Application Note

Pharmaceutical Small Molecules

Agilent

Trusted Answers

Taking Advantage of the Orthogonal Selectivities of Agilent InfinityLab Poroshell 120 C18 Columns for Method Development at Low pH

Author

Anne Mack
Agilent Technologies, Inc.

Abstract

In this application note, resolution is discussed with respect to LC method development. A theoretical plot demonstrates the variables that drive chromatographic separations, with selectivity being the most significant contributor. Using a simple formic acid and acetonitrile gradient, the unique selectivities of four Agilent InfinityLab Poroshell 120 C18 columns is demonstrated with a sample of veterinary drugs.

Introduction

Superficially porous particle LC columns are a popular tool in liquid chromatography. These columns are more efficient at lower pressure in comparison to their totally porous particle column counterparts.[1] This is primarily due to a shorter mass-transfer distance and substantially narrower particle size distribution in the column.[2]

The most popular particle size for superficially porous particle columns is 2.5 to 3 µm. These particles produce similar efficiency to traditional sub-2 µm columns while generating approximately 50% of the backpressure. High efficiency can contribute to resolving closely eluting peaks, while low backpressure allows for flexibility with LC instrumentation.

Agilent currently offers 12 bonded-phase chemistries on their 2.7 µm InfinityLab Poroshell 120 particles for use with reversed-phase LC separations. Four of these phases are C18s, each with unique separation abilities. This study demonstrates that not all C18s are the same, and shows the value in having multiple selectivity options during LC method development.

Experimental

An Agilent 1290 Infinity II LC with an Agilent Ultivo LC/TQ was used in this experiment. The system was modified from its standard configuration to have lower system volume and dispersion. Table 1 shows the configuration details. Four LC columns were used in this experiment and are listed in Table 1. Tables 2 to 4 show the LC and TQ method parameters.

The nine compounds analyzed in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA), and prepared as a mixed solution of 0.01 mg/mL each in water. All compounds are illustrated in Figure 1.

Formic acid (p/n G2453-85060) and LC/MS-grade acetonitrile (p/n G2453-85050) were obtained from Agilent. Water was 0.2 µm-filtered, 18 molecular weight from a Milli-Q system (Millipore, Burlington, MA, USA).

Table 1. System configuration.

Agilent 1290 Infinity II LC System Configuration	
Agilent 1290 Infinity II Flexible Pump (G7104A)	• Degasser • Seal wash pump • 35 µL solvent mixer: Agilent Jet Weaver, 35 µL/100 µL (p/n G4220-60006) • Firmware: B.07.23 [0009]
Agilent 1290 Infinity II Vialsampler (G7129B)	• Sample thermostat (p/n G7167-60101) • Metering parameter: seat assembly PEEK 0.12 mm, sample loop 20 µL, analytical head 20 µL • Autosampler → heater: capillary, stainless steel, 0.12 × 105 mm, SL/SL (p/n 5500-1238) • Vial, screw top, amber with write-on spot, certified, 2 mL, 100/pk (p/n 5182-0716) • Cap, screw, blue, PTFE/red silicone septa, 100/pk (p/n 5182-0717) • Vial insert, 250 µL, glass with polymer feet, 100/pk (p/n 5181-1270) • Firmware: D.07.23 [0009]
Agilent InfinityLab LC Series Integrated Column Compartment (G7130A)	• Integral type: G7129B • 3.0 µL heat exchanger • Heater → column: A-Line quick-connect assembly, 105 mm, 0.075 mm (p/n 5067-5961) • Column → flow cell: capillary, stainless steel, 0.075 × 220 mm, SV/SLV (p/n 5067-4784) • Firmware: B.07.23 [0009]
Agilent Ultivo LC/TQ (G6465A)	• Agilent Jet Stream ESI Source
Agilent 1290 Infinity II Diode Array Detector (G7117B)	• Ultralow dispersion Max-Light cartridge flow cell, 10 mm, 0.60 µL (p/n G4212-60038) • UV lamp (p/n 5190-0917) • Firmware: D.07.23 [0009]
Agilent LC Columns	• Agilent InfinityLab Poroshell 120 CS-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-942) • Agilent InfinityLab Poroshell 120 SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902) • Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902) • Agilent InfinityLab Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-702)

Table 2. UHPLC method parameters.

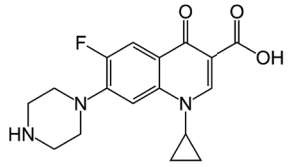
Parameter	Value
Mobile Phases	A: water B: acetonitrile C: 2% formic acid in water
Elution Conditions	0.4 mL/min, 0 to 95% B in 15 min, with 5% C held constant throughout the analysis
Column Temperature	30 °C
Injection Volume	0.05 µL
Sample	0.01 mg/mL standard, prepared in water; compounds shown in Figure 1
Detection	LC/MS/MS: See Tables 3 to 4

Table 3. LC/TQ source method parameters.

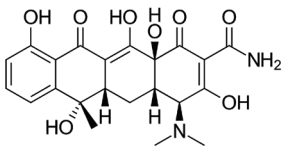
MS Source	Set Point
Gas Temperature	150 °C
Gas Flow	12 L/min
Nebulizer	20 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	5 L/min
Capillary Voltage	2,000 V

Table 4. LC/TQ acquisition method parameters for veterinary drugs.

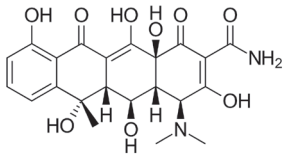
Compound Name	Precursor (*m/z*)	Product (*m/z*)	Fragmentor (V)	CE (V)	Polarity
Ciprofloxacin	332	314.3	100	20	Positive
Enrofloxacin	360.1	342.4	150	15	Positive
Erythromycin	734.68	158.2	150	20	Positive
Oxacillin	402.4	160.3	75	7	Positive
Oxytetracycline	461.1	426.4	125	10	Positive
Penicillin-G	335.17	176	125	7	Positive
Sulfamerazine	265.18	92.1	100	20	Positive
Sulfamethazine	279	124.3	125	20	Positive
Tetracycline	445.2	410.3	125	10	Positive



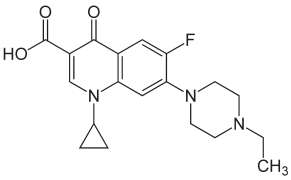
Ciprofloxacin



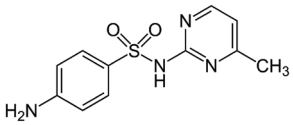
Oxytetracycline



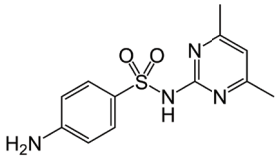
Tetracycline



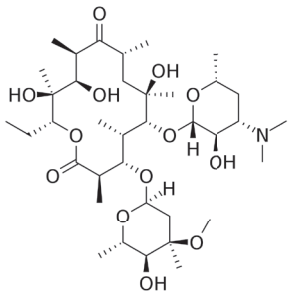
Enrofloxacin



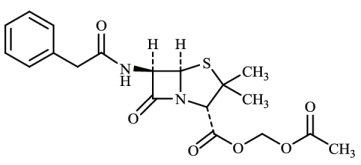
Sulfamerazine



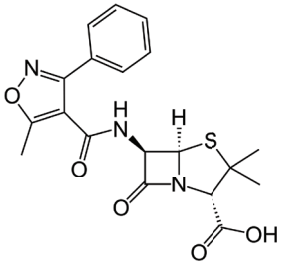
Sulfamethazine



Erythromycin



Penicillin-G



Oxacillin

Figure 1. Compounds of interest.

Results and discussion

Chromatographic resolution is a common separation criterion for LC method developers. Achieving baseline resolution of all analytes allows for accurate integration and quantitation when using nonselective detectors, such as diode array, fluorescence, refractive index, and evaporative light scattering. Even for more sophisticated detectors, such as mass spectrometers, achieving chromatographic resolution is still valuable in the event of isobaric pairs, or to prevent ion suppression from coeluting species.

To get a better understanding of resolution, the resolution equation is shown in Figure 2. Figure 2 demonstrates how efficiency, selectivity, and retention all drive chromatographic resolution. However, they do so to varying degrees, as shown in Figure 3.

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution — Efficiency — Selectivity — Retention

Figure 2. Resolution equation.

Figure 3 shows theoretical resolution values, which were generated by varying one chromatographic factor at a time, while holding the other two constant. The purpose of this plot is to show the isolated contribution of each variable on resolution. The three variables in question are k', plates, and alpha.

The retention factor, k', of an analyte is one driver of resolution. Retention can be increased by decreasing the strength of the mobile phase; for reversed-phase LC, this means reducing the organic content. As analytes are retained longer, resolution may be increased. However, we can see from Figure 3 that increasing k' from 2 to 12 only increases the theoretical resolution from 1 to 1.5.

Plates, or efficiency, can be increased by increasing the length of the LC column or decreasing the particle size. Figure 3 shows an increase in plates from 5,000 to 25,000—this would be the equivalent of increasing column length by a factor of five. Again, this large increase in N only raises our theoretical resolution slightly from 1 to 2.5.

Now, when alpha, or selectivity, is considered, we can see the main driver for resolution. In Figure 3, alpha is only increased from 1.1 to 2.1. However, a significant impact to our theoretical resolution can be seen, as it increases from one to more than six. Selectivity is the largest contributing factor to chromatographic resolution. To alter selectivity, the chemistry of the chromatographic system must change—that is, the mobile phase or the column stationary phase. For the mobile phase, the type of organic solvent—acetonitrile versus methanol—can impact selectivity. Mobile phase pH can also impact selectivity, as shown in Agilent application note Using pH as a Method Development Tool with Agilent InfinityLab Poroshell 120 CS-C18 (publication number 5994-2274EN).[3]

Changing the column stationary phase is another way to alter selectivity and potentially improve resolution. Figure 4 demonstrates that even multiple variations of C18-bonded phases can be different enough to change elution order and impact chromatographic resolution.

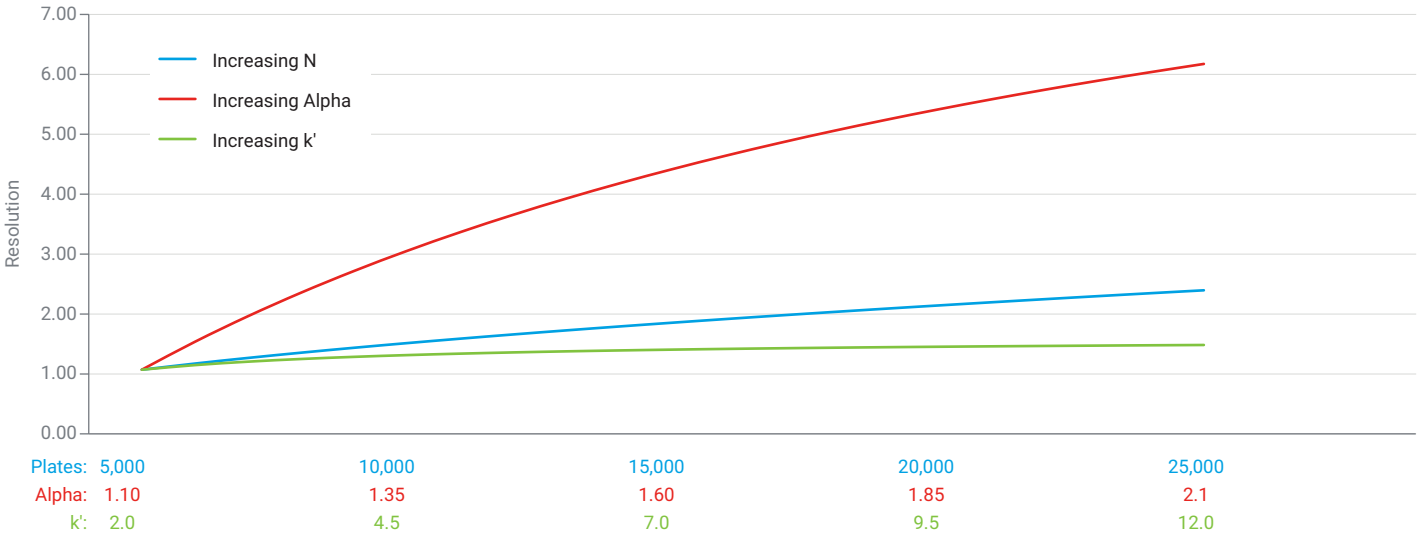


Figure 3. Factors that influence resolution.

Figure 4 demonstrates the orthogonal selectivity of four InfinityLab Poroshell 120 C18 columns with a sample of veterinary drugs with a low-pH mobile phase. Screening multiple columns with a simple formic acid and acetonitrile gradient, as done in this work, is a common way to initiate LC method development. The more complex a sample is, the less likely it is that a column screen will immediately give a baseline resolution of all compounds of interest. Often, additional method development will be required. However, starting method development this way can systematically guide an appropriate column choice for the analysis.

The InfinityLab Poroshell 120 CS-C18 gave the most orthogonal selectivity in the example shown, with the most desirable separation. Given that many pharmaceutical compounds are basic, this is not surprising. The charged surface chemistry on the CS-C18 is designed to provide the user with unique retention of bases, as well as excellent peak shape and loadability when using weak ionic mobile phases, such as formic acid.

Conclusion

The simple formic acid and acetonitrile gradient used in the previously mentioned separations is a great starting point for method development. Combined with high-efficiency columns, like those in the InfinityLab Poroshell 120 family, this simple method can be used to quickly evaluate multiple column stationary phases, which can improve chances to retain and resolve all analytes.

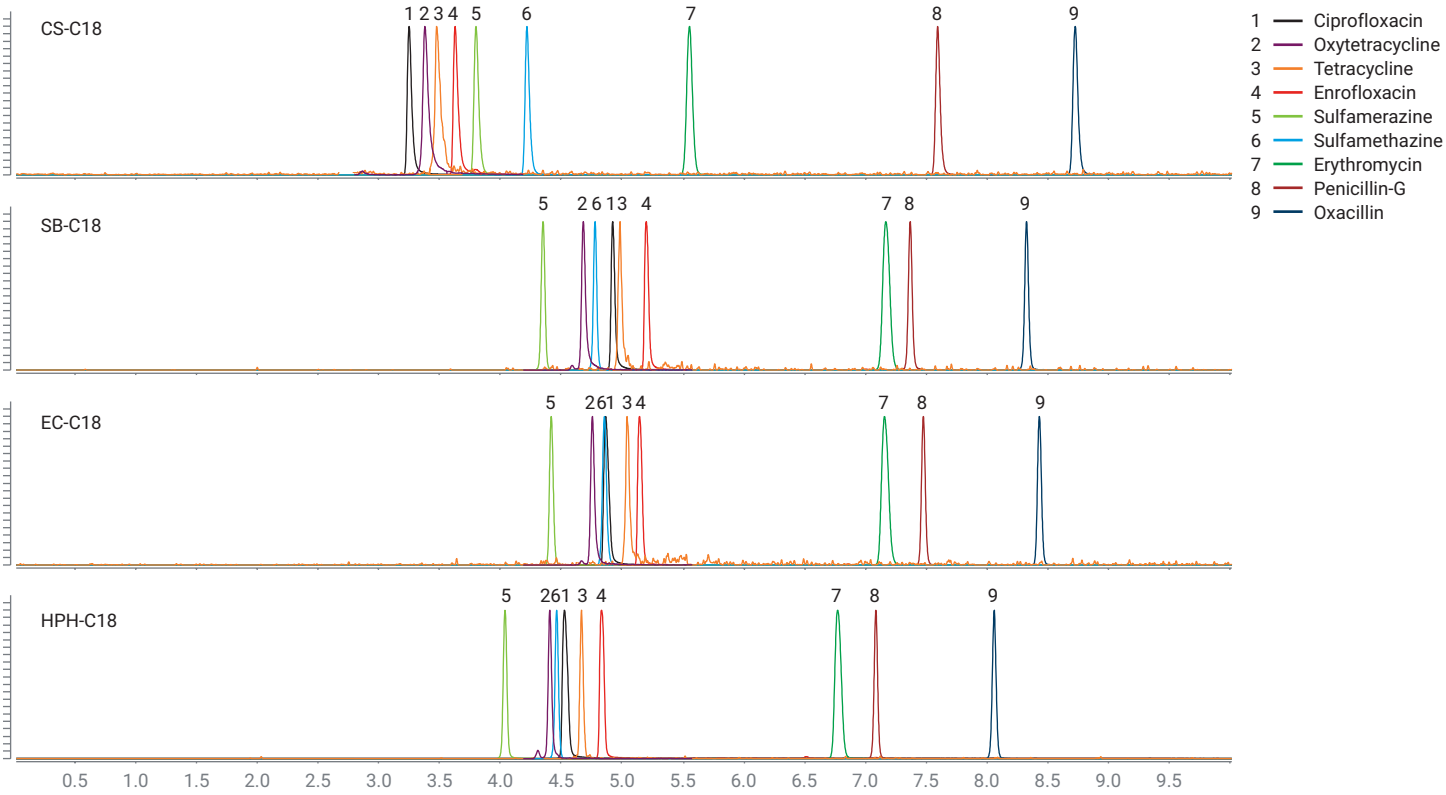


Figure 4. Four different Agilent InfinityLab Poroshell 120 C18 phases give unique selectivity for veterinary drugs with a low-pH mobile phase.

References

1. Gratzfield-Hugsen, A.; Naegele, E. Maximizing Efficiency Using Agilent InfinityLab Poroshell 120 Columns. *Agilent Technologies application note*, publication number 5990-5602EN, **2016**.
2. Meyer, V. R. Practical High-Performance Liquid Chromatography. Fourth Edition, p. 34. Wiley, **2004**.
3. Mack, A. Using pH as a Method Development Tool with Agilent InfinityLab Poroshell 120 CS-C18. *Agilent Technologies application note*, publication number 5994-2274EN, **2020**.

www.agilent.com/chem

DE.0273611111

Printed in the USA, September 4, 2020
5994-2358EN

Agilent
Trusted Answers