figure 12. Solvent scouting on an Agilent ZORBAX Eclipse Plus C18 column at 40 °C.

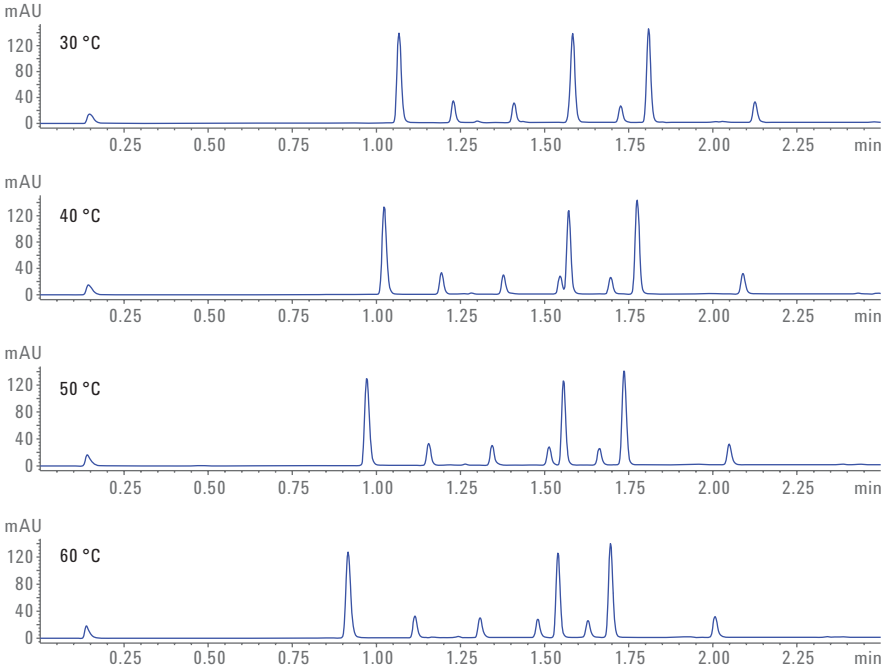


Figure 13. Temperature scouting to evaluate temperature effects on selectivity using an Agilent ZORBAX Eclipse Plus C18 column with water and acetonitrile.

To facilitate data analysis, especially when running large campaigns containing many sample injections, preconfigured report templates can be used with the Intelligent Reporting tool of the Agilent OpenLAB CDS ChemStation Edition. These reports list the chromatographic runs either by detected peaks, or resolution. In addition, the report creates a bubble plot, enabling the user to quickly identify the most promising results, and facilitate the search for the optimal separation. Figure 14 and Figure 15 show bubble plots for all injections at 50 °C, displaying the maximal number of peaks found (Figure 14) as well as the summed resolution (Figure 15). The size of the bubbles represents the number of peaks found (Figure 14) or the amount of summed resolution (Figure 15). Both bubble plots are displayed as maximal retention time on the Y-axis and injections on the X-axis.

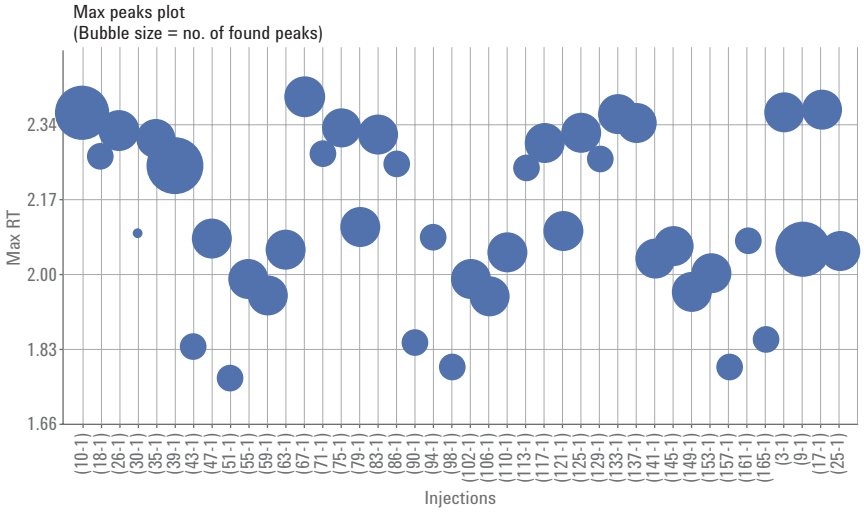


Figure 14. Maximal peak amount found per injection at 50 °C, displayed as a bubble plot.

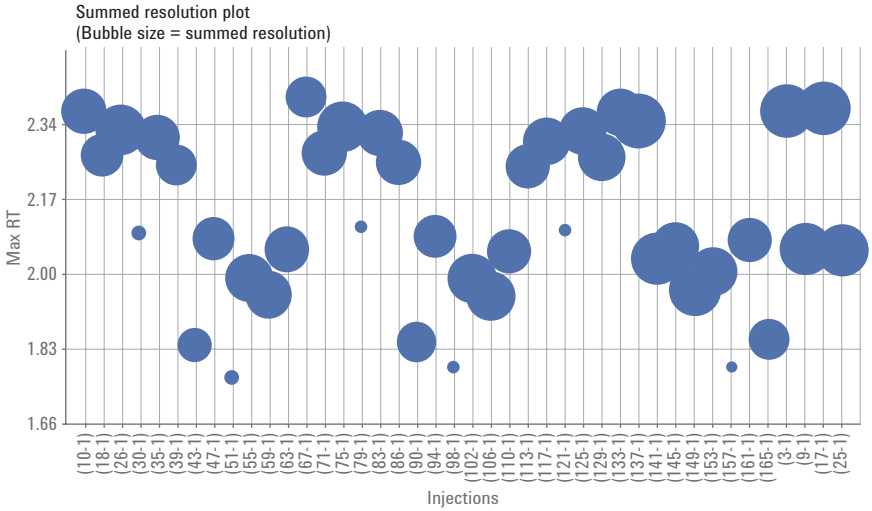


Figure 15. Maximal summed resolution found per injection at 50 °C, displayed as a bubble plot.

After complete data evaluation, the combination of the ZORBAX Eclipse Plus C18 column at 60 °C using water and acetonitrile as mobile phases was identified as one of the most optimal separation conditions (Figure 16).

Conclusion

This Technical Overview demonstrates how the combination of the Agilent 1290 Infinity II Method Development Solution and the Agilent Method Scouting Wizard facilitates automated scouting of stationary and mobile phases. The Method Scouting Wizard enables creation of column, solvent, gradient, and temperature scouting runs in any possible combination. The created scouting sequence runs all combinations, automatically including solvent flush methods, column equilibration, and column storage methods. The Agilent 1290 Infinity II Multicolumn Thermostat is part of the system, and can host up to eight columns. The Agilent 1290 Infinity II Flexible Pump, enhanced with two solvent-selection valves, can deliver up to 26 different solvents. In combination, more than 1,000 different scouting conditions are possible. Data analysis is facilitated using preconfigured report templates, accelerating the search for the optimal separation conditions.

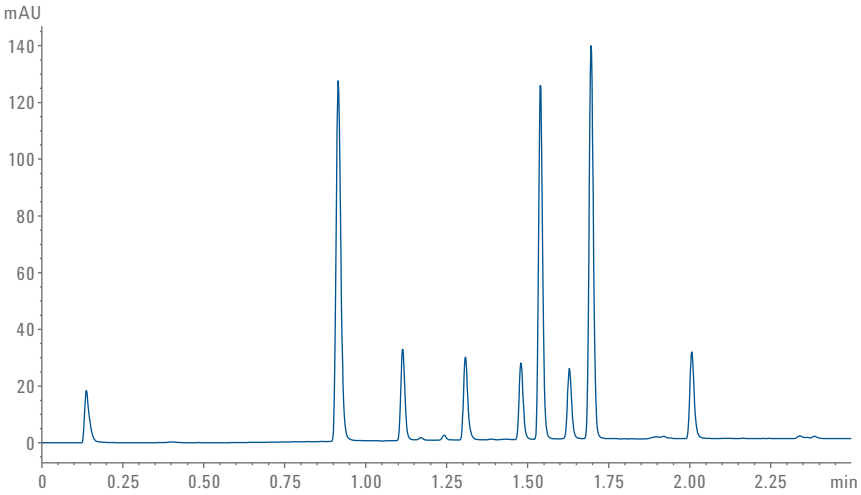


Figure 16. One of the most optimal separations found after evaluation of the scouting campaign. Using water and acetonitrile as eluents at 60 °C on an Agilent ZORBAX Eclipse Plus C18 column.

Reference

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MARKET-SPECIFIC APPLICATIONS

Analytical Method Development for USP Related Compounds in Paclitaxel Using an Agilent Poroshell 120 PFP

Application Note

Clinical Research

Author

Rongjie Fu

Agilent Technologies (Shanghai) Co. Ltd

Abstract

An analytical method for the analysis of USP related compounds in paclitaxel was run on a superficially porous Agilent Poroshell 120 PFP 4.6 × 250 mm, 4 µm column. The method repeated the United States Pharmacopeia test for related compounds in paclitaxel. The analytical method was then transferred to a 3.0 × 100 mm, 2.7 µm Poroshell 120 PFP with significant solvent and time saving. Both columns met all system suitability requirements.

Introduction

Paclitaxel, a drug also known as Taxol and Onxol, was first isolated from the bark of the Pacific yew tree, *Taxus brevifolia* in 1967. After the 1990s, synthetic methods and plant cell fermentation technology were used for its production. United States Pharmacopeia (USP) includes three different HPLC analytical methods for related compounds, based on the source of the paclitaxel. The method for the related compounds analysis in paclitaxel from natural sources uses a PFP column for the analysis [1].

The Agilent Poroshell 120 PFP (pentafluorophenyl) stationary phase can give extra retention and selectivity for positional isomers of halogenated compounds. These PFP columns can also be used for selective analysis of nonhalogenated compounds, such as polar compounds containing hydroxyl, carboxyl, nitro, or other polar groups. This selectivity is enhanced when the functional groups are on an aromatic or other rigid ring system [2].

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We describe a method developed for the USP related compounds analysis with a 4 µm Poroshell 120 PFP column transferred to a 2.7 µm Poroshell 120 PFP column. This approach delivered significant time and solvent savings.

Materials and Methods

All reagents and solvents were HPLC or analytical grade. The standards were purchased from USP. Glacial acetic acid, methanol, and acetonitrile were purchased from J&K Scientific Ltd, Beijing. The standard and assay solutions were prepared according to the USP monograph for paclitaxel. The HPLC analysis was performed with an Agilent 1290 Infinity LC system, including an:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A).

Columns

- Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 689975-902)
- Agilent Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm (p/n 695975-308)
- Agilent Poroshell 120 PFP, 4.6 × 250 mm, 4 µm (p/n 690970-408).

Results and Discussion

Selectivity comparison

The Poroshell 120 PFP stationary phase can give extra retention and selectivity for positional isomers of halogenated compounds. The column successfully separated the isomers in lapatinib in a previous note [4]. PFP columns can also be used for selective analysis of nonhalogenated compounds, especially for functional groups on an aromatic or other rigid ring system. Paclitaxel and its impurities, as examples of such types of compound, were separated on Poroshell 120 PFP and EC-C18 columns (Figure 1). Both columns showed different selectivity. The PFP column had short retention, but with good resolution between the impurities and paclitaxel. However, EC-C18 had long retention, but did not fully resolve impurity B and paclitaxel.

Conditions

Columns:	Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 689975-902) Agilent Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm (p/n 695975-308)
Sample:	Paclitaxel, impurity A and impurity B in methanol containing 0.5% acetic acid
Mobile phase:	Water:acetonitrile (55:45)
Temp:	30 °C
Flow rate:	1.5 mL/min for 4.6 × 100 mm column, 0.64 mL/min for 3.0 × 100 mm column
Inj vol:	4 µL for 4.6 × 100 mm column, 2 µL for 3.0 × 100 mm column
Detection:	UV, 227 nm

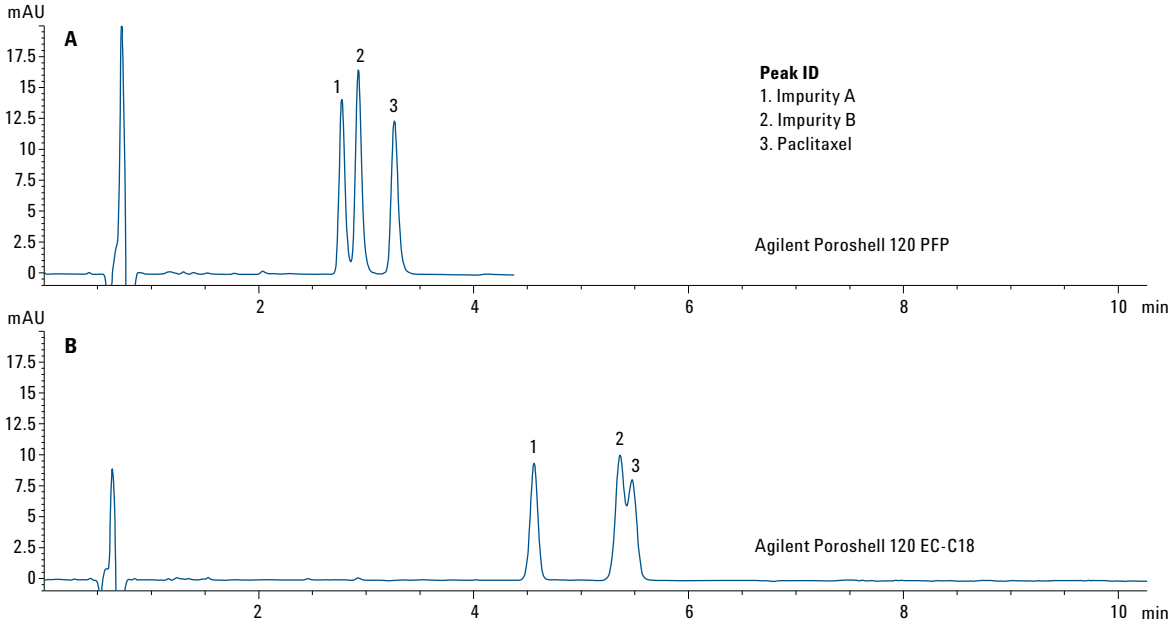

Figure 1. Selectivity comparison for separating paclitaxel and its impurities on Agilent Poroshell 120 PFP and EC-C18 columns.

System suitability test

The USP includes test 1 for paclitaxel labeled as isolated from natural sources. The chromatographic conditions for related compounds in paclitaxel require that "The liquid chromatograph is equipped with a 227-nm detector and a 4.6 mm × 25 cm column that contains 5-µm packing L43" [1].

In this trial, we first used a Poroshell 120 PFP, 4.6 × 250 mm, 4 µm column under LC conditions specified in USP methods. Figure 2 shows the system suitability analysis for the related compounds analysis on the column. The chromatograms show that the resolution between impurities A and B, impurity B and paclitaxel were good enough to meet the system suitability (shown in Table 1).

Conditions

Columns:	Agilent Poroshell 120 PFP, 4.6 × 250 mm, 4 µm (p/n 690970-408)
Sample:	Paclitaxel, impurity A and impurity B in methanol containing 0.5% acetic acid
Mobile phase:	A: water B: acetonitrile; 0-35 min, 35% A; 35-60 min, 35% A-80% A; 60-70 min, 80% A- 35% A; 70-80 min, 35% A
Temp:	30 °C
Flow rate:	2.6 mL/min
Inj vol:	10 µL
Detection:	UV, 227 nm

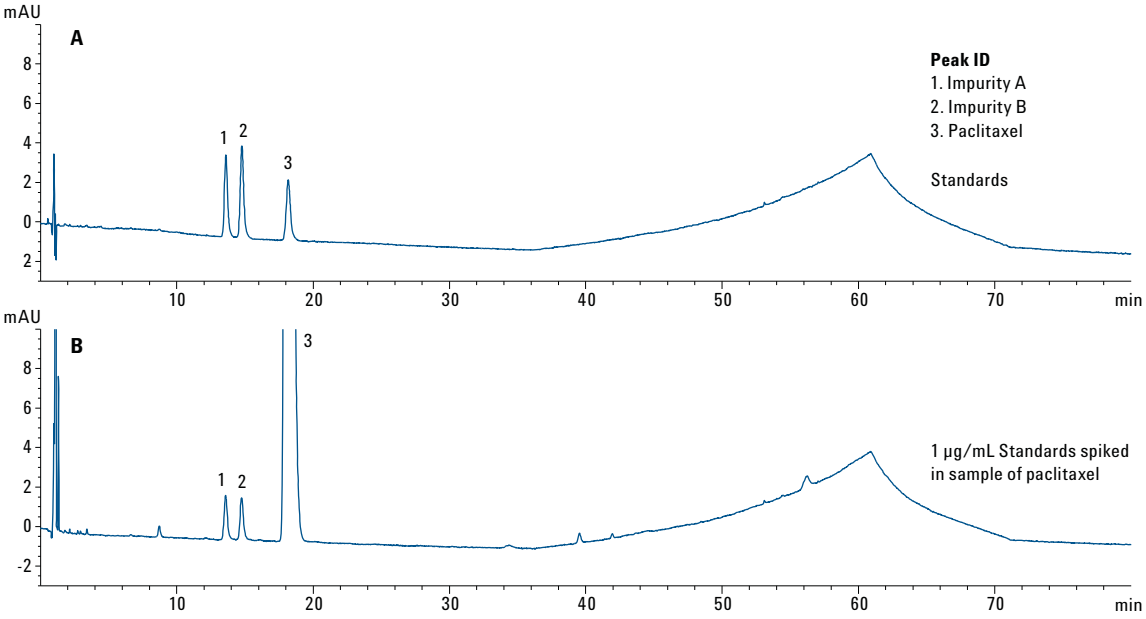

Figure 2. Chromatograms of standards and spiked sample of paclitaxel on an Agilent Poroshell 120 PFP, 4.6 × 250 mm, 4 µm column.

Table 1. USP chromatographic system suitability requirements and measured values for related compounds in paclitaxel.

USP requirements	Agilent Poroshell 120 PFP, 4.6 × 254 mm, 4 µm	Agilent Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm
The relative retention times are about 0.78 for paclitaxel-related compound A and 0.86 for paclitaxel-related compound B.	T_RA = 0.75 T_RB = 0.81	T_RA = 0.75 T_RB = 0.81
The resolution, R, between paclitaxel-related compound A and paclitaxel-related compound B is not less than 1.0.	$Rs_{1,2}$ = 2.7	$Rs_{1,2}$ = 2.2
The relative standard deviation for replicate injections is not more than 2.0%.	RSD = 0.78%	RSD1 = 0.59%

Method transfer

The analytical method was then transferred to a Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm column (Figure 3). The analysis was performed in 32 minutes, down from 80 minutes on the original column. The resolution of impurity A and impurity B was 2.2, compared to 2.7 on the 4.6 × 250 mm column. The narrow-bore column significantly saved time and solvents, and still produced results that met the USP system suitability requirements. The USP chromatographic system suitability requirements were all measured according to the USP related compounds analysis in paclitaxel on both columns. Table 1 lists the USP system requirements and measured values on the columns. The methods on the columns met all the USP chromatographic system requirements.

Conditions

Column:	Agilent Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm (p/n 695975-308)
Sample:	Paclitaxel, impurity A and impurity B in methanol containing 0.5% acetic acid
Mobile phase:	A: water B: acetonitrile; 0-14 min, 35% A; 14-24 min, 35% A- 80% A; 24-28 min, 80% A- 35% A; 28-32 min, 35% A
Temp:	30 °C
Flow rate:	1.1 mL/min
Inj vol:	2 µL
Detection:	UV, 227 nm

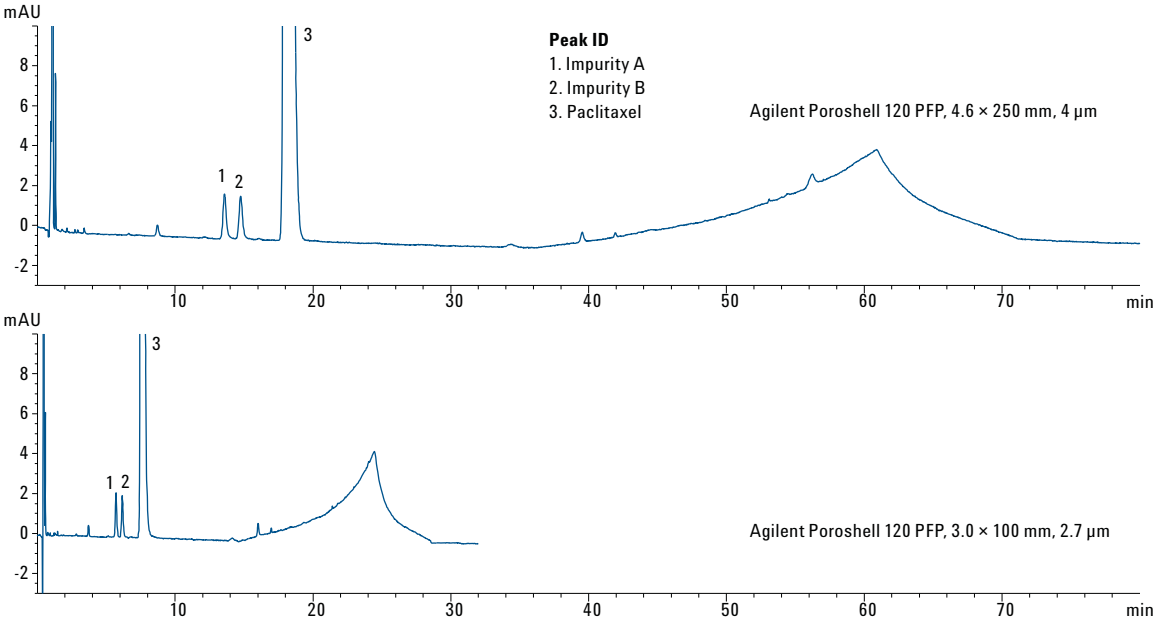

Figure 3. Chromatograms of a spiked sample for paclitaxel on Agilent Poroshell 120 PFP, 4.6 × 250 mm, 4 µm column and 3.0 × 100 mm, 2.7 µm columns.

According to USP37 NF32S1 guidelines after 1 August 2014, changes in length, column inner diameter, and particle size for gradient separations are not allowed. Therefore, to realize the benefits of speed and resolution offered by the Poroshell 120 PFP columns some method development will be required. Several experimental parameters must be tested. These include robustness, linearity, accuracy, precision, limit of detection, limit of quantitation, analytical specificity/selectivity, range, and ruggedness. An Agilent application note describes a step-by-step approach to method development [4].

Conclusions

This application shows that the Agilent Poroshell 120 PFP, 4 µm column is suitable for USP related compounds analysis of paclitaxel using the USP method conditions. With changes to the method, a narrow bore 3.0 × 100 mm column decreases analysis time by 60% with significant solvent saving.

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Agilent Technologies

Fast Screening Methods for Steroids by HPLC with Agilent Poroshell 120 Columns

Application Note

Pharma, BioPharma, and Clinical Research

Author

William Long

Agilent Technologies, Inc.

Introduction

Steroids are a type of lipid derived from cholesterol. The main feature of steroids is the ring system of 3 cyclohexanes and 1 cyclopentane in a fused ring system, as shown in Figure 1. There are a variety of functional groups that may be attached. The main feature, as in all lipids, is the large number of carbon-hydrogens, which makes steroids non-polar [1].

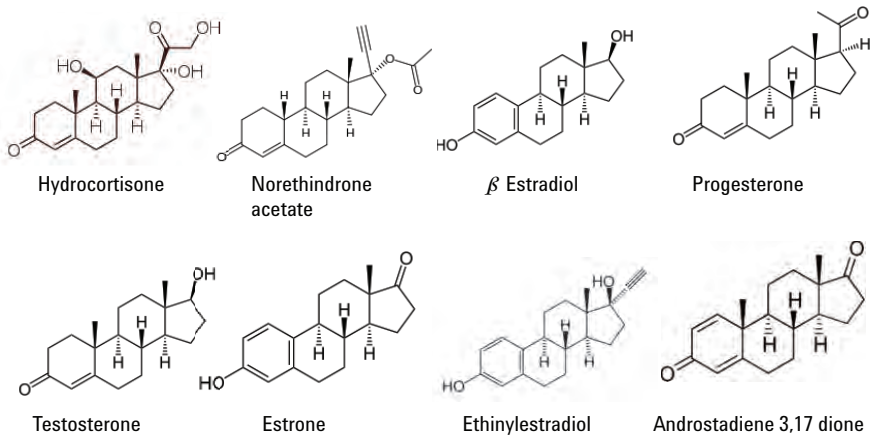

Figure 1. Structures of selected steroids.

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Using Selectivity to Enhance Separation of Steroids

When considering the best way to increase chromatographic resolution, it can be useful to consider the resolution equation, which relates efficiency, selectivity, and retention faction.

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

To obtain high resolution, the 3 terms must be maximized. An increase in N, the number of theoretical plates, by lengthening the column, leads to an increase in retention time and increased band broadening. This may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the particle size of the stationary phase particles. Superficially porous particles, such as Agilent Poroshell 120, achieve 90% of the efficiency of 1.8 µm materials with considerably lower pressure.

The selectivity factor, α, can also be manipulated to improve separations. Changing selectivity is the variable that can have the largest impact on any separation. Selectivity can be increased by:

- Changing mobile phase composition
- Changing column temperature
- Changing composition of stationary phase

Selectivity is the most powerful tool to optimize separations in HPLC. This parameter is changed by using different bonded phases, including C18, C8, polar embedded, and phenyl bonded phases, or by changing the mobile phase. In this work, Poroshell 120 columns and the Agilent 1200 SL Method Development Solution were used to quickly evaluate method development choices for the analysis of steroids. The short column length and high efficiency provided short analysis times and rapid equilibration leading to fast investigations of selectivity.

Experimental

The Agilent 1260 Infinity Series LC Multi-Method Solution was used. This system consisted of:

- 1260 Infinity Binary Pump (G1312B)
- 1290 Infinity Thermostatted Column Compartment (G1316C)
- 1260 Infinity High Performance Autosampler (G1367E)
- 1290 Infinity Diode-Array Detector (G4212A), equipped with 10 mm MaxiLight cartridge flow cell
- G6140 Single Quadrupole Mass Spectrometer.

The Agilent 1260 Infinity Series LC Multi-Method Solution is a highly flexible system that can be used for up to 4 (100 mm) columns. In addition, the Agilent ChemStation Method Scouting Wizard automates the setup of methods and sequences to screen the available combinations of columns, solvents, predefined gradients, and temperatures. In this work, 4 Agilent Poroshell 120 columns were used:

- Agilent Poroshell 120 StableBond SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902)
- Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
- Agilent Poroshell 120 Bonus-RP, 2.1 × 100 mm, 2.7 µm (p/n 685775-901)
- Agilent Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm (p/n 695775-912)

The TCC was fitted with a 6 position/6 port selection valve. This is a new Quick Change Valve mounted on a slide-out rail to make plumbing and maintenance more convenient. Port 1 was connected to a StableBond C18 column, and port 2 was connected to an EC-C18 column. Port 3 was connected to a Bonus-RP column, port 4 to a Phenyl-Hexyl column, and port 6 to a bypass connecting capillary.

The solvent passing into each column was heated using 1 of 4 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 could be screened using this system. The mobile phase was methanol or acetonitrile with 0.1% formic acid and water with 0.1% formic acid. An acetonitrile/water mixture (50%/50% v/v) was used to rinse the modifiers from the columns and allow proper column storage. Agilent ChemStation version B.04.02 was used to control the instrument and process the data.

The compounds examined included hydrocortisone, norethindrone acetate, estradiol, progesterone, testosterone, estrone, ethinylestradiol, and boldione, which were all purchased from Sigma Aldrich. Structures and details are shown in Figure 1 and Table 1. All samples were prepared at 10 mg/mL in acetonitrile and were diluted in water to a final concentration of 0.1 mg/mL.

Column choice to enhance selectivity

The columns were chosen to improve selectivity in the separation. They included a highly end capped column recommended as a first choice in method development (Poroshell 120 EC- C18), and a non end capped C18 (Poroshell 120 StableBond SB-C18) that could have interaction with silanol groups to provide an alternative C18 selectivity using neutral to low pH mobile phases. A polar-embedded amine column (Poroshell 120 Bonus-RP) and a phenyl-hexyl column (Poroshell 120 Phenyl-Hexyl) were also used. Phenyl bonded phases are known for their improved selectivity for aromatic compounds.

A polar-embedded group inserted into the hydrophobic C14 alkyl chain allows the Bonus-RP phase on totally porous Poroshell 120 to minimize interaction of polar samples with silanols, providing symmetrical peaks for a wide variety of applications. This phase is especially useful at neutral pH where amines can interact strongly with ionized silanols. The polar-embedded group also helps to wet the hydrophobic chains and prevents phase collapse in highly aqueous mobile phases.

Poroshell 120 Bonus-RP can be used for many of the same separations as a C18 column while avoiding some of the disadvantages of C18, such as poor wettability in high aqueous mobile phases. In addition, it is much more retentive for those molecules that can interact by hydrophobic interactions and also by H-bonding with the amide group. Compared to alkyl only phases, Bonus-RP has enhanced retention and selectivity for phenols, organic acids, and other polar solutes due to strong H-bonding between polar group (H-bond acceptor) and H-bond donors, like phenols and acids. Bonus-RP gives retention slightly less than a C18 allows, for easy column comparison without the need to change mobile phase conditions. The Bonus-RP phase gives different selectivity than C18 for polar compounds. It is also compatible with 100% water.

The Phenyl-Hexyl phase has unique reversed-phase selectivity, especially for polar aromatics and heterocyclic compounds, derived from analyte interaction with the aromatic ring of the bonded phase and its delocalized electrons. Poroshell 120 Phenyl-Hexyl can be orthogonal to both C18 and Bonus-RP phases. More retention and selectivity will often be observed for solutes with aromatic electron-withdrawing groups such as fluorine or nitro groups [2,3,4].

Table 1. Steroid nomenclature and molecular characteristics.

Common name	IUPC name	Molecular formula	Molecular weight
Hydrocortisone	Cortisol	$C_{21}H_{30}O_5$	362.460
Norethindrone acetate	(17α)-17-ethynyl-3-oxoestr-4-en-17-yl acetate	$C_{22}H_{28}O_3$	340.456
β Estradiol	(17β)-estra-1,3,5(10)-triene-3,17-diol	$C_{18}H_{24}O_2$	272.38
Progesterone	Pregn-4-ene-3,20-dione	$C_{21}H_{30}O_2$	314.46
Testosterone	(8R,9S,10R,13S,14S,17S)- 17-hydroxy-10,13-dimethyl- 1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one	$C_{19}H_{28}O_2$	288.42
Ethinylestradiol	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol	$C_{20}H_{24}O_2$	296.403
Androstadiene 3,17 dione (boldione)	(8R,9S,10R,13S,14S)-10,13-dimethyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-dione	$C_{19}H_{24}O_2$	284.39
Estrone	3-hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren-17- one	$C_{18}H_{22}O_2$	270.366

Poroshell 120 Phenyl-Hexyl columns deliver unique selectivity for compounds with aromatic groups, providing superior resolution for these samples. Poroshell 120 Phenyl-Hexyl can also provide optimum separations of moderately polar compounds where typical alkyl phases (C18 and C8) do not provide adequate resolution. Acetonitrile tends to decrease the π–π interactions between aromatic and polarizable analytes and the phenyl-hexyl stationary phases, but methanol enhances those same interactions, giving both increased retention and changes in selectivity [5]. This does not mean that acetonitrile should not be used with a phenyl bonded phase or that it might not provide an acceptable separation, but methanol is more likely to deliver the additional selectivity that is desired from a phenyl phase.

Results and Discussion

As can be seen in Figure 2, the separation of all 8 compounds was attempted on all columns surveyed. The Poroshell 120 EC-C18 and Poroshell 120 Phenyl-Hexyl columns showed very similar profiles, although the elution on the Phenyl Hexyl column was faster. This could indicate that the π–π interactions on the Phenyl-Hexyl column were being reduced by the acetonitrile. The overlap of estradiol and androstadiene was less severe on the Phenyl-Hexyl column. The Poroshell 120 SB-C18 column delivered a very different separation, resolving estradiol but losing resolution on ethinylestradiol and estrone. This could be due to the exposed silanols on the SB-C18 phase or to some additional shape selectivity derived

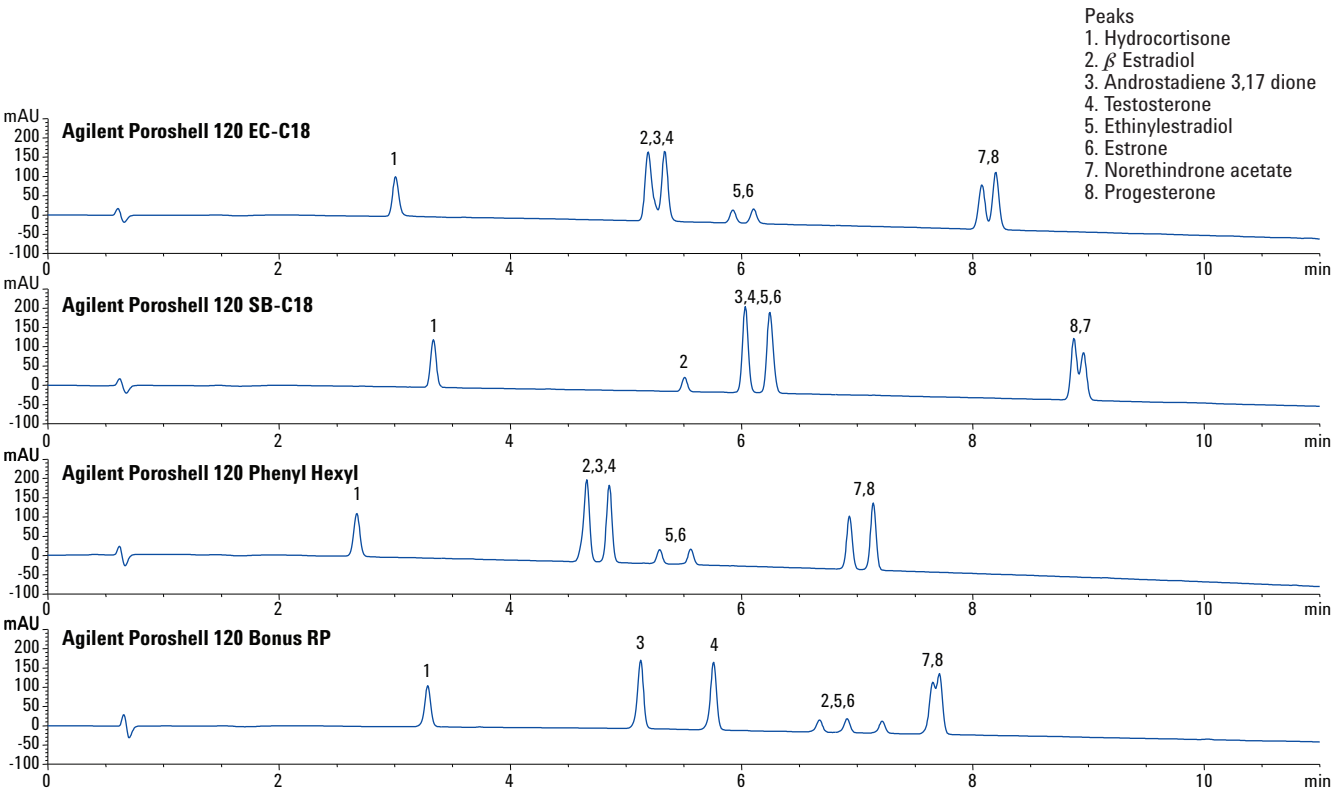

Figure 2. Separation of steroids using Agilent Poroshell 120 columns with acetonitrile.

Conditions

Columns:	Agilent Poroshell 120, 2.1 × 100 mm
Flow rate:	0.4 mL/min
Gradient:	25-80% MeCN/10 min (0.1% formic acid in water and MeCN)
Temperature:	25 °C
Detection:	DAD 260,80 ref = off

from the di-isobutyl side chains on the SB-C18 phase. Some additional work is needed to determine this. The Poroshell 120 Bonus-RP phase almost separates all 8 compounds, and when using acetonitrile, it would provide the best method development option for further development.

In Figure 3, the separation was carried out using methanol at slightly elevated temperature (40 °C). In this case, the 2 C18 phases (Poroshell 120 EC-C18 and Poroshell 120 SB-C18) yielded nearly identical chromatographic profiles. Some additional retention was seen on the SB-C18 phase due to some silanol interaction. The Poroshell 120 Bonus-RP chromatogram had 3 overlapping peak pairs, which would likely make further method development difficult in methanol. However, the Poroshell 120 Phenyl-Hexyl phase resolved 8 compounds at better than baseline resolution.

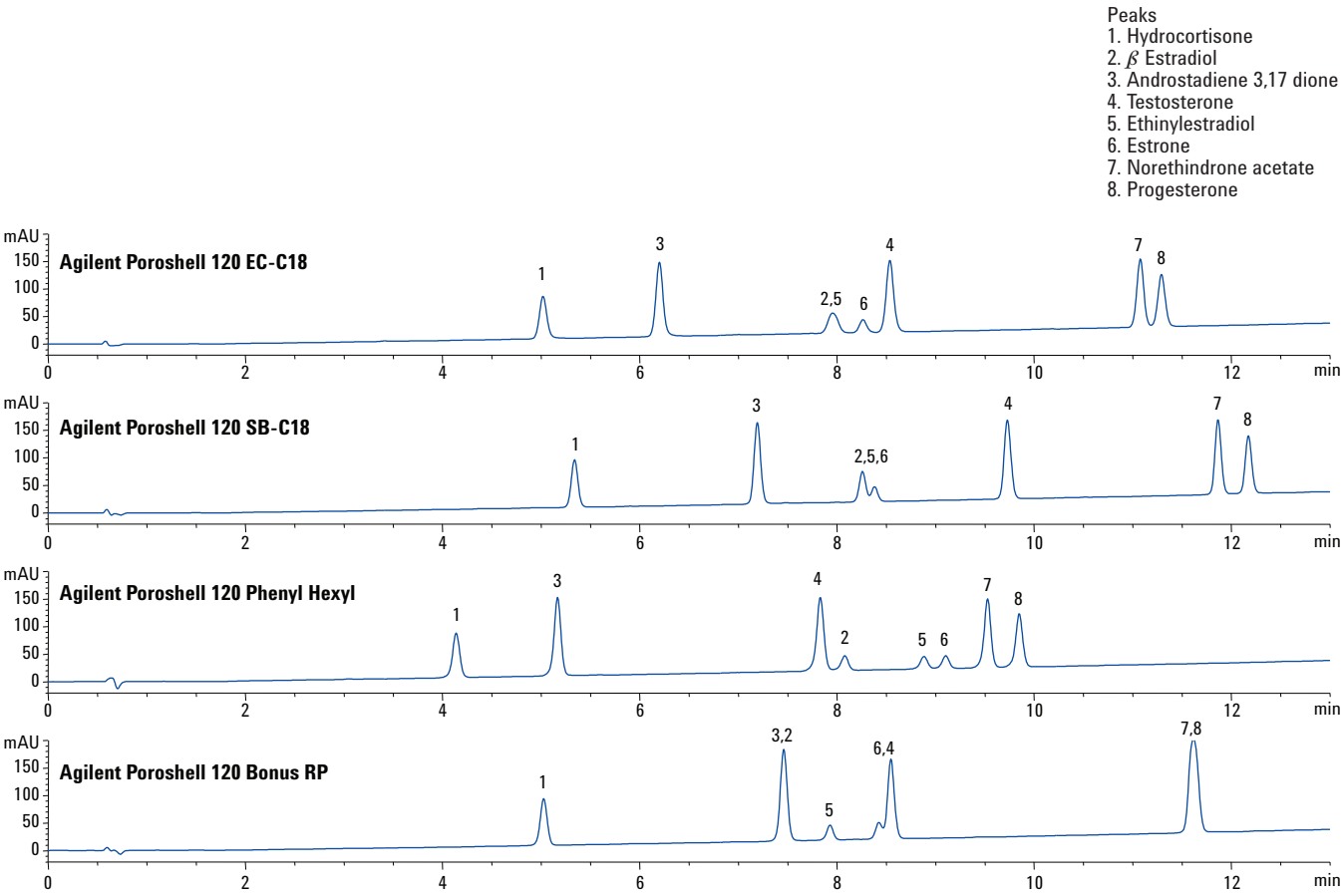

Figure 3. Separation of steroids using Agilent Poroshell 120 columns with methanol.

Conditions

Columns:	Agilent Poroshell 120, 2.1 × 100 mm
Flow rate:	0.4 mL/min
Gradient:	40-80% MeOH/14 min (0.1% formic acid in water and MeOH)
Temperature:	40 °C
Detection:	DAD 260, 80 ref = off

Conclusions

Analysis problems can be quickly resolved by including survey methods with generic gradients as part of the method development scheme. This work used steroids as an example, and showed how phases and organic modifiers, such as acetonitrile and methanol, could develop different selectivity that could be used to optimize the separation. In this case, the widely used C18 phases, as found on Poroshell 120 EC-C18 and SB-C18 columns, did not provide adequate separation. Using an alternative selectivity column such as Poroshell 120 Bonus-RP in acetonitrile or Poroshell 120 Phenyl Hexyl yielded better results, and could be used for several thousand samples.

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Agilent Technologies

Selectivity Comparison of Agilent Poroshell 120 Phases in the Separation of Butter Antioxidants

Application Note

Food Testing & Agriculture

Author

Rongjie Fu
Agilent Technologies
(Shanghai) Co. Ltd.

Abstract

The selectivity of various phases of Agilent Poroshell 120, including EC-C18, SB-C18, SB-Aq, Bonus-RP, and Phenyl-Hexyl, were compared in the separation of nine artificial antioxidants. The method was then optimized on an Agilent Poroshell 120 SB-Aq column for these compounds in butter samples with a water (with acetic acid modifier) and acetonitrile mobile phase. The method separated the antioxidants very well and was suitable for quantitative analysis.

Introduction

Foods, such as oils and fats, containing unsaturated fatty acids can easily cause lipid oxidation leading to rancidity, odor problems, and a decrease of their nutritional value. Synthetic ascorbyl palmitate and phenolic antioxidants are often added to foods to prevent oxidation of these unsaturated fatty acids. Single or combinations of antioxidants are permitted to enhance the antioxidative effect in food, but excessive consumption can cause some health problems in humans. For example, 2,4,5-trihydroxybutyrophenone has mutagenic effects, butylated hydroxyanisole and 2,6-di-tert-butyl-4-methylphenol may be carcinogenic, and propyl gallate can cause kidney damage [1].

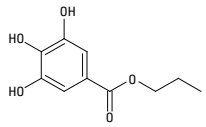
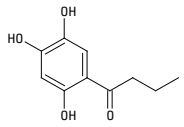
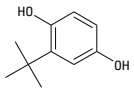
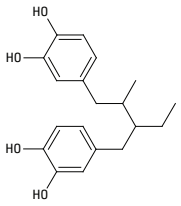
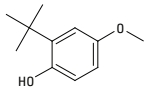
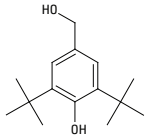
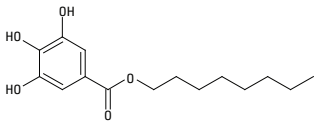
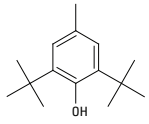
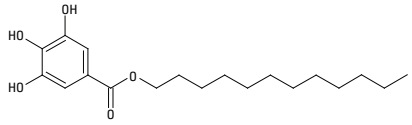
Regulatory agencies from both Europe and the US have imposed maximum levels for some antioxidants while others have been forbidden [2]. Therefore, the determination of antioxidants in foods and food components is important. Table 1 lists nine antioxidants that are most commonly added to foods along with their structures and abbreviations.

Currently, a regulatory HPLC method in China is used for the determination of antioxidants in fats. This method uses columns 15 to 25 cm long with an internal

diameter of 4.6 mm, packed with 5 µm C18 bonded silica particles, and a mobile phase composed of acetic acid (eluent A) and methanol (eluent B). Unfortunately, this method is quite slow with an analysis time over 30 minutes [3]. QC laboratories in fat-processing industries are looking for a way to shorten analysis time, because some of the antioxidants have been found to suffer significant degradation in solvents at room temperature. Thus, a fast, accurate, and rugged method is desirable for economical and practical reasons.

This application note describes the analysis of nine antioxidants in butters using the Agilent 1290 Infinity LC with Poroshell 120 columns. Selectivity of various phases was compared for separation. The Poroshell 120 SB-Aq column was chosen for method development for its better selectivity and retention time versus other phases, and then the method was optimized to fit the analysis of various butter samples.

Table 1. Artificial antioxidants used in this study.

Peak No.	Name	CAS	Structure
1	Propyl gallate (PG)	121-79-9	
2	2,4,5-Trihydroxybutyrophenone (THBP)	1421-63-2	
3	2-Tert-butylhydroquinone (TBHQ)	1948-33-0	
4	Nordihydroguaiaretic acid (NDGA)	500-38-9	
5	Butylated hydroxyanisole (BHA)	25013-16-5	
6	Ionox-100	88-26-6	
7	Octyl gallate (OG)	1034-01-1	
8	2,6-Di-tert-butyl-4-methylphenol (BHT)	128-37-0	
9	Dodecyl gallate (DG)	1166-52-5	

Materials and Methods

HPLC analysis was performed with the Agilent 1290 Infinity LC, including an Agilent 1290 Infinity Binary Pump (G4220A), an Agilent 1290 Infinity Autosampler (G4226A), an Agilent 1290 Infinity Thermostatted Column Compartment (G1316C), and an Agilent 1290 Infinity Diode Array Detector (G4212A).

Columns

Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)

Agilent Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm (p/n 685975-302)

Agilent Poroshell 120 SB-Aq, 3.0 × 100 mm, 2.7 µm (p/n 685975-314)

Agilent Poroshell 120 Bonus-RP, 3.0 × 100 mm, 2.7 µm (p/n 695968-301)

Agilent Poroshell 120 Phenyl-Hexyl, 3.0 × 100 mm, 2.7 µm (p/n 695975-312)

The stock solution of standard's mixture was prepared in methanol at 0.1 mg/mL individually. The standard solutions for linearity were diluted from the stock solution in a series of concentrations including 0.2, 0.5, 1, 2, 5, and 10 ppm in acetonitrile:isopropanol (50:50 v:v).

The butters were purchased in the supermarket, and originated from the US, Switzerland, Australia, and New Zealand. Sample solutions were prepared according to the Chinese regulatory method [3]. For the spiked samples, a stock solution of the antioxidants in the solvent was added prior to extraction. The extraction was carried out by weighing 1.0 g of butter and adding 5.0 mL of *n*-hexane saturated with acetonitrile. This mixture was vortexed to dissolve the butter, was added to 10 mL of acetonitrile saturated with *n*-hexane, was vortexed again for 1 minute, and was allowed to stand until a separate layer developed. The acetonitrile layer was collected and extracted once more with acetonitrile saturated with *n*-hexane. The collected acetonitrile portions were mixed together and concentrated with a flow of nitrogen to a volume of 1 mL, and then made up to 2 mL with isopropanol. These solutions were filtered using Agilent 0.2 µm regenerated cellulose membrane filters (p/n 5064-8222) before transfer into autosampler vials for injection.

Results and Discussion

Selectivity comparison

Poroshell 120 columns were packed with superficially porous particles, which provided performance similar to the sub-2 µm particles but with a 40 to 50% lower pressure than columns with sub-2 µm particles. The recent introduction of new stationary phases available on Poroshell 120 columns made them useful for method development by changing selectivity.

Using a variety of bonded phases to try sequentially for method development demonstrated the different selectivity easily gained from the columns. Figure 1 and Figure 2 are overlays of five different reversed phases with acetic acid:methanol and acetic acid:acetonitrile mobile phases. All gave a symmetrical peak shape, with the exception of the Poroshell 120 Bonus-RP with methanol organic phase. This might have been caused by the amide group embedded in the bonded phase of Poroshell Bonus-RP, which has a strong H-bonding attraction with acidic analytes when methanol is present in the mobile phase.

The differences in selectivity between the five columns were due to the differences in bonding chemistry, such as the type of bonding, the end capping, and the amount and type of silanols on the silica surface. Other factors that influence selectivity, including mobile phase composition, temperature, and pH were identical during the investigation. These five bonded phases were all based on 2.7-µm Poroshell 120 superficially porous silica. They included an EC-C18 column, highly end capped that gave the best overall peak shape; SB-C18 and SB-Aq non-end capped columns, which had interaction with silanol groups and provided alternative selectivity to C18 phases; Bonus-RP, a polar embedded amine column that gave unique selectivity; and a Phenyl-Hexyl bonded column, which had improved selectivity for aromatic compounds.

Under a mobile phase of acetonitrile/1.5% acetic acid, the Phenyl-Hexyl and EC-C18 did not resolve peaks 6 and 7 well . SB-C18 and SB-Aq both resolved all nine peaks, but SB-C18 gave longer retention and less resolution between peaks 6 and 7 than SB-Aq. When changing the mobile phase to methanol:1.5% acetic acid, the elution order changed on all columns with poor separation for several peaks, but SB-Aq still separated all nine compounds well. In a careful comparison between the two mobile phases on the SB-Aq column, acetonitrile:1.5% acetic acid gave even better resolution and higher performance for all the compounds. Therefore, the Poroshell 120 SB-Aq was chosen for further method development with the mobile phase of acetonitrile:1.5% acetic acid.

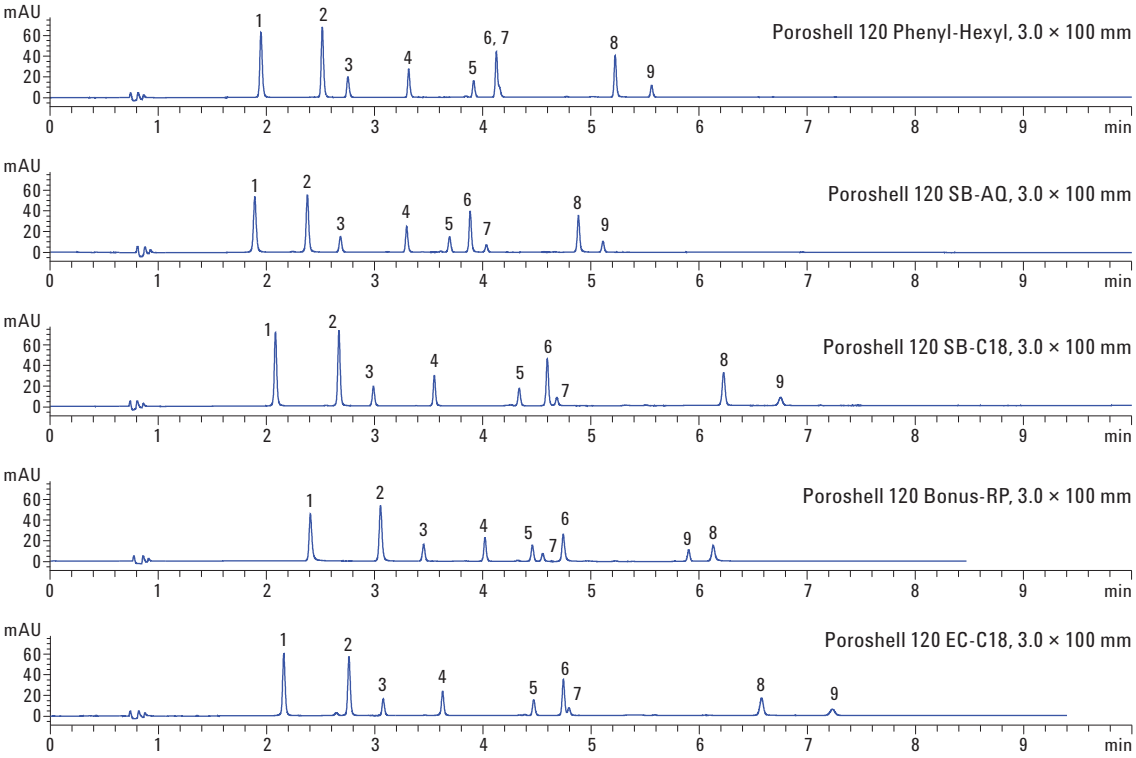


Figure 1. Chromatograms of nine antioxidants in acetonitrile:1.5% acetic acid on various Agilent Poroshell 120 phases showing different selectivity.

Conditions, Figure 1

Eluent:	A 1.5% acetic acid, B ACN	
Injection volume:	2 µL of 10 ppm mixture in 10% methanol	
Flow rate:	0.6 mL/min	
Gradient:	Time (min)	% B
	0	25
	0.5	25
	5	80
	8	80
Temperature:	40 °C	
Detector:	UV, 280 nm	

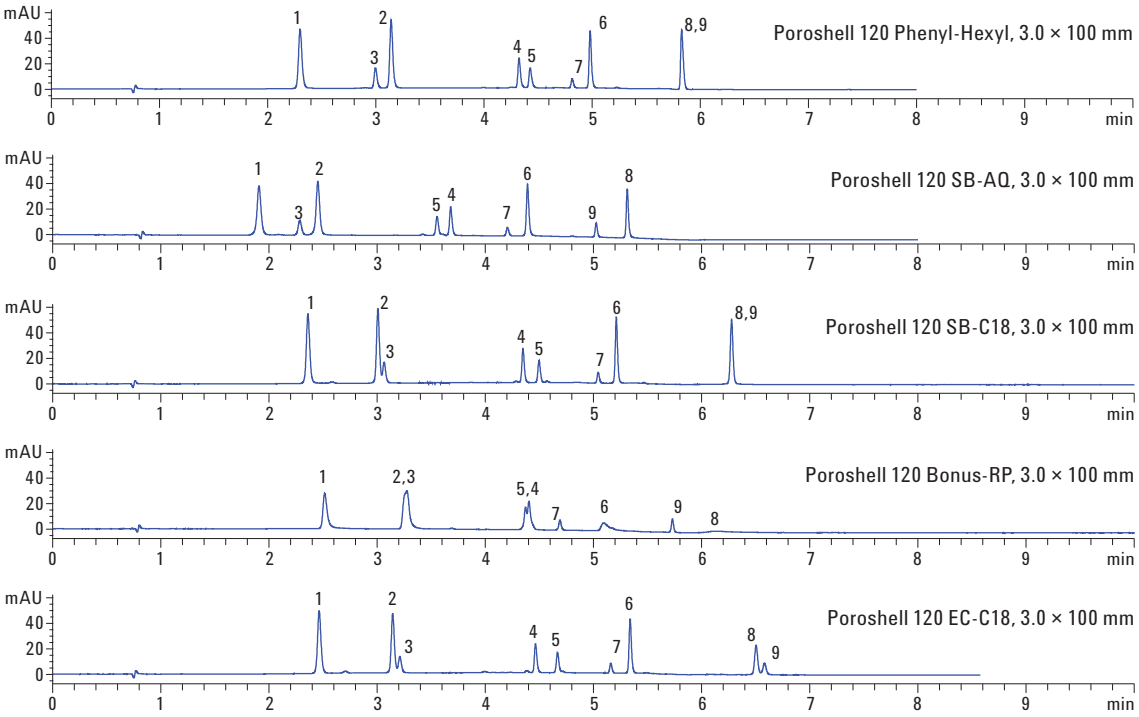


Figure 2. Chromatograms of nine antioxidants in methanol:1.5% acetic acid on various Agilent Poroshell 120 phases showing different selectivity.

Conditions, Figure 2

Eluent:	A 1.5% acetic acid, B methanol	
Injection volume:	2 µL of 10 ppm mixture in 10% methanol	
Flow rate:	0.6 mL/min	
Gradient:	Time (min)	% B
	0	40
	1	40
	5	80
	8	80
Temperature:	40 °C	
Detector:	UV, 280 nm	

Method development on Poroshell 120 SB-Aq

The method should be modified according to the above description on Poroshell 120 SB-Aq with acetonitrile:1.5% acetic acid mobile phase, because the real samples (butter) are more complex than the standards. For good separation when dealing with interference components in butter, the gradient was adjusted to get ideal resolutions of target antioxidants. Figure 3 shows the chromatogram of the nine antioxidants separated under the modified gradient at 10 ppm. All the compounds had a symmetrical peak shape, and the eluted time of the last peak was extended to 8.5 minutes from 5.2 minutes under the original gradient. It was necessary for the real sample separation to use a slightly longer gradient to get ideal separations.

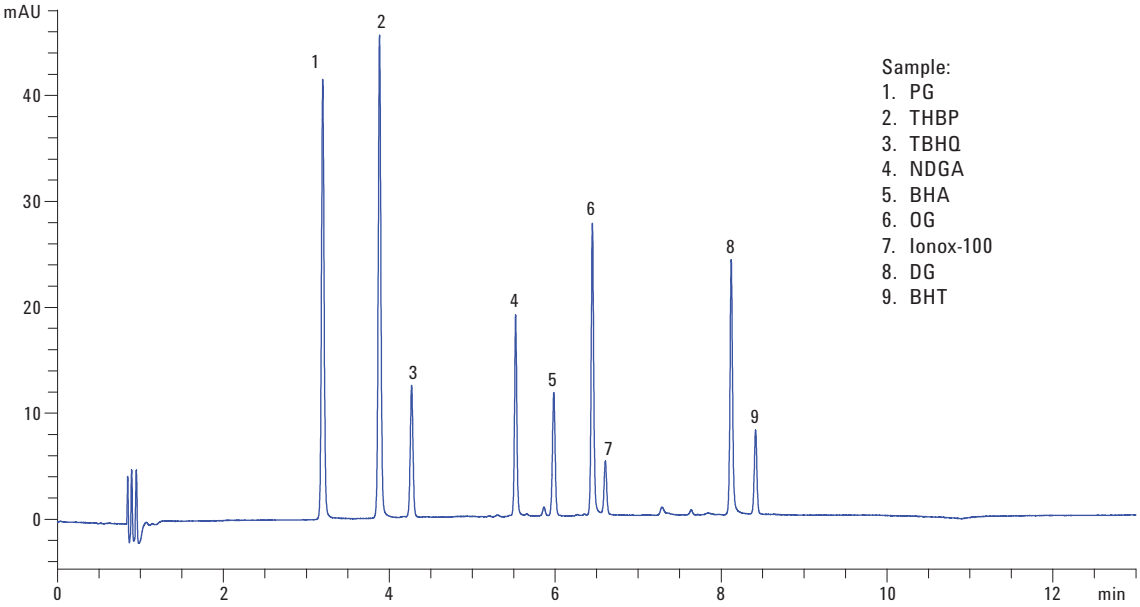


Figure 3. Chromatogram of 10 ppm standards' separation with an Agilent Poroshell 120 SB-Aq column.

Conditions, Figure 3

Eluent:	A 1.5% acetic acid, B ACN	
Injection volume:	2 µL of 10 ppm mixture in 50% ACN/50% IPA	
Flow rate:	0.6 mL/min	
Gradient:	Time (min)	% B
	0	10
	9	75
	10	95
Stop time:	15 minutes	
Temperature:	40 °C	
Detector:	UV, 280 nm	

The data of correlation of linearity and limit of detection (LOD) by UV at 280 nm is shown in Table 2. The coefficients of linearity were excellent for all nine compounds. The LODs were calculated with a signal-to-noise ratio of 3. The LOD was equal to or below 0.1 ppm for all the antioxidants. This corresponded to approximately 0.2 mg/kg or below in a butter sample.

Stable retention times are important for correct identification of analytes in complex food matrixes. Figure 4 shows overlay chromatograms of eight consecutive injections of 1 ppm standard mixture. The retention time of all the peaks was well reproducible and all the relative standard deviations of peak retention time from eight replicate injections on Poroshell 120 SB-Aq column were less than 0.1%.

Table 2. Correlation of linearity and theoretical LOD of nine antioxidants.

No.	Name	Calibration curve	Range (ppm)	Correlation	LOD (s/n=3) (ppm)
1	PG	Y = 9.144X +0.936	0.2 ~ 10	0.9997	0.046
2	THBP	Y = 10.075X + 1.028	0.2 ~ 10	0.9998	0.039
3	TBHQ	Y = 2.815X + 0.309	0.2 ~ 10	0.9997	0.074
4	NDGA	Y = 3.659X +0.306	0.2 ~ 10	0.9997	0.111
5	BHA	Y = 2.631 X +0.330	0.2 ~ 10	0.9997	0.082
6	OG	Y = 5.796 X +0.550	0.2 ~ 10	0.9997	0.037
7	Ionox-100	Y = 1.132 X + 0.130	0.2 ~ 10	0.9994	0.200
8	DG	Y = 5.208X + 0.112	0.2 ~ 10	0.9995	0.090
9	BHT	Y = 1.709 X +0.463	0.2 ~ 10	0.9992	0.118

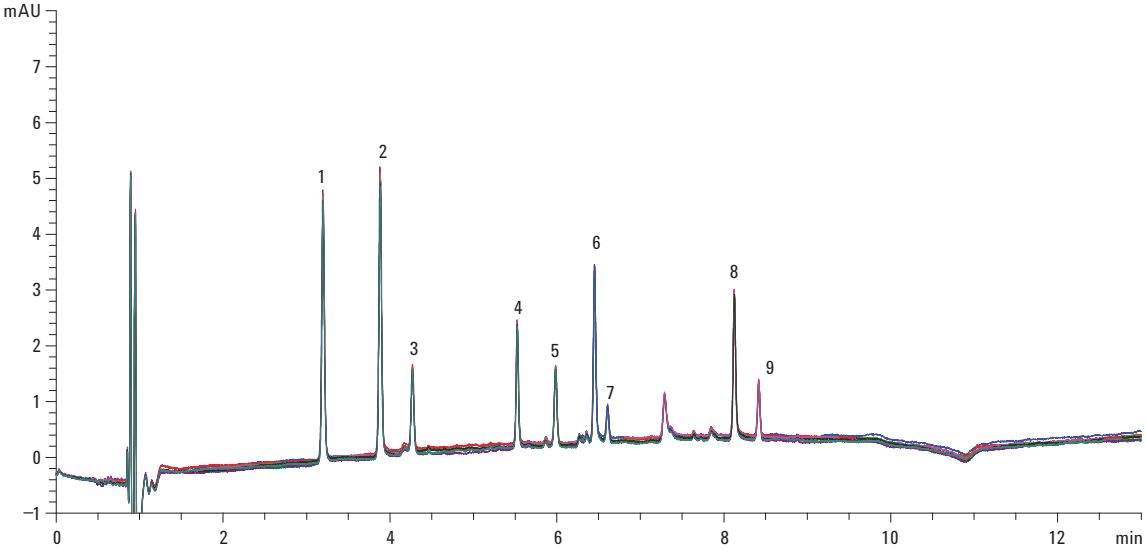


Figure 4. Overlay of chromatograms of eight injections of 1 ppm standards with an Agilent Poroshell 120 SB-Aq column. Conditions same as for Figure 3.

Extracts of five different kinds of butters (Figure 5) and 1 ppm spiked butters (Figure 6) were analyzed to investigate system suitability. Comparing the chromatograms of samples and 1 ppm spiked samples, additional peaks originating from the butter matrix were visible, but there were only a few interferences with the standard peaks. For example, peak 3 was difficult to differentiate from unspiked sample 1 and sample 5, while peak 9 was difficult to differentiate from unspiked sample 2. Though some interferences were found, we can still measure the amounts of antioxidants from the butter at the ppm level using the HPLC method. If a lower level of LOD is needed, a triple-quadrupole mass spectrometer would be a better choice of detector.

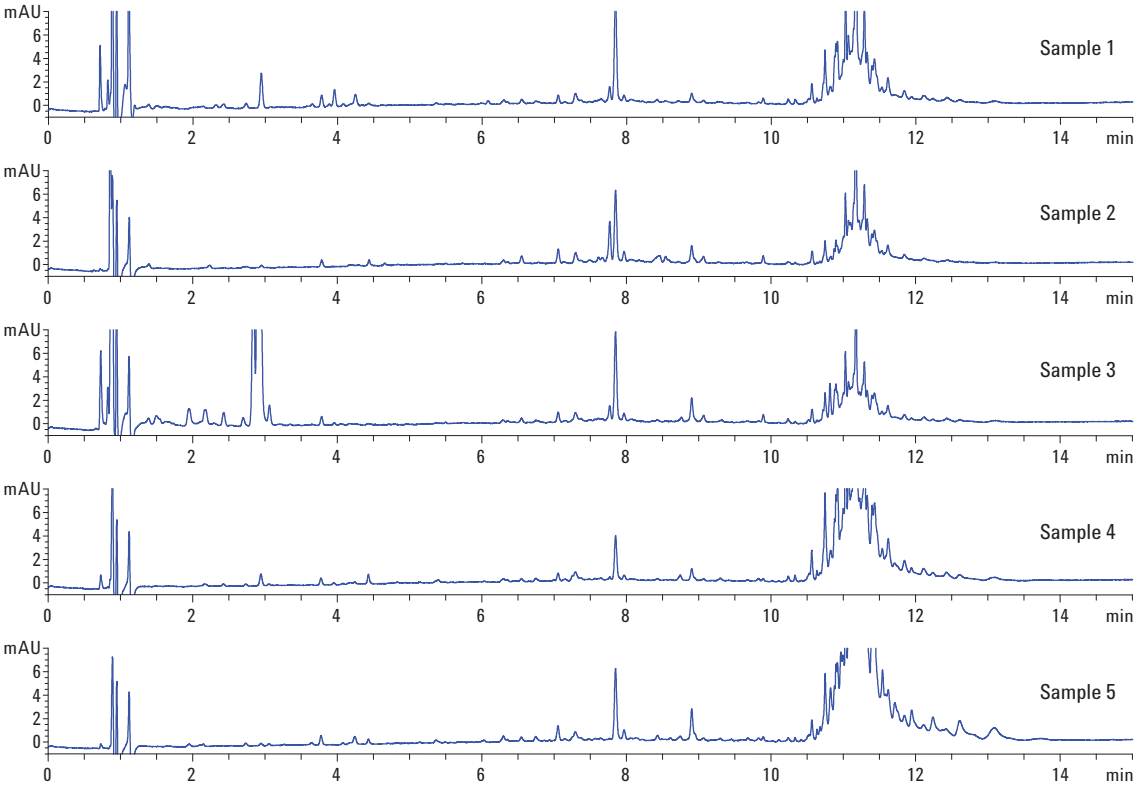


Figure 5. Separation of five different kinds of butter with an Agilent Poroshell 120 SB-Aq column. Conditions same as for Figure 3.

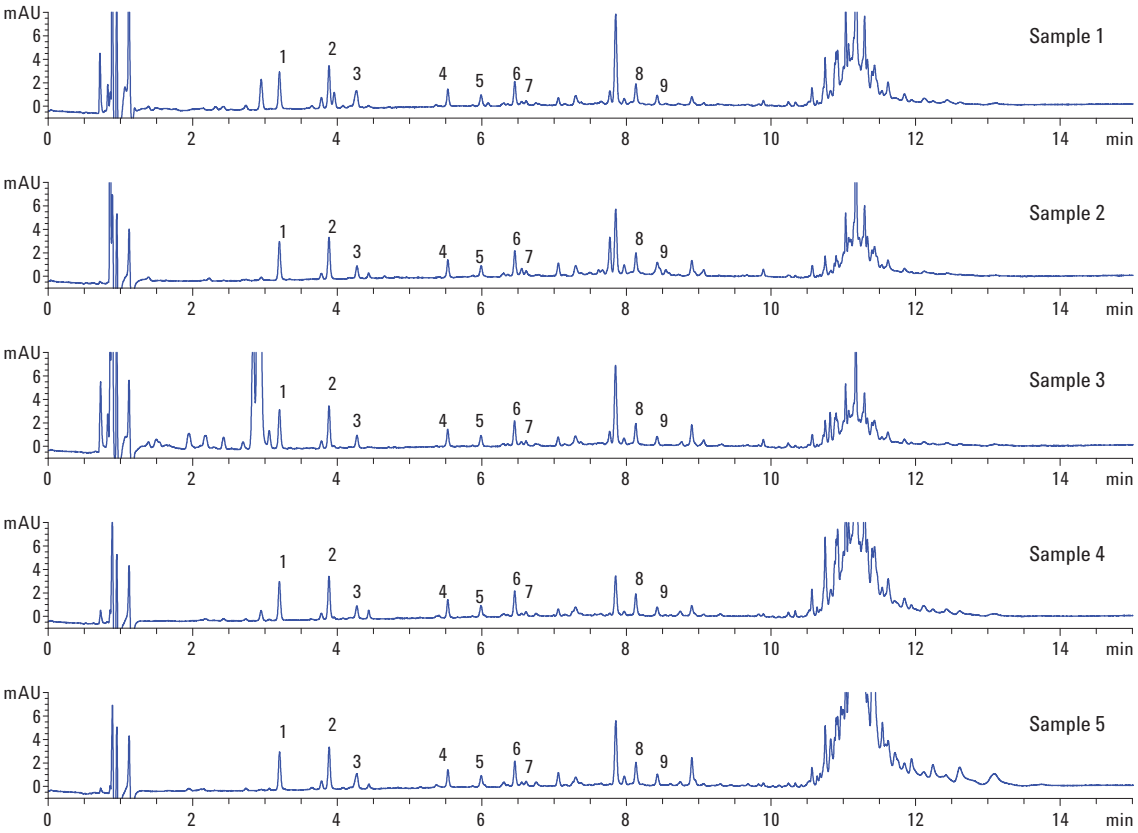


Figure 6. Separation of five different kinds of butter spiked with 1 ppm standards mixture using an Agilent Poroshell 120 SB-Aq column. Conditions same as for Figure 3.

Conclusions

Agilent Poroshell 120 columns are packed with superficially porous particles, which provide high performance similar to the sub-2 µm particles but with a 40 to 50% lower pressure than columns with sub-2 µm particles. The recent introduction of new bonded phases on Poroshell 120 makes them useful in method development by offering unique and differing selectivities across the various chemistries.

Using Poroshell 120 SB-Aq with the Agilent 1290 Infinity LC, a quick analytical method was developed for the determination of antioxidants in butters. The analysis time could be reduced to 15 minutes, one-third of the analysis time with a traditional 5 µm column. Butter samples and spiked butter samples were extracted and the system suitability was investigated. Satisfactory results were achieved at a ppm level. The developed method was suitable for QC laboratories in the food industry for the antioxidant analysis of butter.

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