Transfer and Optimization of Existing Methods for Analysis of Antibiotics in Meat to Agilent Poroshell 120 EC-C18 Columns using MS/MS Detection

Application Note

Food

Authors

William J. Long, Anne E. Mack and
James R. Evans
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19809
USA

Abstract

In this work, a generic gradient method with UV detection is used to evaluate mobile phase choices for fast method optimization of antibiotics analysis in meat, with the ultimate goal of producing a mass spectrometer compatible method. This evaluation included four buffers and two organic choices. The mobile phase combination that yielded the best separation is transferred and optimized to an Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm, 2.7 µm column. Gradient time was decreased from 45 min to 12 min. Time can be further reduced using a 3 mm × 50 mm column, at the cost of some resolution. The method is demonstrated on an Agilent 6410 triple quadrupole LC/MS System coupled with an Agilent 1200 Series Rapid Resolution LC.

Table 1. Method Parameters for Various Column Dimensions

	4.6 × 50 mm Poroshell 120 EC-C18	3.0 × 50 mM Poroshell 120 EC-C18	2.1 × 100 mM Poroshell 120 EC-C18
Mobile Phase	A: Buffer, varies	A: 10 mm ammonium formate pH 3.8	A: 10 mm ammonium formate pH 3.8
	B: Organic, varies	B: Acetonitrile	B: Acetonitrile
Gradient	10-40% B	10-40% B	10-40% B
Gradient Time	12 min	12 min	12 min
Flow Rate	2 mL/min	0.85 mL/min	0.42 mL/min
Injection Volume	0.5 µL	5 µL	2.5 µL or 10 µL
Sample	0.1 mg/mL antibiotics	1 µg/mL antibiotics	1 µg/mL or 10 ng/mL antibiotics
TCC Temperature	30 °C	30 °C	30 °C
Detector	DAD: Sig = 270, 4 nm; Ref = 360, 100 nm	MS/MS: See Table 2	MS/MS: See Table 2

Introduction

Administration of antibiotics is a common practice in chicken, pork, beef and fish farming. Many domestic cattle receive various antibiotics in their feed for the prevention and control of disease caused by fungi and bacteria. Many countries regulate acceptable residue levels of these compounds in agricultural and animal products. In this work, an older method is transferred from a 5 µm, 250 mm column to a new superficially porous column to increase the speed of the analysis and change the method of detection from UV to MS/MS. An increase in throughput of 5 to 10 times is demonstrated, while minimally impacting sample preparation. Since the analysis time is shortened dramatically, time is available for optimization of mobile phase selectivity (pH, buffer types and organic modifier).

Transition methods can be developed by modifying an existing method or starting fresh. In this case, the objective was to develop a new MS-compatible separation from an existing UV separation. Consequently, a change in the mobile phase was required because 0.7 % phosphoric acid is not a desirable solvent for MS detection. A generic screening method using 0.1 % formic acid was investigated, but additional MS-compatible solvent systems were also evaluated. In this work a method is developed by first screening different mobile phase combinations using a short Agilent Poroshell 120 column using UV detection, then transferring that method to an Agilent 6410 triple quadrupole LC/MS System. A major advantage of the Agilent Poroshell 120 EC-C18 is that it uses the same 2 µm frit as the original 5 um column, negating the need for sample preparation method development.

Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm columns have similar performance to 1.8-µm totally porous Agilent ZORBAX Eclipse Plus C18 columns, but since they use 2-µm column frits similar to those found on 5-µm columns, they require no additional sample preparation. This allows for a more seamless method transfer. While some previous work demonstrates the use of Agilent Poroshell 120 columns on older Agilent 1100 systems, they are ideally used on more modern systems such as the Agilent 1200 or 1260 series UHPLC's.

Table 2. MRM Transitions for Antibiotic Compounds.

Compound name	Precursor ion	Product ion	Fragmentor voltage	Collision energy
Sulfamerazine	265	172	100	25
Sulfamerazine	265	108	100	25
Thiamphenicol	338	308	140	10
Thiamphenicol	338	118	140	50
Sulfamethazine	279	124	100	25
Sulfamethazine	279	108	100	30
Furazolidone	226	137	140	25
Furazolidone	226	122	140	25
Sulfamonomethoxine	281	126	100	25
Sulfamonomethoxine	281	108	100	25
Oxolinic acid	262	160	100	40
Oxolinic acid	262	130	100	45
Pyrimethamine	249	198	140	45
Pyrimethamine	249	128	140	60
Sulfadimethoxine	311	156	140	25
Sulfadimethoxine	311	108	140	55
Sulfaquinoxaline	301	129	100	50
Sulfaquinoxaline	301	108	100	40
Difurazone	361	222	100	15
Difurazone	361	154	100	45

Experimental

Method development is based upon the use of a generic gradient. Using a short 4.6 mm × 50 mm Poroshell 120 EC-C18, 2.7 µm column, several different mobile phases can be quickly evaluated. The generic gradient is run at 2.0 mL/min, starts at 10% and proceeds to 40% organic over 12 min. This gradient is later transferred to 2.1 mm × 100 mm and 3 mm × 50 mm columns by changing the gradient according to Equation 1. The three gradients used are listed in Table 1 with MRM transitions shown in Table 2. MS-compatible mobile phases consisting of volatile buffer components such as formic acid, ammonium formate buffer and ammonium acetate buffer are used.

An Agilent 1200 Rapid Resolution LC (RRLC) system was used for this work:

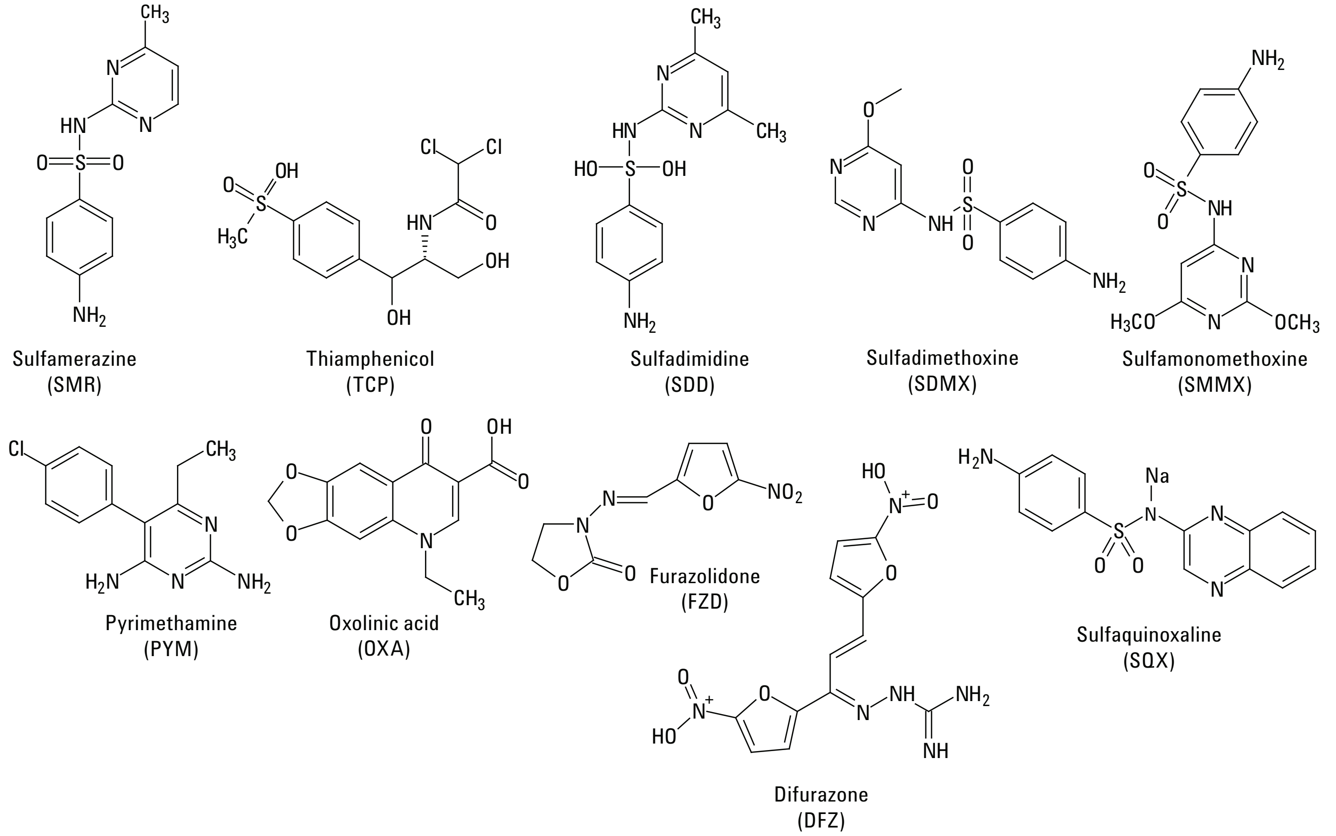
- G1312B Binary Pump SL.
- G1367C Automatic Liquid Sampler (ALS) SL.
- G1316C Agilent 1290 Infinity Thermostatted Column Compartment (TCC) SL.
- G1315C Agilent Diode Array Detector (DAD) SL using a G1315-60024 micro flow cell (3-mm path, 2-µL volume).
- G6410 Agilent Triple Quadrupole LC/MS System with Electrospray (ESI).
- ChemStation version B.04.01 was used to control the HPLC and process the data. Agilent MassHunter Version 2.0 was also used to control the Agilent 6410 Triple Quadrupole LC/MS System, the Agilent 1200 Rapid Resolution LC (RRLC), and to analyze the data.

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

- 4.6 mm × 50 mm, 2.7 µm p/n 699975-902
- 3.0 mm × 50 mm, 2.7 µm p/n 699975-302
- 2.1 mm × 100 mm, 2.7 µm p/n 695775-902

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. Compounds were purchased from Sigma Aldrich (Bellefonte, PA). Additionally, methanol, acetonitrile, ammonium formate, ammonium acetate, formic acid, and glacial acetic acid were purchased from Sigma Aldrich. Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Buffers used in this work were prepared by dissolving an appropriate amount of the ammonium salt to produce a 10 mM solution, adding 950 mL water and titrating the solution with either formic acid (for the ammonium formate buffers) or glacial acetic acid (for the ammonium acetate buffers). The buffer solutions were then brought to a 1 L volume.



Sulfamerazine (SMR)

Thiamphenicol (TCP)

Sulfadimidine (SDD)

Sulfadimethoxine (SDMX)

Sulfamonomethoxine (SMMX)

Pyrimethamine (PYM)

Oxolinic acid (OXA)

Furazolidone (FZD)

Difurazone (DFZ)

Sulfaquinoxaline (SQX)

Figure 1. Compounds of interest.

Results and Discussion

The original method published in 2002 by Kumagai and Onigbinde provides an effective method for the analysis of antibiotics in meat using UV detection. As seen in Figure 2, the method separates the analytes in approximately 45 min. However the nonvolatile phosphoric acid in the mobile phase is not compatible with MS detection.

In many cases, simple scaling of a method will allow for a fast method transfer. In this case, however, a change in the mobile phase was required for LC/MS compatibility. The use of short Poroshell 120 EC-18 4.6 mm × 50 mm, 2.7 µm columns for assessing mobile phase changes has several advantages. One advantage is that they allow quick separations without sacrificing resolving power. In addition, since they are used at 2 mL/min with a generic gradient, the solvent is rapidly purged through the system. This ensures that the solvent screening experiment can be quickly performed by changing solvent bottles, with no concerns about residual solvents in the HPLC pump or the degasser. These columns can be used for LC/MS but typically smaller diameter columns such as 3.0 or 2.1 mm columns are used.

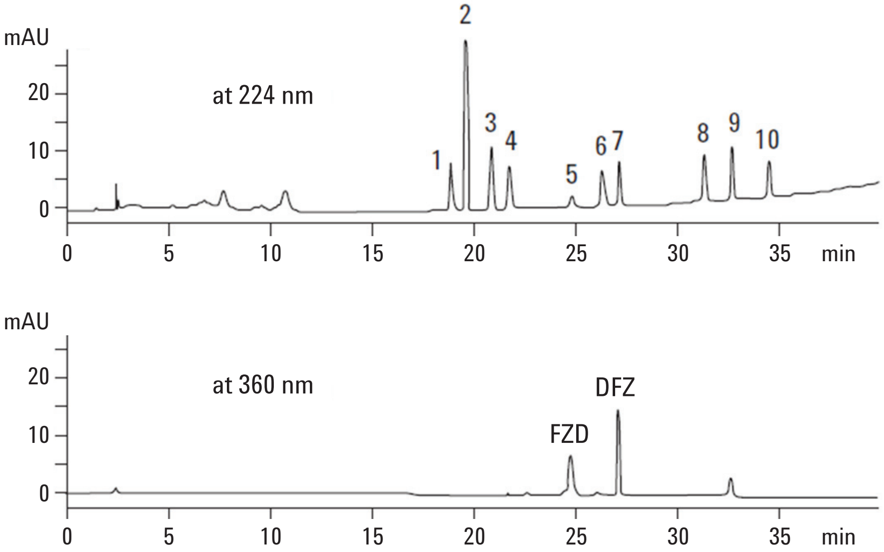
As discussed in reference 5, once a separation has been optimized according to selectivity and retention index, it is possible to further improve the chromatography by varying column length, particle size and flow rate. However the k^* value must be maintained, while varying these column conditions so as not to lose selectivity.

Equation 1: $k^* = (t_gF)/(d/2)^2L(\Delta\%B)$

Where:

t_g is the gradient time
F is the flow rate
L is the column length
d is the column internal diameter
$\Delta\%B$ is the change in organic content across the gradient segment

Original Method Kumagai and Onigbinde 5988-7135 June 2002
Only 338 and 360 wavelengths are shown for brevity.



1	SMR	6	SMMX
2	PYM	7	DFZ
3	TCP	8	SDMX
4	SDD	9	SQX
5	FZD	10	OXA

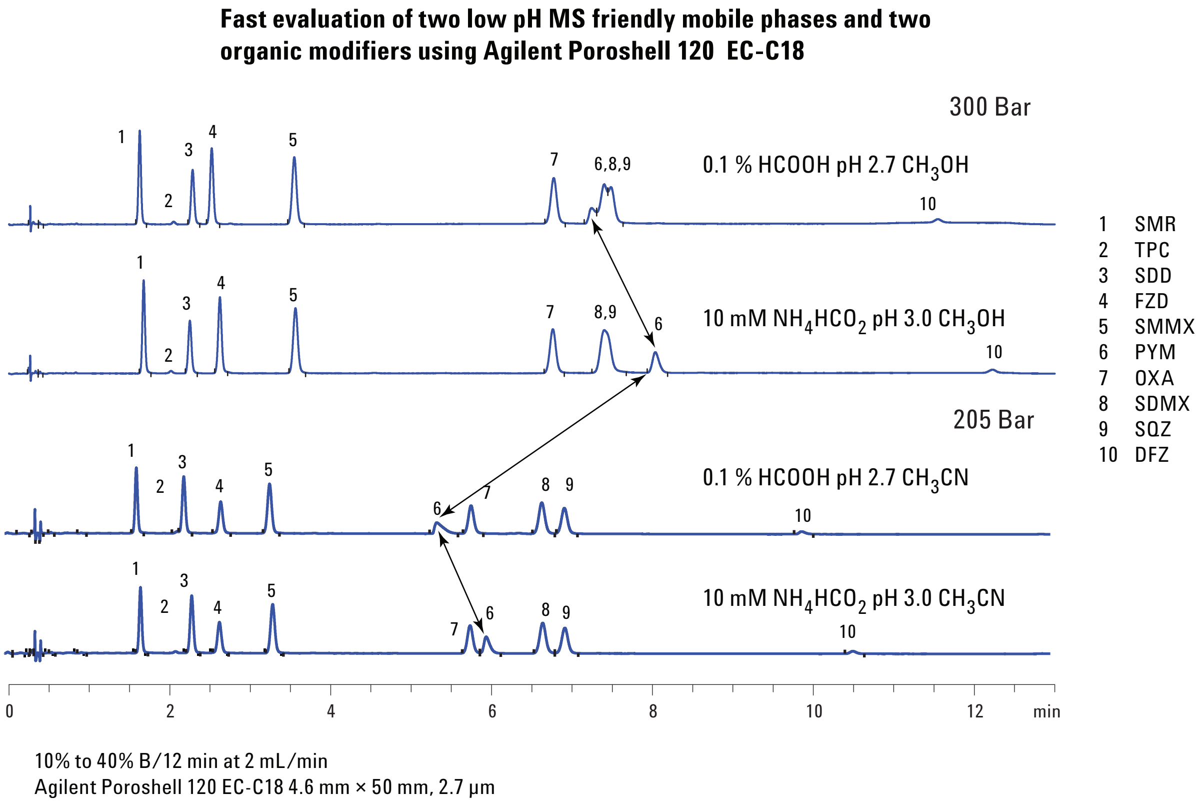
Instrument:	Agilent 1100 Series HPLC
Column:	250 mm × 4 mm id, RP-18 Purospher, 5 µm, p/n 79925PU-584
Mobile phase:	A = 0.7% Phosphoric acid, B = CH_3CN
Gradient:	0.0 min 5% B; 10.0 min 5% B; 40.0 min 65% B; 45.0 min 65% B; Post Time 7.0 min 5% B
Flow rate:	1.0 mL/min
Temperature:	40 °C
Injection volume:	20 µL
Diode array detector:	A-338/10 nm, reference wavelength off B-264/8 nm, reference wavelength off C-360/8 nm, reference wavelength off

Figure 2. Original method produces excellent results on a 250 mm column with UV detection.

As illustrated in Figure 3, generic gradients using methanol or acetonitrile are used to separate the compounds of interest. The gradients using methanol generate 50% higher pressure (300 bar instead of 200 bar). While this is not critical when using a 50 mm column, this does become more important as the length of the column is increased to 100 or 150 mm.

With methanol, the last compound elutes later due to the lower solvent strength. Formic acid, while a convenient mobile phase additive, produces less optimal results than 10 mM ammonium formate buffer (pH =3), particularly for pyrimethamine. In addition to peak shape improvements, elution order changes also occur most notably with pyrimethamine.

Many selectivity improvements and changes can be produced by choice of pH or organic modifier. As noted earlier, the peak shape of many basic compounds are improved when using methanol, however Poroshell 120 EC-C18 yields excellent peak shape for all compounds in this study. By adjusting the pH even slightly, both the elution order and peak spacing can be changed. This is most evident in Figure 3, where methanol and pH act to dramatically change the elution order. For the compounds in this study the best mobile phase combination is found at pH 3.8, ammonium formate with acetonitrile.

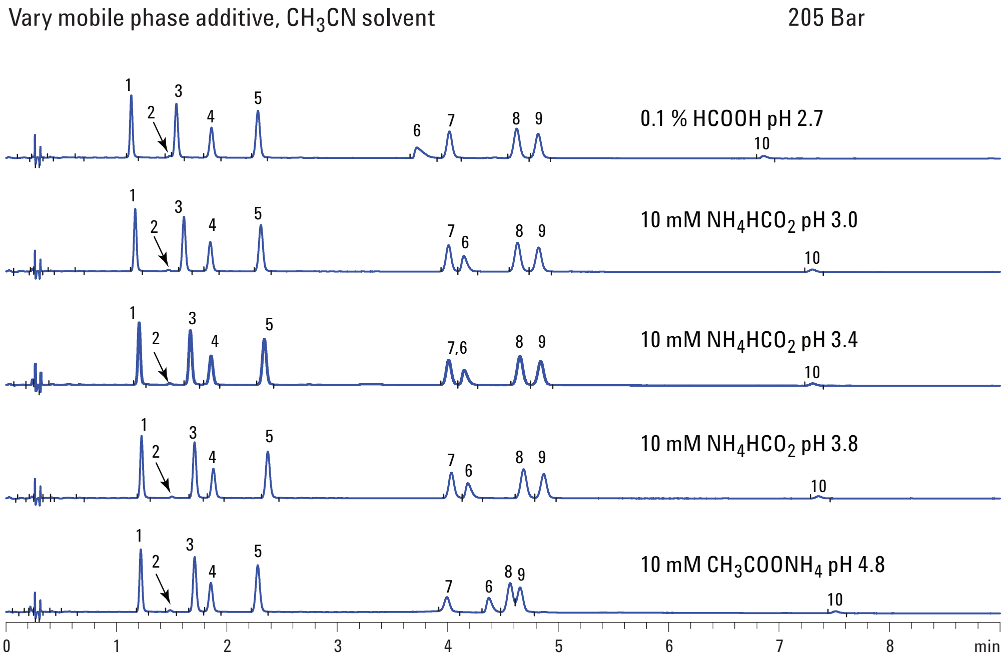


Fast evaluation of two low pH MS friendly mobile phases and two organic modifiers using Agilent Poroshell 120 EC-C18

10% to 40% B/12 min at 2 mL/min
Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm

Figure 3. Comparison of chromatographic conditions: buffer, 0.1 % formic acid, CH_3OH, CH_3CN.

Acetonitrile with ammonium formate buffer yields excellent peak shape and selectivity with pH 3.8 being optimal for these analytes

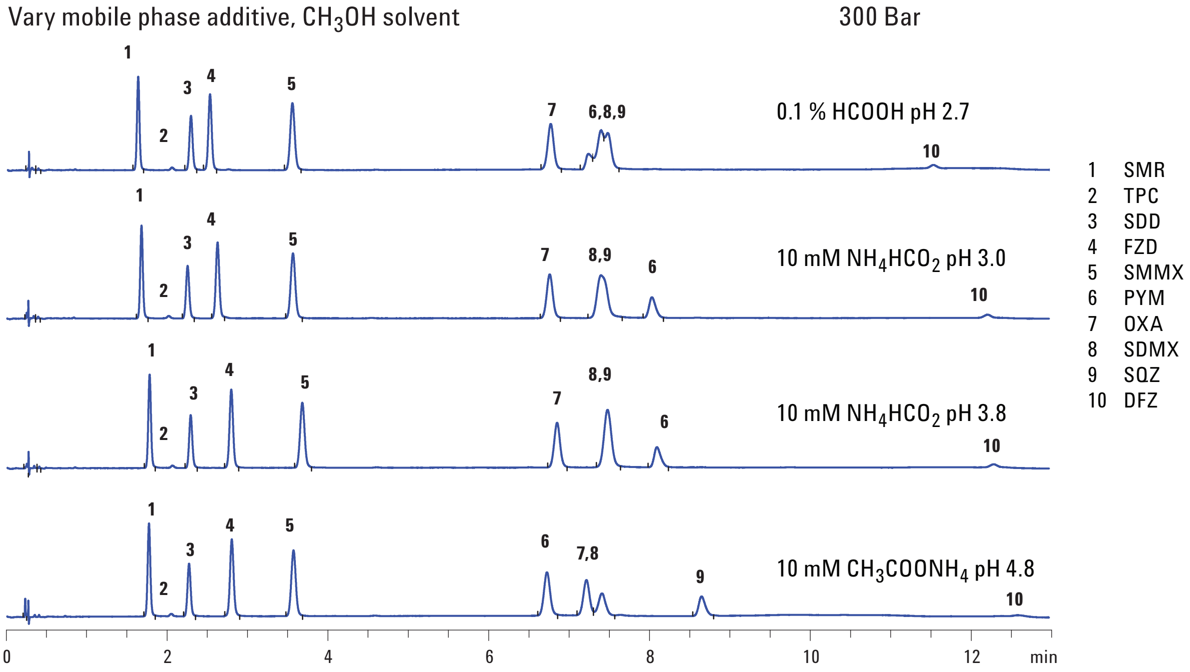


1	SMR
2	TPC
3	SDD
4	FZD
5	SMMX
6	PYM
7	OXA
8	SDMX
9	SQZ
10	DFZ

10% to 40% B/12 min at 2 mL/min
Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm

Figure 4. Comparison of buffers with CH_3CN.

Methanol with ammonium acetate buffer yields excellent peak shape with pH 4.8 being optimal for these analytes



10% to 40% B/12 min at 2 mL/min
Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm

Figure 5. Comparison of buffers with CH_3OH.

Figure 6 illustrates a total ion chromatogram based on the scouting work shown in Figures 3, 4 and 5. Conditions were scaled according to Equation 1 for the 3.0 mm × 50 mm column. This easy change demonstrates that the 3 mm column can be easily used for both conventional UV and more sensitive MS. In addition, a 2.1 mm × 100 mm column is also used with the same gradient with only the flow rate changed. If the gradient had been exactly scaled, the analysis time would have been twice as long, but as illustrated, the resolution is adequate. Figure 7 shows an MRM chromatogram of the antibiotic mixture. The compounds are sufficiently separated even with a large sample volume injected on-column. Conditions are listed in Tables 1 and 2.

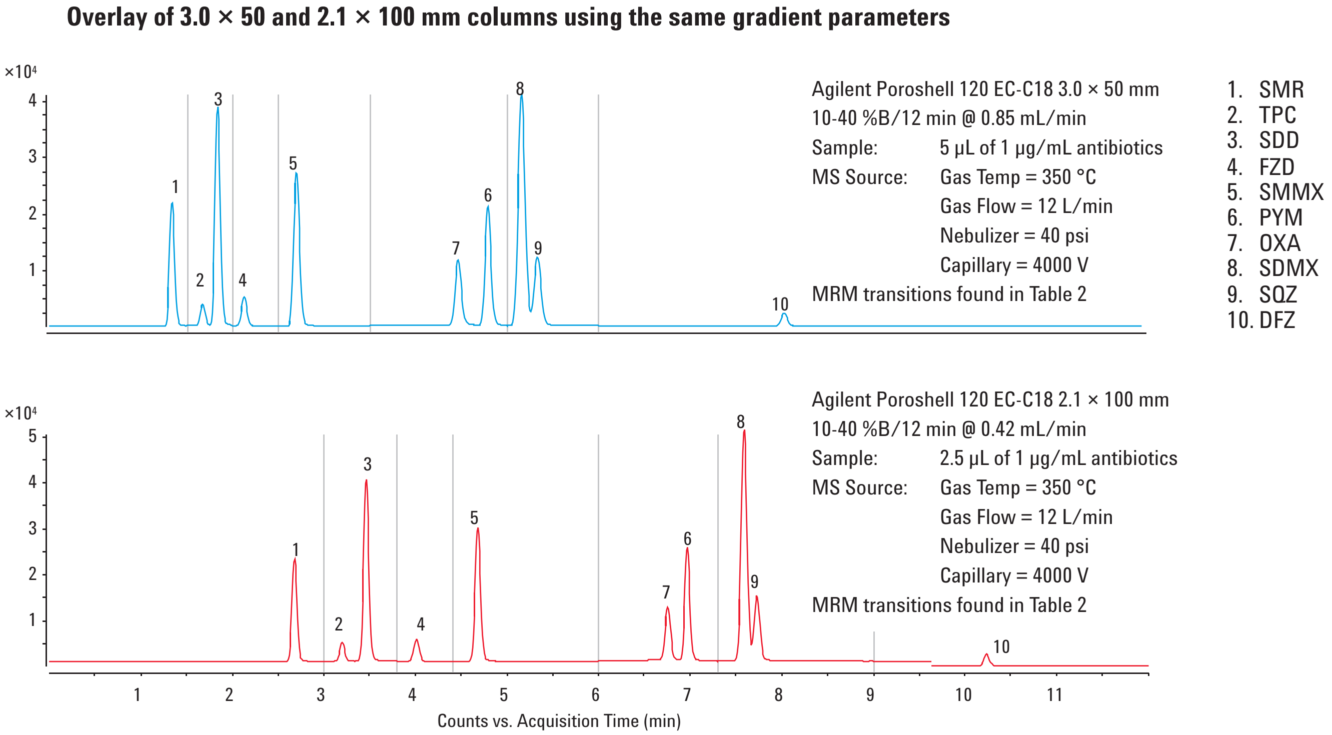
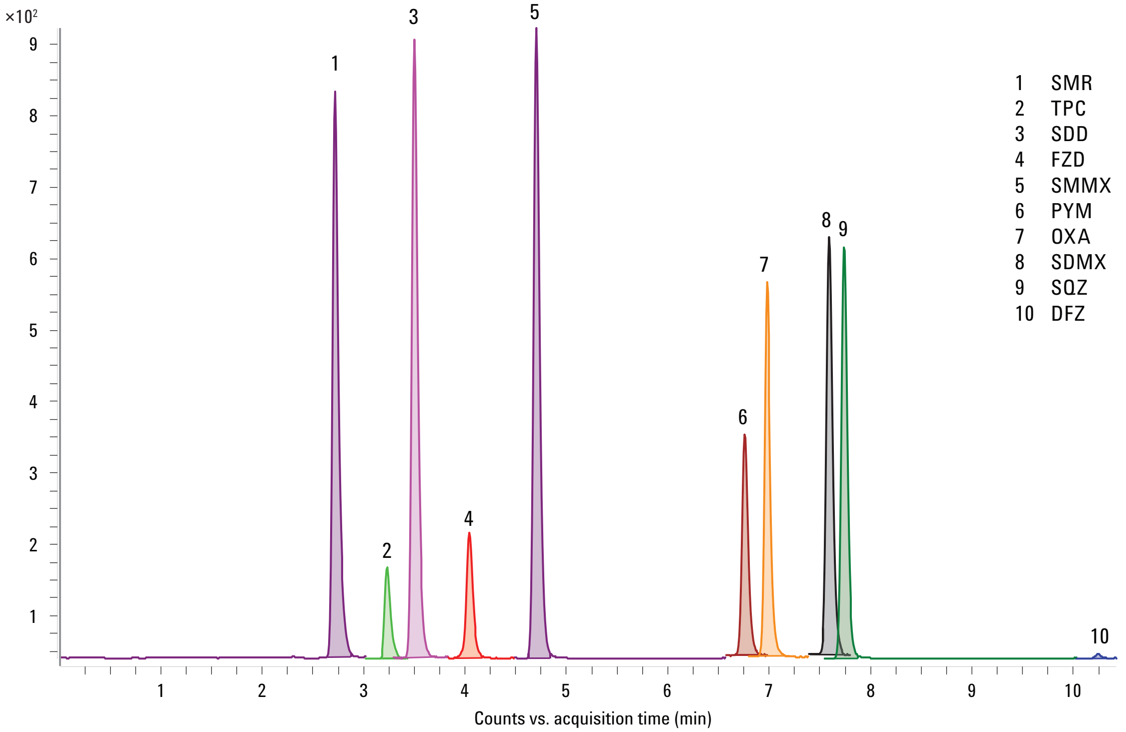

Overlay of 3.0 × 50 and 2.1 × 100 mm columns using the same gradient parameters

Figure 6. Total ion chromatograms of antibiotic mixture on 3 × 50 mm, and 2.1 × 100 mm Agilent Poroshell 120 EC-C18 columns.

Optimized MRM of 10 antibiotics in less than 11 minutes on Agilent Poroshell 120 EC-C18



Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm, 2.7 µm
10% CH_3CN at t_0, ramp to 40% CH_3CN in 12 min (buffer 10 mM NH_4HCO_2 pH 3.8 adjusted with concentrated formic acid), 0.42 mL/min
Sample: 10 uL of 10 ng/mL antibiotics
using dynamic MRM mode on MS/MS

Figure 7. Dynamic MRM of antibiotic mixture on Agilent Poroshell 120.

Conclusions

This work shows that in method migration, modern colums and fast liquid chromatographs make it easier to start fresh. Using a generic gradient on short columns, 10 mobile phase combinations are quickly evaluated. Following basic scaling equations, a method can easily be transferred to a column of another dimension. By optimizing the mobile phase using a UV detector, the method is partially developed on an instrument that may be commonly used in a lab rather than the more expensive and possibly less available instrument that the method will be transferred to.

Poroshell 120 columns are good to use for LC/MS of complex samples at low pressure. Regardless of the analytical power of the triple quadrupole mass spectrometer, a better separation simplifies data analysis, which may shorten cycle time. Baseline separated compounds also allow the mass spectrometer to maximize dwell time for a given peak to yield more accurate and reproducible results. This ensures the best possible quantitation. Additionally, less chance of ion suppression is possible caused by coeluting compounds.

Several additional factors are also demonstrated. Optimal conditions for this mixture are found using the fast scouting method in acetonitrile ammonium formate buffer pH 3.8 (8 min). The analysis also works in methanol with pH 4.8, ammonium acetate (13 min). This could easily be shortened by changing the gradient to elute the last peak more quickly. For example, ramp organic more quickly at the end with a second step; however this would increase pressure further. The use of a "true buffer" such as 10 mM ammonium formate provides better peak shape for bases than a buffering solution such as 0.1 % formic acid at similar pH. The method as shown is chromatographically optimized and work is in progress to optimize detection conditions.

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