

Not just speed—intelligent speed.

Intelligent Speed (IS™) Columns Application Notebook



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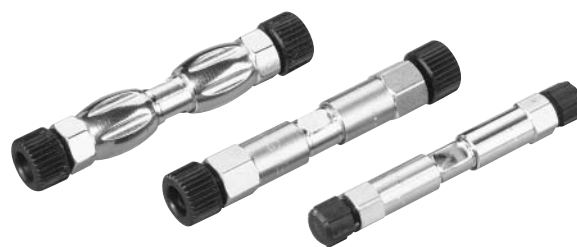
INTRODUCTION

INTELLIGENT SPEED (IS™) COLUMNS

Intelligent Speed (IS™) columns from Waters give today's chemist more than the raw speed they need for high-throughput HPLC.

- Increasing throughput and productivity – more results in less time
- Reducing the overall cost per analysis
- Shortening new product development timelines

Only IS™ columns incorporate the knowledge and intelligence necessary for today's speed requirements while maintaining peak efficiency through optimized column hardware design and packing technology. IS™ columns are available in all Waters innovative chemistries (XTerra®, Symmetry®, SymmetryShield™ and Atlantis™) with a wide variety of stationary phases, particle sizes and internal diameters.



Packing	Particle Size (µm)	Pore Size (Å)	Carbon Load %	End-Capped	Inner Diameter (I.D) (mm)	Length (mm)
XTerra® MS C ₁₈	2.5, 3.5 and 5	125	15.5	Yes	2.1, 3.0, 3.9 and 4.6	20
XTerra® MS C ₈	2.5, 3.5 and 5	125	12.0	Yes	2.1, 3.0, 3.9 and 4.6	20
XTerra® RP ₁₈	3.5 and 5	125	15.0	Yes	2.1, 3.0, 3.9 and 4.6	20
XTerra® RP ₈	3.5 and 5	125	13.5	Yes	2.1, 3.0, 3.9 and 4.6	20
Atlantis™ dC ₁₈	3 and 5	100	12.0	Yes	2.1, 3.0 and 4.6	20
Symmetry® C ₁₈	3.5 and 5	100	19.0	Yes	2.1, 3.0, 3.9 and 4.6	20
Symmetry® C ₈	3.5 and 5	100	11.7	Yes	2.1, 3.0, 3.9 and 4.6	20
SymmetryShield™ RP ₁₈	3.5 and 5	100	17.0	Yes	2.1, 3.0, 3.9 and 4.6	20
SymmetryShield™ RP ₈	3.5 and 5	100	15.0	Yes	2.1, 3.0, 3.9 and 4.6	20

*YMC Intelligent Speed (IS™) columns are available in the Americas.

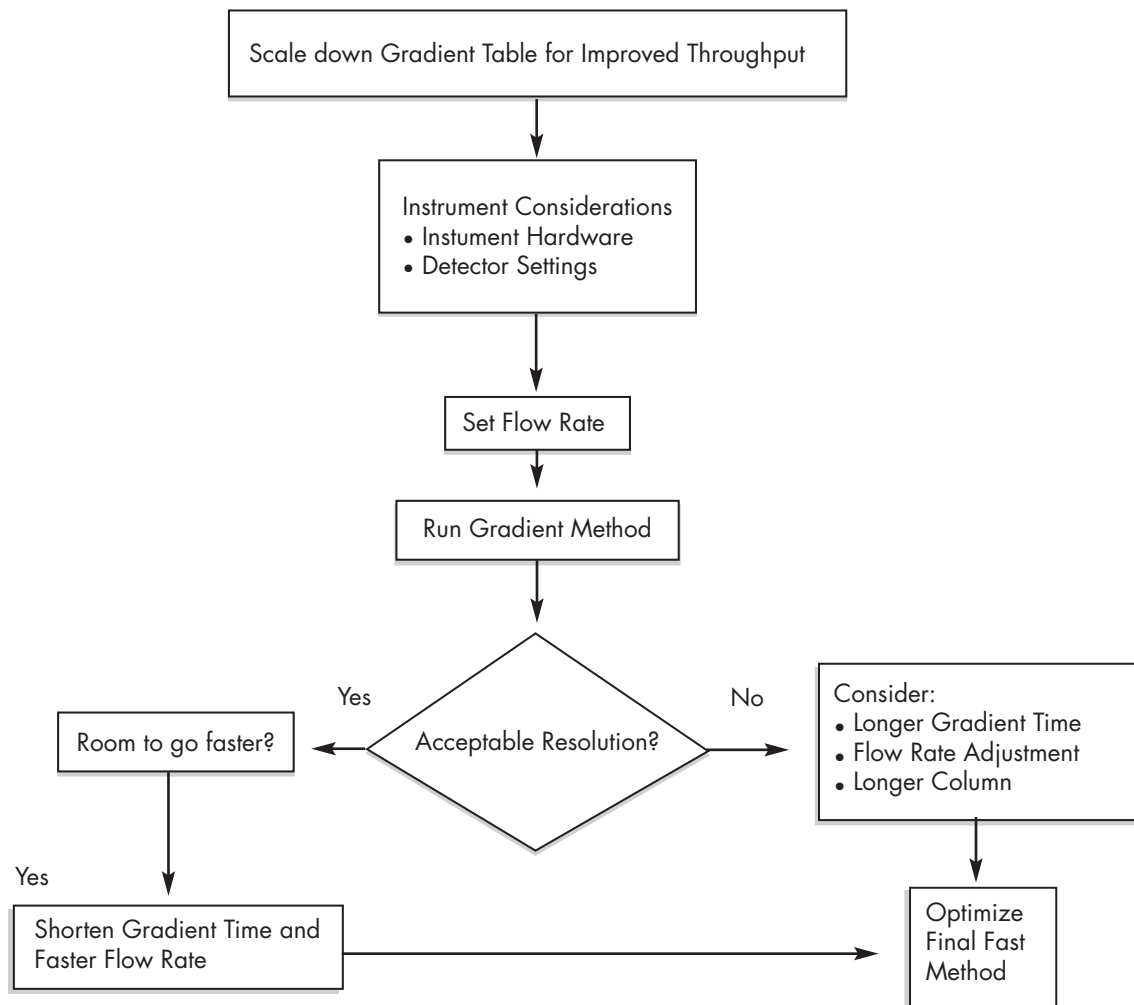
Only Waters provides you with every element critical for successful separations — columns, instrumentation, software and proven methodologies.

INTRODUCTION

FASTER, SHORTER SEPARATIONS WITH INTELLIGENT SPEED (IS™) COLUMNS

The fundamentals for converting slower, longer analytical methods to faster IS™ columns are:

- Scaling down the gradient table
- HPLC instrument and software considerations (requirements for converting to IS™ columns)
- Optimum flow rates
- Cost savings with shorter analysis times



INTRODUCTION

SCALING DOWN THE GRADIENT TABLE FOR IMPROVED THROUGHPUT

Scaling down a separation on a longer length column to a shorter 20 mm *IS*[™] column begins with reducing the gradient times. In scaling the gradient we are scaling the column volume. Keeping the column diameters and flow rates constant, the ratio of the lengths of the columns are multiplied by the gradient time on the longer column to determine the new gradient time.

GRADIENT TABLE

Time (min)	Profile		
	%A	%B	%C
0.0	80	10	10
20.0	50	40	10
21.0	80	10	10
25.0	80	10	10

Our example shows that we have a 20 minute gradient, 25 minute cycle time on a 4.6 x 150 mm column. It has been determined that the optimum flow rate for a 20 minute gradient on this dimension column, with an injection volume of 10 μ L is 1.4 mL/min.

NEW GRADIENT TABLE

Time (min)	Profile		
	%A	%B	%C
0.0	80	10	10
2.7	50	40	10
2.8	80	10	10
3.3	80	10	10

The new gradient table can be calculated using the gradient table equation. The 2.7 minute gradient time has been established. This gradient method was run on the 20mm column with a flow rate of mL/min. Same injection volume as on the 150 mm column.

EQUATION FOR SCALING DOWN THE GRADIENT TABLE

To scale a gradient

$$\frac{L_1}{L_2} \times t_{g1} = t_{g2}$$

L_1 = Long column length

L_2 = Short column length

t_{g1} = Gradient time on long column

t_{g2} = Gradient time on short column

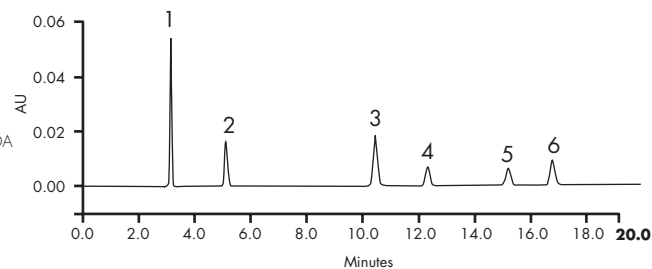
Where column diameters and flow rate remain constant

CONDITIONS

Column: XTerra[®] MS C₁₈, 4.6 x 150 mm, 5 μ m
 Mobile Phase A: Water
 Mobile Phase B: Acetonitrile
 Mobile Phase C: 100mM NH₄HCO₃, pH 10
 Flow Rate: 1.4 mL/min
 Injection Volume: 10 μ L
 Sample Concentration: 20 μ g/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Sampling Rate: 5pts/sec
 Filter: 0 (no filter)
 Instrument: Alliance[®] 2695 with 966 PDA

Compounds

1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N,N-Dimethylaniline

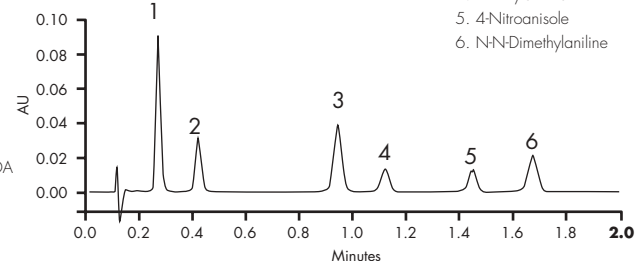


CONDITIONS

Column: XTerra[®] MS C₁₈, 4.6 x 20 mm, *IS*[™], 3.5 μ m
 Mobile Phase A: Water
 Mobile Phase B: Acetonitrile
 Mobile Phase C: 100mM NH₄HCO₃, pH 10
 Flow Rate: 3.0 mL/min
 Injection Volume: 10 μ L
 Sample Concentration: 20 μ g/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Instrument: Alliance[®] 2695 with 966 PDA

Compounds

1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N,N-Dimethylaniline



INTRODUCTION

INSTRUMENT AND DETECTOR CONSIDERATIONS USING INTELLIGENT SPEED (IS™) COLUMNS

OPTIMIZE HPLC INSTRUMENT BY REDUCING THE SYSTEM VOLUME

To reduce system volume from the injector to the column and from the column to the detector.

(for IS™ 4.6 x 20 mm columns, the recommended system volume should be less than 100µL)

- Use small I.D. tubing
- For larger I.D. IS™ columns (for example, 4.6 x 20 mm, 0.009 inch I.D. stainless steel tubing or 0.010 inch I.D. PEEK tubing is recommended. (The standard tubing for Waters Alliance® 2695 HPLC system is 0.009 inch stainless steel tubing).
- Minimize all tubing lengths — remove any excess tubing and extra connections (unions, tees).
- Use precise tubing connections (factory pre-cut tubing is recommended).

OPTIMIZE THE DATA COLLECTION SYSTEM

Data Acquisition Rate – To insure optimal results with narrow peaks, the data acquisition rate may need to be increased in order to insure more reproducible results. It is recommended that for reproducible quantization of chromatographic peaks, a minimum of 10 points is required. Generally, higher acquisition rates will also provide greater sensitivity for detecting and quantitating lower level degradants and impurities.

Filter Response (Time Constant) Setting – Increasing the filter decreases the baseline noise. However, increasing the filter also reduces the sensitivity. Run some experiments with your software and detector to determine the best settings.

Waters Alliance® 2695 HPLC System

The information obtained for this application notebook was completed using Waters Alliance® HPLC systems with Empower™ Software. Only Waters provides you with every element critical for successful separations—columns, instrumentation, software, and proven methodologies.

Please check with your Waters Representative for additional information on Waters complete solution using Intelligent Speed (IS™) columns on the Waters Alliance® 2695 HPLC System. *For additional information on the Waters Alliance® HPLC Systems, please see Appendix B & C.*

SET FLOW RATE

Higher flow rates produce the best peak capacities on IS™ columns. According to the Van Deemter equation, as flow rate increases, there is a point where the narrowest peaks can be achieved. Based on our experiments conducted in the Waters laboratories, a 4.6 x 20 mm IS™, 3.5 µm column, with a 4 minute gradient time, 3mL/min offers us excellent peak capacities, low back pressures, short runtimes and lower solvent consumptions than the higher flow rates. The recommended starting flow rates for a 4 minute gradient using Intelligent Speed (IS™) columns are:

RECOMMENDED FLOW RATE FOR IS™ COLUMNS

COLUMN DIMENSIONS	FLOW RATE
4.6 x 20 mm	3.0 mL/min
3.9 x 20 mm	2.16 mL/min
3.0 x 20 mm	1.28 mL/min
2.1 x 20 mm	0.68 mL/min

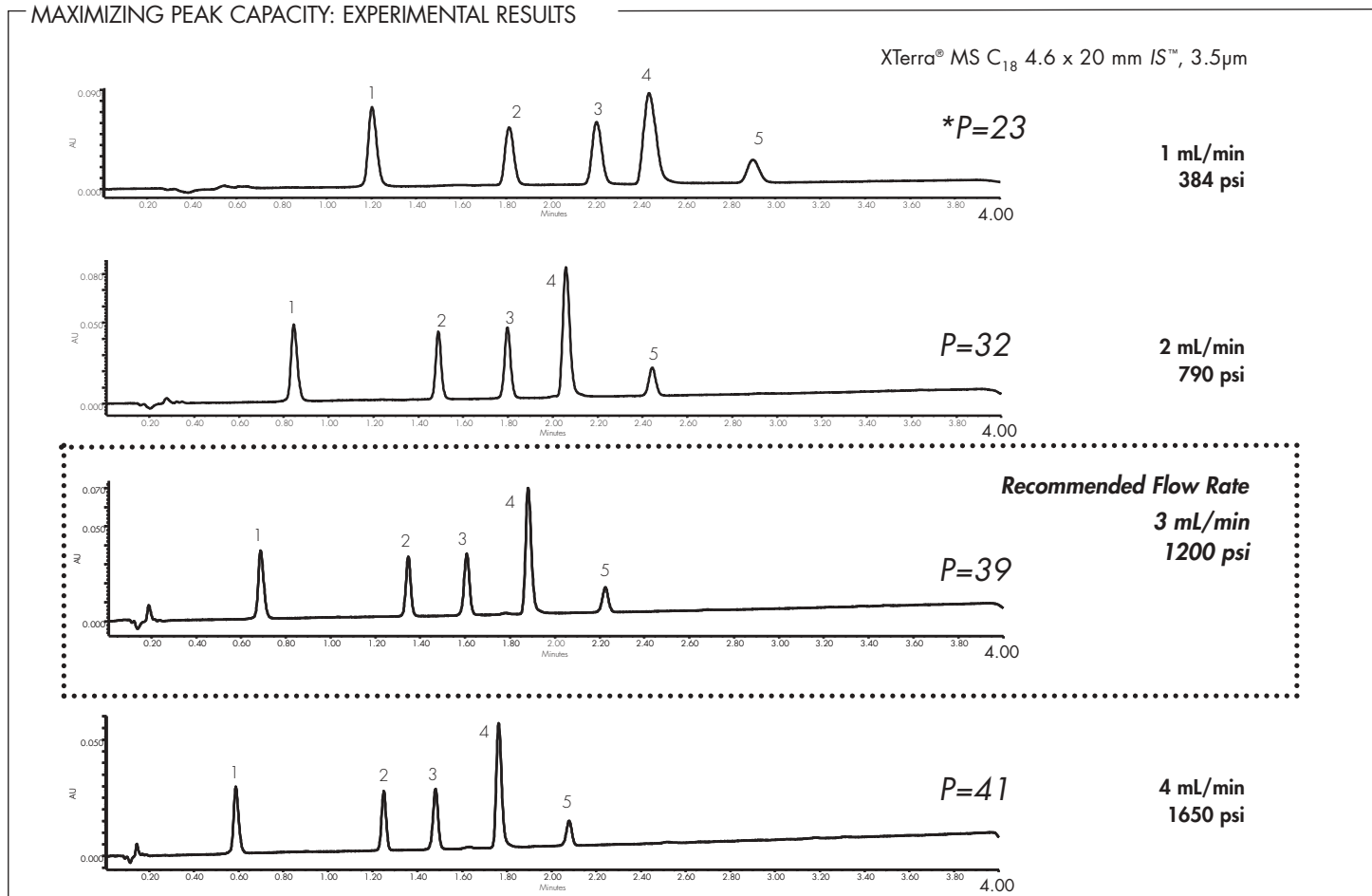
Note: Further optimization of the flow rate may be required depending on your separation.



Waters Alliance® 2695 HPLC System

INTRODUCTION

MAXIMIZING PEAK CAPACITY: EXPERIMENTAL RESULTS



*P= Peak Capacity

COST ANALYSIS

You can realize significant cost savings when reducing analysis times using IS™ columns. With a separation on the 150 mm length column with 25 minute cycle time, it would take nearly 3 months to analyze the samples. Using the IS™ columns (20 mm length) the same set of samples would only take 14 days.

The solvent cost includes the cost of Acetonitrile (ACN), plus the cost of disposal of ALL of the solvent waste. The combined total is nearly \$2300 in the example using the 150 mm column, but only about \$800 for the 20 mm IS™ column. A savings of \$1507 for one study!

Additional cost savings can also be determined through improved instrument utilization, labor costs, etc.

CALCULATION OF SOLVENT SAVINGS

Assume that 5000 samples need to be analyzed for a study

	4.6 x 150 mm	4.6 x 20 mm IS™
Cycle time	25	4
Total time for 5000 samples (hours)	2083 (87 days)	333 (14 days)
Flow rate (mL/min)	1.4	3
Total solvent consumption (L)	175	60
Amount ACN Consumed (L) (~ 25% is ACN)	43.75	15
Cost for ACN (\$ 42.50/L)	\$1860	\$640
Cost for waste disposal (~ \$2.50/L)	\$438	\$151
Total solvent costs	\$2298	\$791

Cost savings of \$1507 in solvents alone

INTRODUCTION

BENEFITS OF NARROWBORE CHROMATOGRAPHY

HPLC system changes may be required to scale down to 2.1 x 20 mm columns. However there are several benefits that should be recognized:

- Lower flow rates for columns with narrow inner diameters (equal to or less than 2.1 mm)
- Narrowbore columns allow for the direct flow of eluent into a mass spectrometer — eliminating the need for flow splitting.
- Narrowbore columns increase sensitivity — therefore smaller injections can be made and smaller amounts of material can be quantified.

Successful narrowbore chromatography requires further optimization of the HPLC instrumentation.

2.1 X 20 mm IS™ COLUMNS CAN FURTHER REDUCE COST

It is important to note that if we are changing the inner diameter of the columns, then we need to scale the cross sectional areas of the columns and multiply by the flow rate to obtain our new flow rate.

TO SCALE A FLOW RATE FOR DIFFERENT INTERNAL DIAMETERS

$$\frac{(d_2)^2}{(d_1)^2} \times F_1 = F_2$$

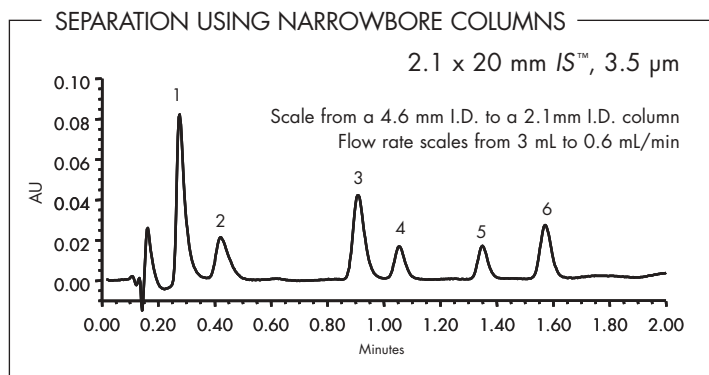
d_1 = Diameter of original column
 d_2 = Diameter of second column
 F_1 = Flow rate on original column
 F_2 = Flow rate on second column

Original flow rate on a 4.6 mm I.D. column = 3 mL/min

The flow rate on a 2.1 mm I.D. column:

$$\frac{2.1^2}{4.6^2} \times 3 \text{ mL/min} = 0.63 \text{ mL/min}$$

Additional benefit is increased sensitivity



We can save further on the solvents by running the separation on a 2.1 mm I.D. column. We scale the flow rate to 0.6/mL. We can do the calculations for the shorter column and now have negligible costs in solvents — a savings of over \$2000!

REDUCE SYSTEM VOLUME FOR NARROWBORE COLUMNS

To Reduce System Volume for 2.1 x 20 mm IS™ columns from 80 μL to ~ 25 μL:

- Optimize Detector flow cell (use a microbore flow cell)
- Use 0.005 inch I.D. tubing
- Minimize all tubing lengths — remove any excess tubing and extra connections (unions, tees). (We recommend that the tubing length from the column to the detector be as short as possible).
- Use precise tubing connections (factory pre-cut tubing is recommended).
- Flow splitters can cause additional bandspreading, broadened peaks.

CALCULATION OF SOLVENT SAVINGS IN NARROWBORE COLUMNS

	4.6 x 150 mm	2.1 x 20mm IS™
Cycle time	25	4
Total time for 5000 samples (hours)	2083 (87 days)	333 (14 days)
Flow rate	1.4	0.6
Total solvent consumption (L)	175	9
Amount ACN consumed (L) (~ 25% is ACN)	43.75	2.25
Cost for ACN (\$42.50/L)	\$1860	\$96
Cost for waste disposal (~\$2.50/L)	\$438	\$23
Total solvent costs	\$2298	\$119

Cost savings of \$2179

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ANILINES

CONDITIONS

Column: XTerra® MS C₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N: 186001891)

Mobile Phase A: Water

Mobile Phase B: Acetonitrile

Mobile Phase C: 100 mM NH₄HCO₃, pH 10

Flow Rate: 3.0 mL/min

Gradient:

Time (min)	%A	%B	%C
0.0	90	0	10
4.0	60	30	10

Injection Volume: 10 µL

Sample concentration: 20 µg/mL

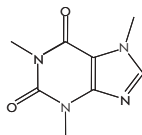
Temperature: Ambient

Detection: UV @ 254 nm

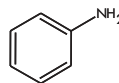
Instrument: Alliance® 2795 with 996 PDA

Compounds

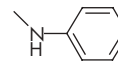
1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N,N-Dimethylaniline



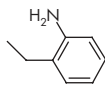
Caffeine



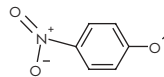
Aniline



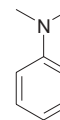
N-Methylaniline



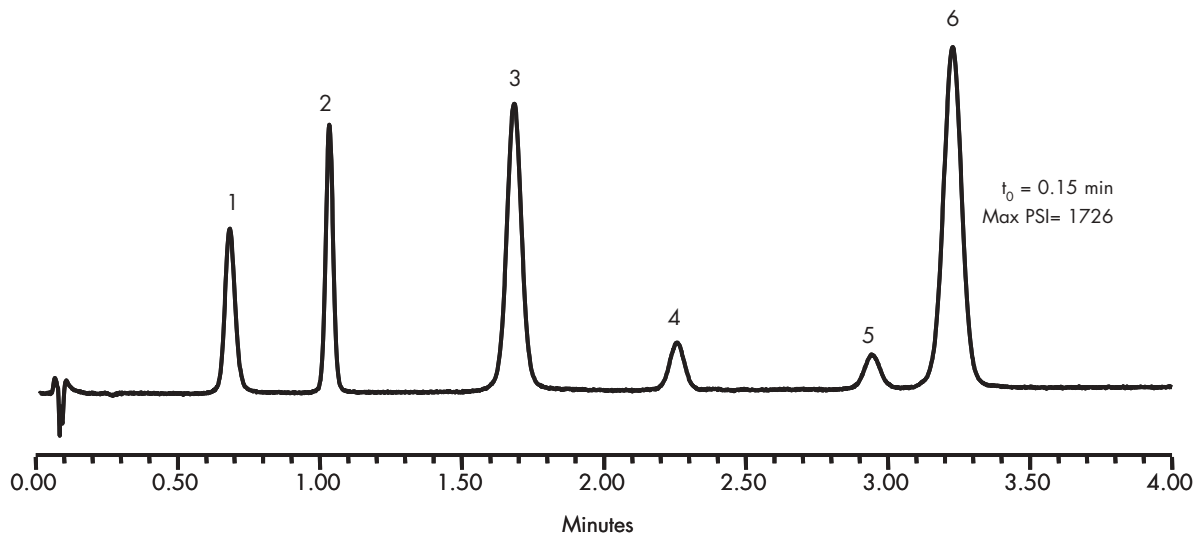
2-Ethylaniline



4-Nitroanisole



N,N-Dimethylaniline



ANILINES

CONDITIONS

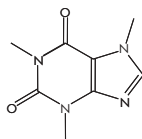
Column: XTerra® MS C₁₈, 3.0 x 20 mm IS™, 3.5 µm, (P/N: 186001974)
 Mobile Phase A: Water
 Mobile Phase B: Acetonitrile
 Mobile Phase C: 100 mM NH₄HCO₃, pH 10
 Flow Rate: 2.0 mL/min
 Gradient:

Time (min)	%A	%B	%C
0.0	80	10	10
2.0	50	40	10
2.2	80	10	10
3.0	80	10	10

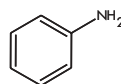
Injection Volume: 5.0 µL
 Sample concentration: 20 µg/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Instrument: Alliance® 2695 with 2996 PDA

Compounds

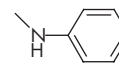
1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N,N-Dimethylaniline



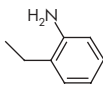
Caffeine



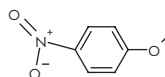
Aniline



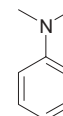
N-Methylaniline



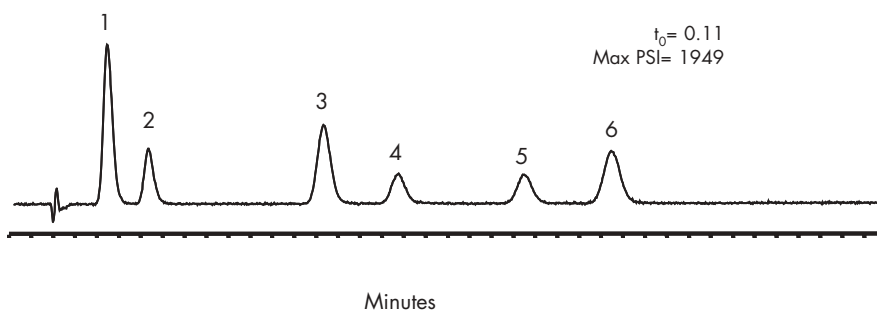
2-Ethylaniline



4-Nitroanisole



N,N-Dimethylaniline



CATECHINS

CONDITIONS

Column: Symmetry® C₁₈, 4.6 x 20 mm IS™,
3.5 µm, (P/N: 186002090)
SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™,
3.5 µm, (P/N: 186002092)

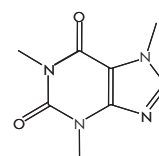
Mobile Phase A: 0.1% HCOOH in Water
Mobile Phase B: 0.1% HCOOH in Methanol
Flow Rate: 3.0 mL/min
Gradient:

Time (min)	Profile	
	%A	%B
0.0	100	0
4.0	60	40

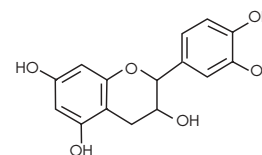
Injection Volume: 10 µL
Sample concentration: 20 µg/mL
Temperature: 30°C
Detection: UV @ 280 nm
Instrument: Alliance® 2795 with 996 PDA

Compounds

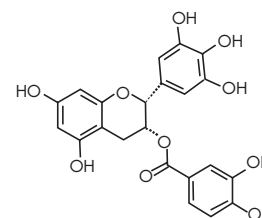
1. Caffeine
2. Epicatechin
3. Epigallocatechin Gallate



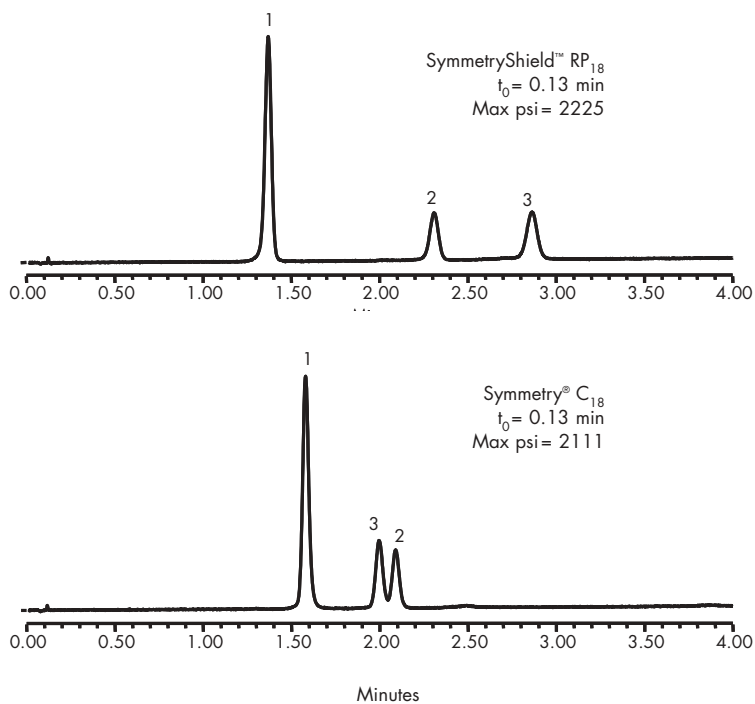
Caffeine



Epicatechin



Epigallocatechin Gallate



DRUGS OF ABUSE

CONDITIONS

Column: XTerra® RP₁₈, 2.1 x 20 mm IS™, 3.5 µm, (P/N: 186001925)

Mobile Phase A: Water

Mobile Phase B: Acetonitrile

Mobile Phase C: 0.8 mL/min

Flow Rate: 0.8 mL/min

Gradient:

Time (min)	%A	%B	%C
0.0	80	10	10
2.0	5	95	10

Injection Volume: 5 µL

Sample Concentration: MDA and MDMA 40 µg/mL, all others 20 µg/mL

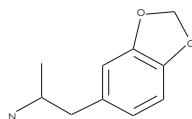
Temperature: 50°C

Detection: UV @ 235 nm

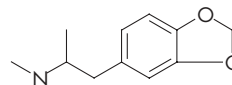
Instrument: Alliance® 2795 with 996 PDA

Compounds

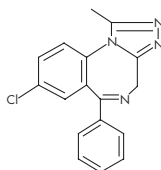
- | | |
|---|----------|
| 1. 3,4-Methylenedioxyamphetamine (MDA) | 40 µg/mL |
| 2. 3,4-Methylenedioxymethamphetamine (MDMA) | 40 µg/mL |
| 3. Alprazolam | 20 µg/mL |
| 4. Flunitrazepam | 20 µg/mL |
| 5. Desmethyldiazepam | 20 µg/mL |
| 6. Diazepam | 20 µg/mL |



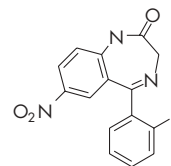
3,4-Methylenedioxyamphetamine



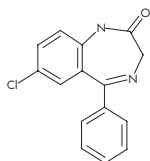
3,4-Methylenedioxymethamphetamine



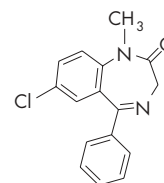
Alprazolam



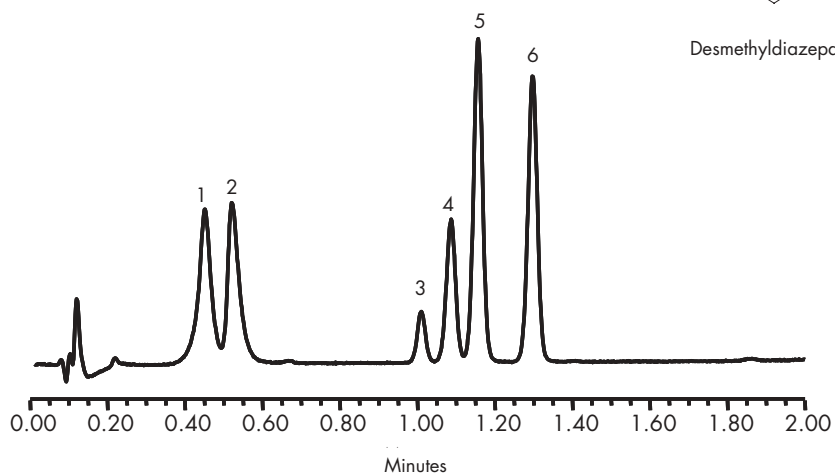
Flunitrazepam



Desmethyldiazepam



Diazepam



NALIDIXIC ACID ANTIBIOTICS ISOCRATIC

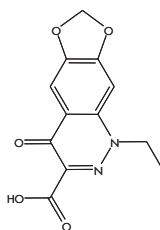
4.6 x 20 mm IS™ separation

CONDITIONS

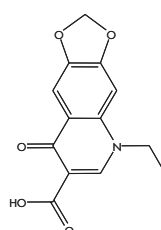
Column: Atlantis™ dC₁₈, 4.6 x 20 mm IS™,
3 μm, (P/N:186002062)
Mobile Phase A: Water
Mobile Phase B: Methanol
Mobile Phase C: Acetonitrile
Mobile Phase D: 1% HCOOH, pH 2.23
Flow Rate: 4 mL/min
Isocratic Mobile-
Phase Composition: 55% A, 30% B, 5% C, 10% D
Injection Volume: 5 μL
Sample concentration: 5 μg/mL
Temperature: 30°C
Detection: UV @ 254 nm
Instrument: Waters Alliance® 2965 Separations Module
with Waters 2996 PDA

Compounds

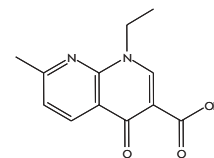
1. Cinoxacin
2. Oxolinic Acid
3. Nalidixic Acid



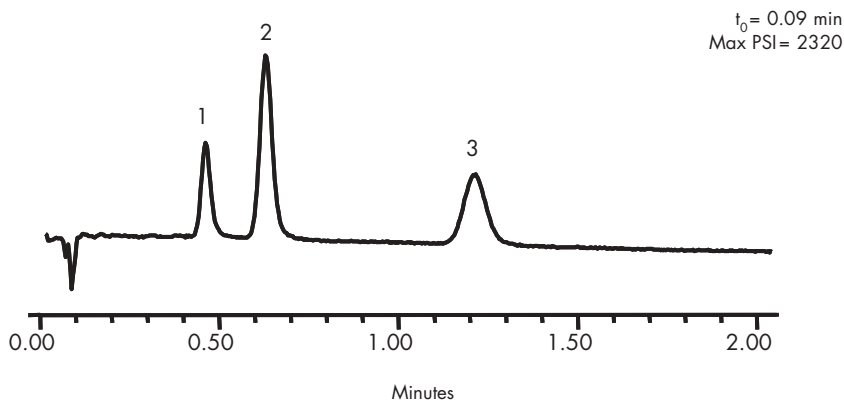
Cinoxacin



Oxolinic acid



Nalidixic acid



NALIDIXIC ACID ANTIBIOTICS BY LC/MS

5.0 minute gradient, 2.1 x 20 mm IS™ separation

LC CONDITIONS

Column: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3.0 µm, (P/N: 186002058)

Mobile Phase A: Water
Mobile Phase B: Methanol
Mobile Phase C: 1% HCOOH in Water
Flow Rate: 0.2 mL/min

Gradient:

Time (min)	%A	%B	%C
0.0	60	30	10
5.0	40	50	10

Injection Volume: 5 µL
Sample concentration: 10 µg/mL
Temperature: 30°C
Instrument: Alliance® 2795 and Waters ZQ™

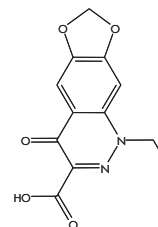
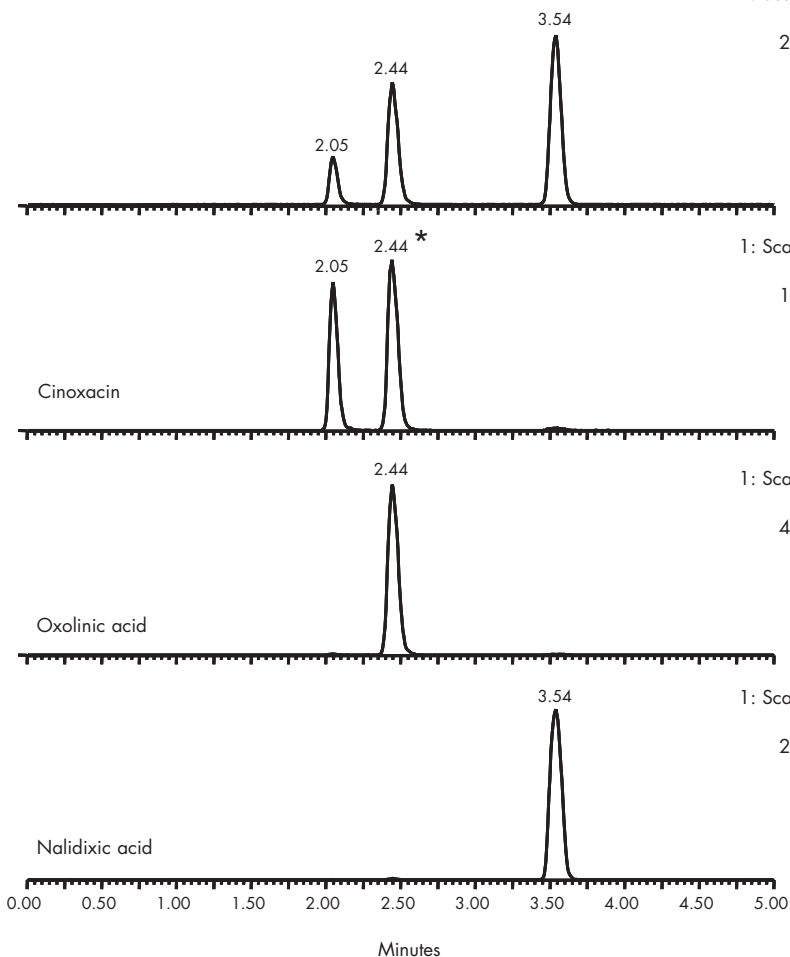
MS CONDITIONS

Waters ZQ™
ES+
Capillary (kV) 3.5
Cone (V) 5.0
Extractor 3.0
RF Lens 0.1
Source Temp (°C) 150
Desolvation Temp (°C) 400
Cone Gas Flow (L/Hr) 50
Desolvation Gas Flow (L/Hr) 500
LM Resolution 15
HM Resolution 15
Ion Energy 0.5
Multiplier (V) 650

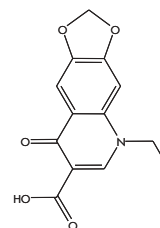
Compounds	MW
1. Cinoxacin	262.2
2. Oxolinic Acid	261.2
3. Nalidixic Acid	232.2

The top figure is the total ion current, followed by the extracted ion signals for each of the three analytes.

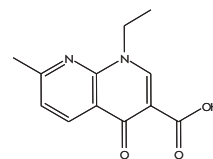
*The "extra" peak in the cinoxacin panel is the isotope from oxolinic acid.



Cinoxacin



Oxolinic acid



Nalidixic acid

NALIDIXIC ACID ANTIBIOTICS BY LC/MS

3.0 minute gradient, 2.1 x 20 mm IS™ Separation

LC CONDITIONS

Column: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3.0 μm, (P/N: 186002058)

Mobile Phase A: Water

Mobile Phase B: Methanol

Mobile Phase C: 1% HCOOH in Water

Flow Rate: 0.4 mL/min

Gradient:

Time (min)	%A	%B	%C
0.0	60	30	10
3.0	40	50	10

Injection Volume: 2 μL

Sample concentration: 10 μg/mL

Temperature: 30°C

Instrument: Alliance® 2795 and Waters ZQ™

MS CONDITIONS

Waters ZQ™

ES+

Capillary (kV) 3.5

Cone (V) 5.0

Extractor 3.0

RF Lens 0.1

Source Temp (°C) 150

Desolvation Temp (°C) 400

Cone Gas Flow (L/Hr) 50

Desolvation Gas Flow (L/Hr) 500

LM Resolution 15

HM Resolution 15

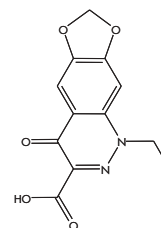
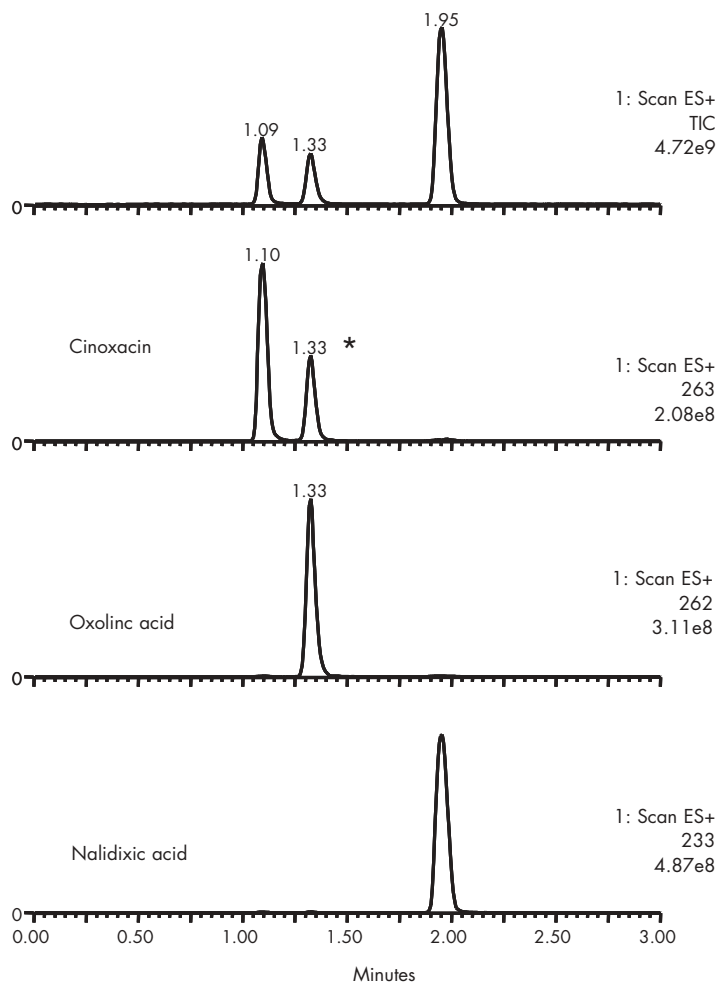
Ion Energy 0.5

Multiplier (V) 650

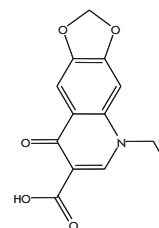
Compounds	MW
1. Cinoxacin	262.2
2. Oxolinic Acid	261.2
3. Nalidixic Acid	232.2

The top figure is the total ion current, followed by the extracted ion signals for each of the three analytes.

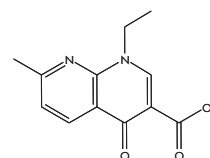
*The "extra" peak in the cinoxacin panel is the isotope from oxolinic acid.



Cinoxacin



Oxolinic acid



Nalidixic acid

NALIDIXIC ACID ANTIBIOTICS BY LC/MS

2.0 minute gradient, 2.1 x 20 mm IS™ Separation

CONDITIONS

Column: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3.0 µm, (P/N: 186002058)

Mobile Phase A: Water

Mobile Phase B: Methanol

Mobile Phase C: 1% HCOOH in Water

Flow Rate: 0.4 mL/min

Gradient:

Time (min)	%A	%B	%C
0.0	60	30	10
2.0	40	50	10

Injection Volume: 2 µL

Sample concentration: 10 µg/mL

Temperature: 30°C

Instrument: Alliance® 2795 and Waters ZQ™

MS CONDITIONS

Waters ZQ™

ES+

Capillary (kV) 3.5

Cone (V) 5.0

Extractor 3.0

RF Lens 0.1

Source Temp (°C) 150

Desolvation Temp (°C) 400

Cone Gas Flow (L/Hr) 50

Desolvation Gas Flow(L/Hr) 500

LM Resolution 15

HM Resolution 15

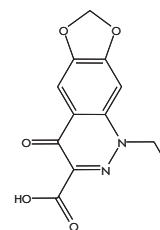
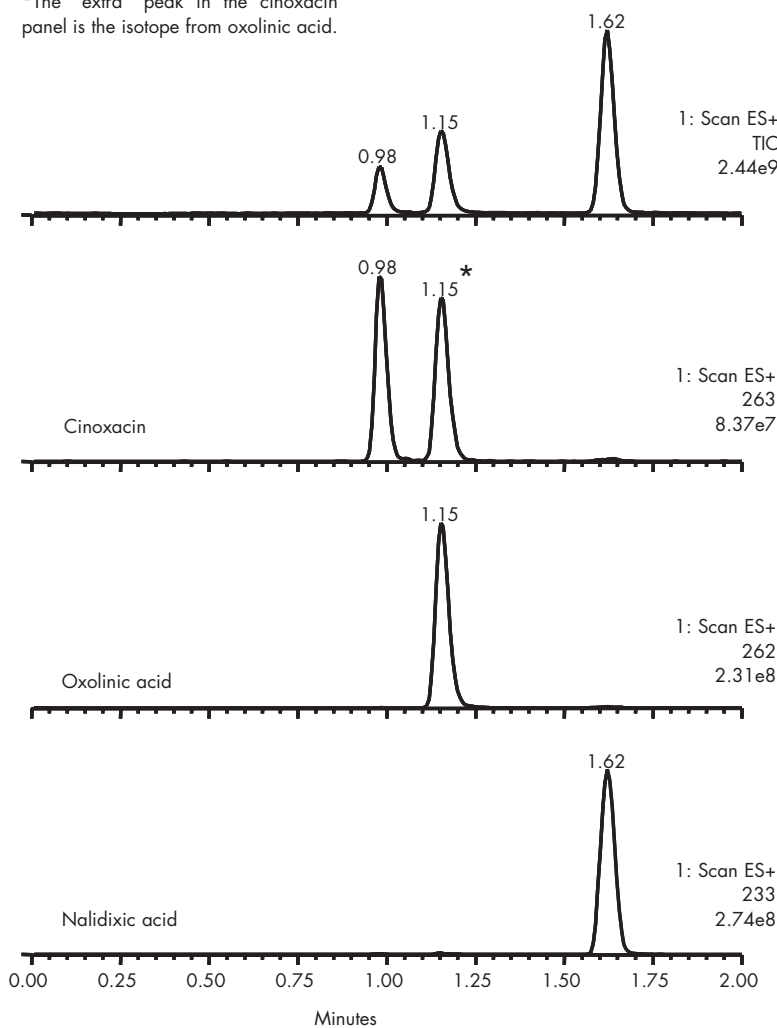
Ion Energy 0.5

Multiplier (V) 650

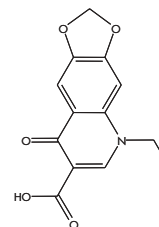
Compounds	MW
1. Cinoxacin	262.2
2. Oxolinic Acid	261.2
3. Nalidixic Acid	232.2

The top figure is the total ion current, followed by the extracted ion signals for each of the three analytes.

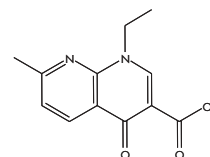
*The "extra" peak in the cinoxacin panel is the isotope from oxolinic acid.



Cinoxacin



Oxolinic acid



Nalidixic acid

NALIDIXIC ACID ANTIBIOTICS BY LC/MS

1.0 minute gradient, 2.1 x 20 mm IS™ Separation

CONDITIONS

Column: Atlantis™ dC₁₈, 2.1 x 20 mm IS™,
3.0 μm, (P/N: 186002058)

Mobile Phase A: Water
Mobile Phase B: Methanol
Mobile Phase C: 1% HCOOH in Water
Flow Rate: 0.4 mL/min
Gradient:

Time (min)	%A	%B	%C
0.0	50	40	10
1.0	30	60	10

Injection Volume: 2 μL
Sample concentration: 10 μg/mL
Temperature: 30°C
Instrument: Alliance® 2795 and Waters ZQ™

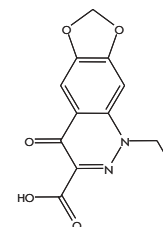
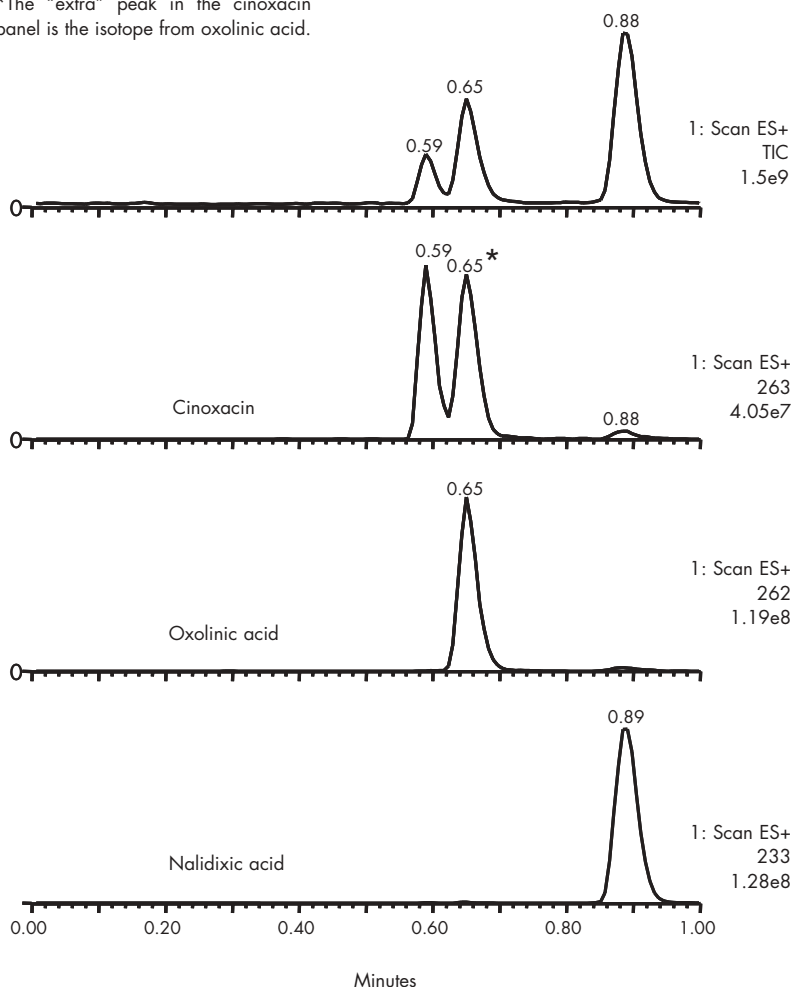
MS CONDITIONS

Waters ZQ™
ES+
Capillary (kV) 3.5
Cone (V) 5.0
Extractor 3.0
RF Lens 0.1
Source Temp (°C) 150
Desolvation Temp (°C) 400
Cone Gas Flow (L/Hr) 50
Desolvation Gas Flow(L/Hr) 500
LM Resolution 15
HM Resolution 15
Ion Energy 0.5
Multiplier (V) 650

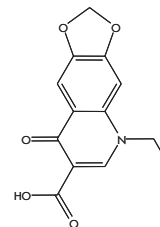
Compounds	MW
1. Cinoxacin	262.2
2. Oxolinic Acid	261.2
3. Nalidixic Acid	232.2

The top figure is the total ion current, followed by the extracted ion signals for each of the three analytes.

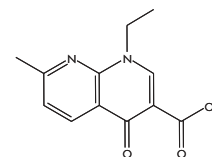
*The "extra" peak in the cinoxacin panel is the isotope from oxolinic acid.



Cinoxacin



Oxolinic acid



Nalidixic acid

PHARMACEUTICAL COMPOUNDS IN PLASMA

2.1 x 20 mm IS™ Separation

LC CONDITIONS

Column: XTerra® MS C₁₈, 2.1 x 20 mm IS™, 3.5 µm, (P/N: 186001923)
 Mobile Phase A: 10 mM NH₄HCO₃, pH 10
 Mobile Phase B: Methanol
 Flow Rate: 0.4 mL/min
 Gradient:

Time (min)	%A	%B
0.0	100	0
5.0	5	95

Injection Volume: 20 µL
 Sample concentration: 5 µg/mL
 Temperature: Ambient
 Detection: MS
 Instrument: Waters 277 Sample Manager, Waters 1525 Binary HPLC Pump and Waters Micromass® Quattro Ultima

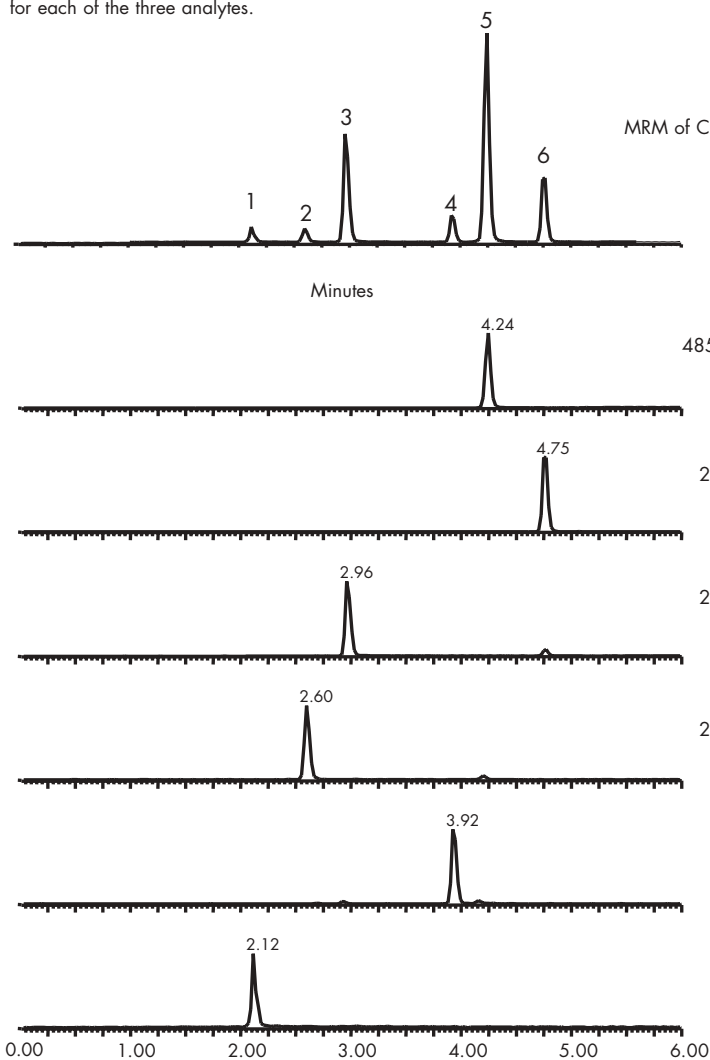
MS CONDITIONS

Quattro Ultima™
 ES+ MRM
 Cone (V): 5.0
 Capillary (kV): 3.5
 Source (°C): 150
 Desolvation (°C): 400
 Cone gas flow (L/hr): 50
 Desolvation gas flow (L/hr): 550
 LM resolution 1&2: 13.5
 HM resolution 1&2: 13.5
 Ion Energy 1: 0.4
 Ion Energy 2: 0.8
 Multiplier (V): 650

SAMPLE PREPARATION

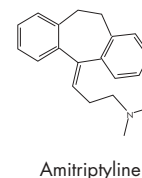
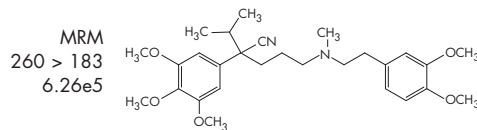
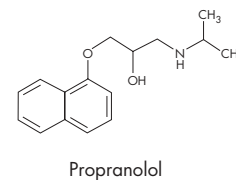
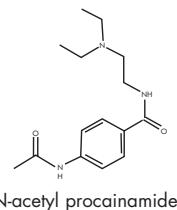
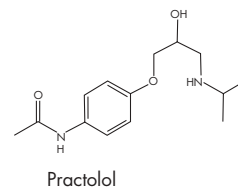
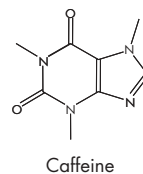
SPE: Oasis® HLB µElution Plate, (P/N: 186001828BA)
 P/N: 186001828BA
 Condition: 200 µL Methanol
 Equilibrate: 200 µL Water
 Load: 150 µL Spiked rat plasma with 2% H₃PO₄, diluted with 1:1 Water
 Wash: 200 µL 5% Methanol in Water
 Elute: 50 µL Methanol
 Dilute: 100 µL Water

The top figure is the total ion current, followed by the extracted ion signals for each of the three analytes.



Compounds (MRM Transitions, Cone voltage (V), CID (eV))

- Caffeine (195 > 138; 20; 20)
- Practolol (267 > 190.1; 30; 20)
- N-acetyl procainamide (278 > 205.1; 25; 20)
- Propranolol (260 > 183; 25; 18)
- Methoxyverapamil (485.2 > 165.1; 45; 30)
- Amitriptyline (278 > 233.1; 25; 17)



CONDITIONS

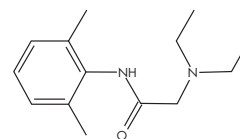
Column: XTerra® MS C₁₈, 2.1 x 20 mm IS™,
2.5 µm, (P/N: 186001921)
Mobile Phase A: 10 mM CH₃COOHN₄, pH 5.0/ACN (97/3)
Mobile Phase B: 10 mM CH₃COOHN₄, pH 5.0/ACN (10/90)
Flow Rate: 0.6 mL/min; 1.0 mL/min
Gradient:

Time (min)	%A	%B
0.0	95	5
1.7	5	95

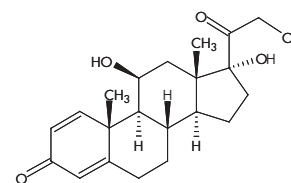
Injection Volume: 10 µL
Sample concentration: 20 µg/mL
Temperature: 30°C; 60°C
Detection: UV @ 210 nm
Instrument: Alliance® 2795 with 996 PDA

Compounds

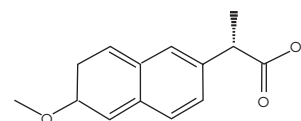
1. Lidocaine
2. Prednisolone
3. Naproxen
4. Amitriptyline
5. Ibuprofen



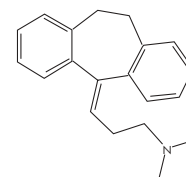
Lidocaine



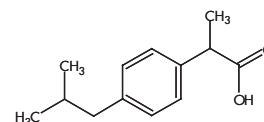
Prednisolone



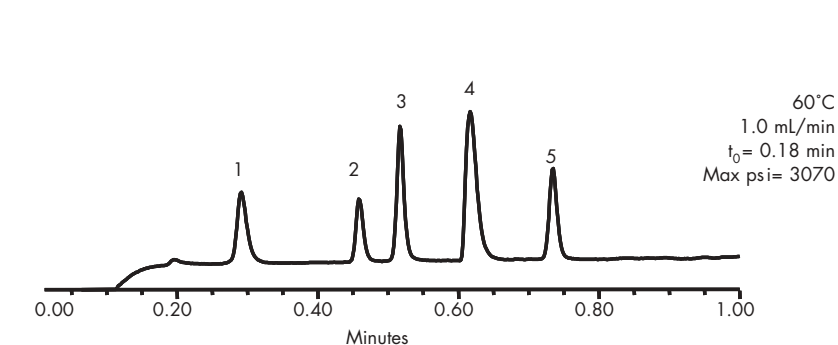
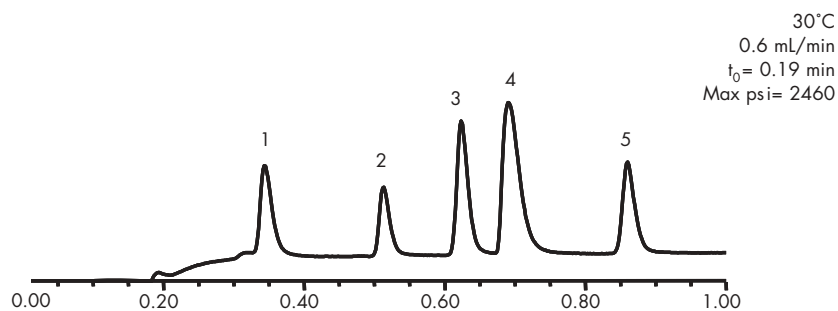
Naproxen



Amitriptyline



Ibuprofen



PHENONES

CONDITIONS

Column: Atlantis™ dC₁₈, 4.6 x 20 mm IS™, 3 μm, (P/N: 186002062)
 SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N: 186002092)
 Symmetry® C₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N: 186002090)
 XTerra® MS C₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N: 186001891)

Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Acetonitrile
 Mobile Phase C: 1% HCOOH in Water
 Flow Rate: 3.0 mL/min

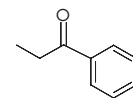
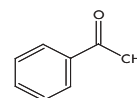
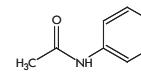
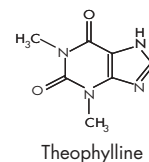
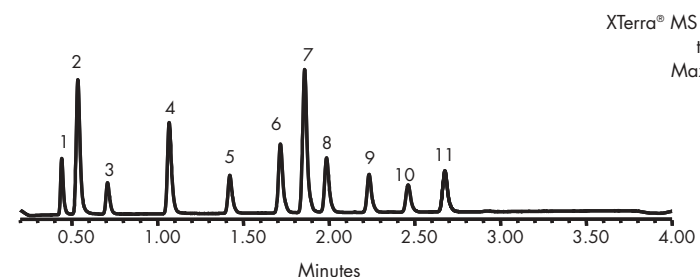
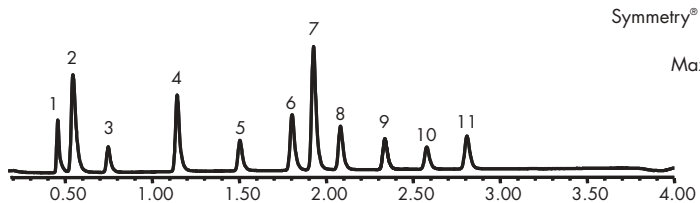
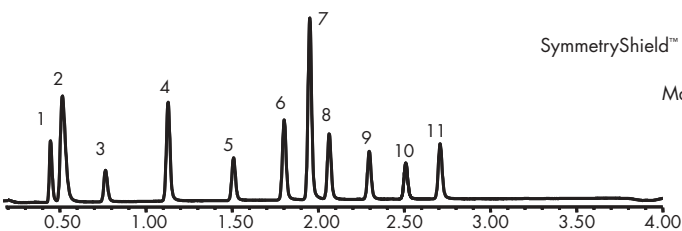
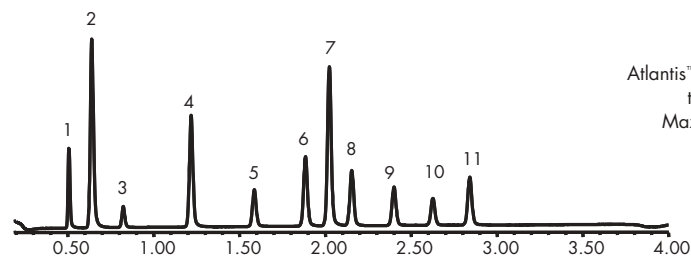
Gradient:

Time (min)	%A	%B
0.0	100	0
4.0	0	100

Injection Volume: 10 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Instrument: Alliance® 2795 with 996 PDA

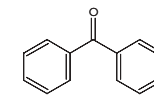
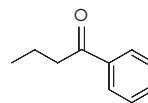
Compounds

- | | |
|------------------|--------------------|
| 1. Theophylline | 7. Benzophenone |
| 2. 2-Acetylfuran | 8. Valerophenone |
| 3. Acetanilide | 9. Hexanophenone |
| 4. Acetophenone | 10. Heptanophenone |
| 5. Propiophenone | 11. Octanophenone |
| 6. Butyophenone | |



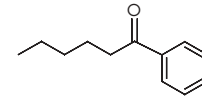
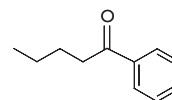
Acetophenone

Propiophenone



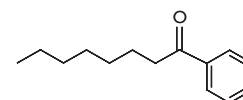
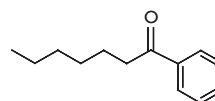
Butyophenone

Benzophenone



Valerophenone

Hexanophenone



Heptanophenone

Octanophenone

PHENONES

CONDITIONS

Columns: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3 µm, (P/N: 186002058)
 SymmetryShield™ RP₁₈, 2.1 x 20 mm IS™, 3.5 µm, (P/N: 186002068)
 Symmetry® C₁₈, 2.1 x 20 mm IS™, 3.5 µm, (P/N: 186002066)
 XTerra® MS C₁₈, 2.1 x 20 mm IS™, 3.5 µm, (P/N: 186001923)

Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Acetonitrile
 Flow Rate: 0.6 mL/min

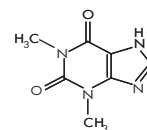
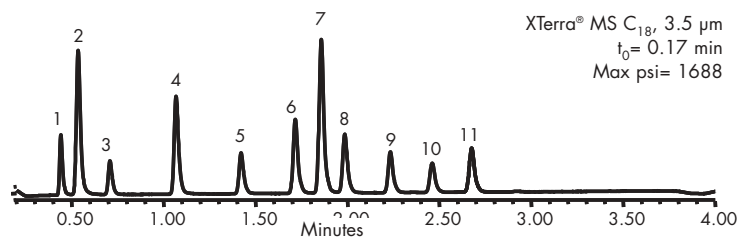
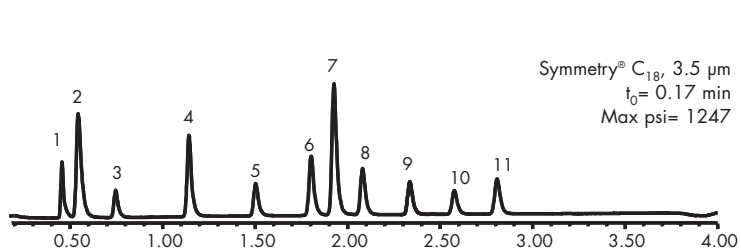
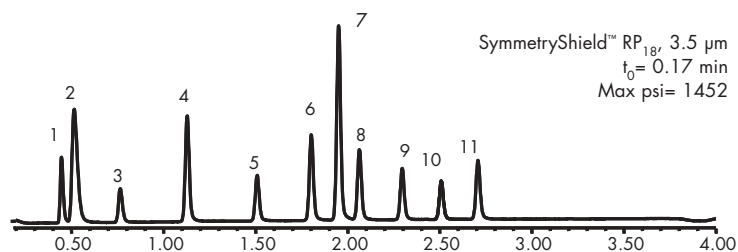
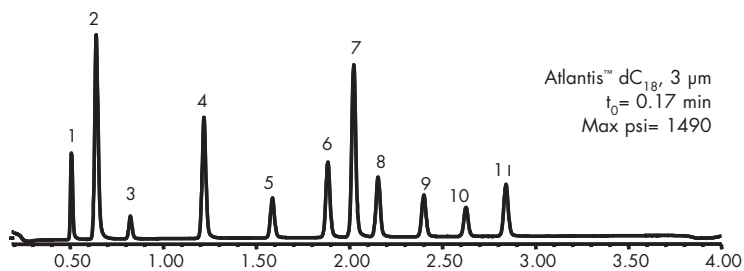
Gradient: Time Profile
 (min) %A %B
 0.0 100 0
 4.0 0 100

Injection Volume: 5 µL
 Sample concentration: 20 µg/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

1. Theophylline
 2. 2-Acetylfuran
 3. Acetanilide
 4. Acetophenone
 5. Propiophenone

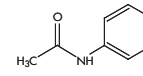
6. Butyrophenone
 7. Benzophenone
 8. Valerophenone
 9. Hexanophenone
 10. Heptanophenone
 11. Octanophenone



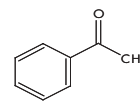
Theophylline



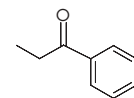
2-Acetylfuran



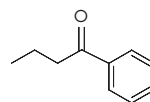
Acetanilide



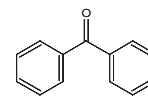
Acetophenone



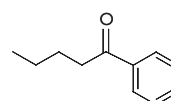
Propiophenone



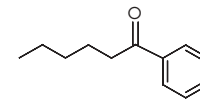
Butyrophenone



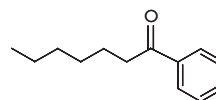
Benzophenone



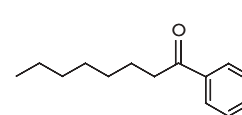
Valerophenone



Hexanophenone



Heptanophenone



Octanophenone

POLAR AND NON-POLAR COMPOUNDS TEST MIX

4.6 x 20 mm IS™ Separation

CONDITIONS

Columns: Atlantis™ dC₁₈, 4.6 x 20 mm IS™, 3 μm, (P/N: 186002062)
 SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N: 186002092)
 Symmetry® C₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N: 186002090)
 XTerra® MS C₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N: 186001891)

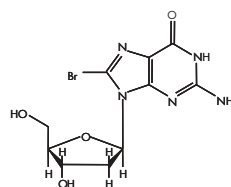
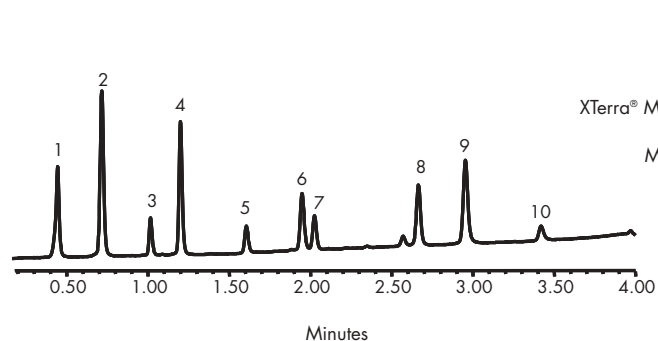
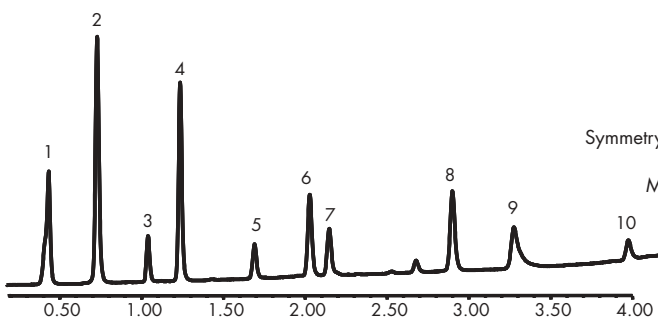
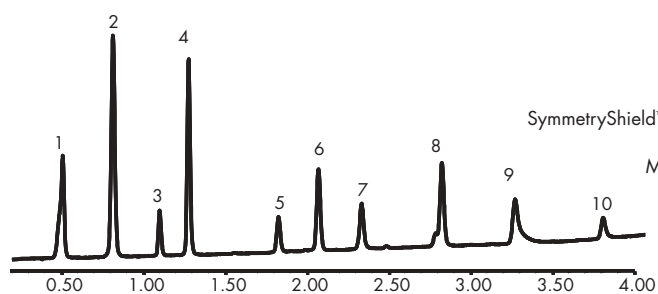
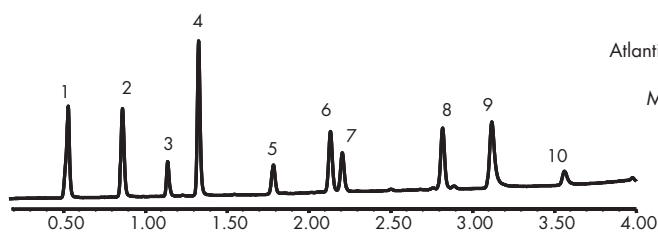
Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Acetonitrile
 Mobile Phase C: 1% HCOOH in Water
 Flow Rate: 3.0 mL/min

Gradient: Time (min) Profile %A %B
 0.0 100 0
 4.0 0 100

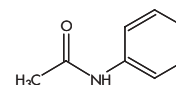
Injection Volume: 10 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

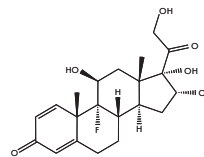
1. 8-Bromoguanosine
2. Acetanilide
3. Triamcinolone
4. Hydrocortisone
5. 2-Amino-7-chloro-5-oxo-5H-[1]benzopyrano[2,3-b]pyridine-3-carbonitrile
6. 6α-Methyl-17α-hydroxyprogesterone
7. 3-Aminofluoranthene
8. 2-Bromofluorene
9. Perylene
10. Naphtho(2,3-a)pyrene



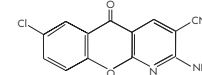
8-Bromoguanosine



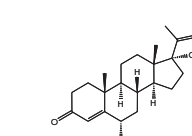
Acetanilide



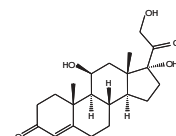
Triamcinolone



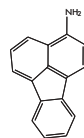
2-Amino-7-chloro-5-oxo-5H-[1]benzopyrano[2,3-b]pyridine-3-carbonitrile



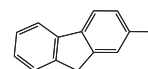
6α-Methyl-17α-hydroxyprogesterone



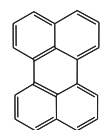
Hydrocortisone



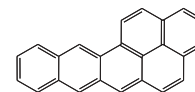
3-Aminofluoranthene



2-Bromofluorene



Perylene



Naphtho(2,3-a)pyrene

POLAR AND NON-POLAR COMPOUNDS TEST MIX

2.1 x 20 mm IS™ Separation

CONDITIONS

Columns: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3 μm, (P/N: 186002058)
 SymmetryShield™ RP₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N: 186002068)
 Symmetry® C₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N: 186002066)
 XTerra® MS C₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N: 186001923)

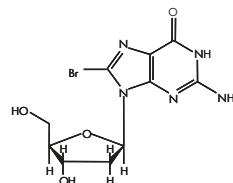
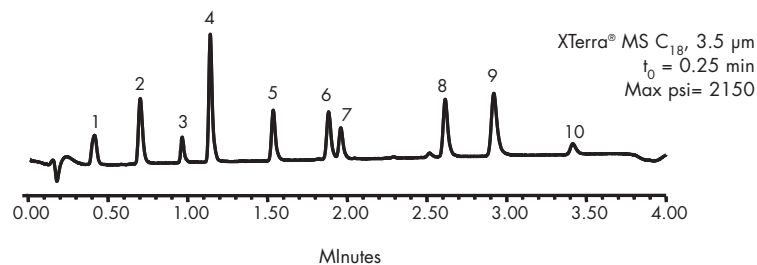
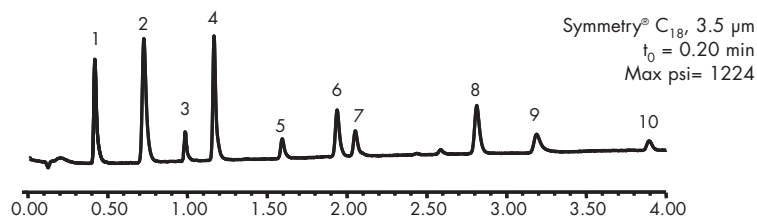
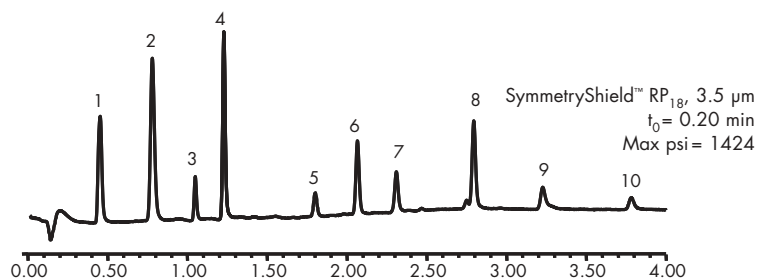
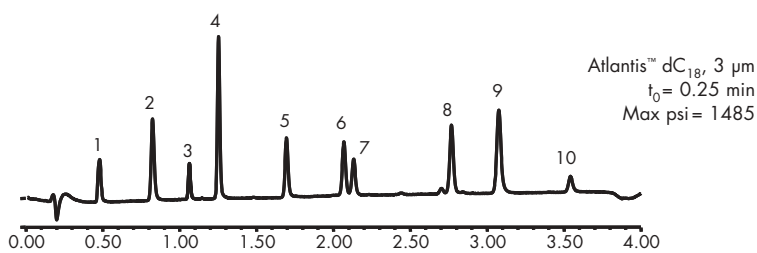
Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Acetonitrile
 Flow Rate: 0.6 mL/min

Gradient: Time Profile
 (min) %A %B
 0.0 100 0
 4.0 0 100

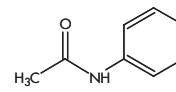
Injection Volume: 5 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 254 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

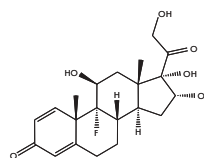
1. 8-Bromoguanosine
2. Acetanilide
3. Triamcinolone
4. Hydrocortisone
5. 2-Amino-7-chloro-5-oxo-5H-[1]benzopyrano[2,3-b]pyridine-3-carbonitrile
6. 6α-Methyl-17α-hydroxyprogesterone
7. 3-Amino-9-fluorenone
8. 2-Bromofluorene
9. Perylene
10. Naphtho(2,3-a)pyrene



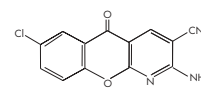
8-Bromoguanosine



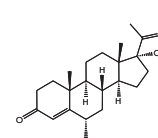
Acetanilide



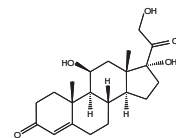
Triamcinolone



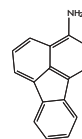
2-Amino-7-chloro-5-oxo-5H-[1]benzopyrano[2,3-b]pyridine-3-carbonitrile



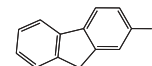
6α-Methyl-17α-hydroxyprogesterone



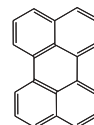
Hydrocortisone



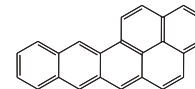
3-Amino-9-fluorenone



2-Bromofluorene



Perylene



Naphtho(2,3-a)pyrene

PROFENS, GRADIENT

CONDITIONS

Columns: XTerra® RP₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186001893)
 XTerra® MS C₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186001891)
 SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186002092)
 Symmetry® C₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186002090)
 Atlantis™ dC₁₈, 4.6 x 20 mm IS™, 3 µm, (P/N:186002062)

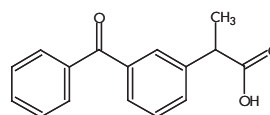
Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Acetonitrile
 Flow Rate: 3.0 mL/min

Gradient: Time Profile
 (min) %A %B
 0.0 65 35
 2.0 60 40

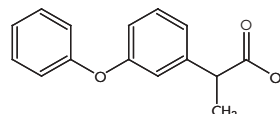
Injection Volume: 10 µL
 Sample concentration: 20 µg/mL
 Temperature: 30°C
 Detection: UV @ 224 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

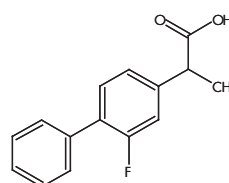
1. Ketoprofen
2. Fenoprofen
3. Flurbiprofen
4. Ibuprofen



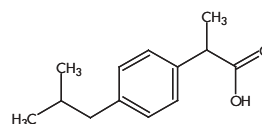
Ketoprofen



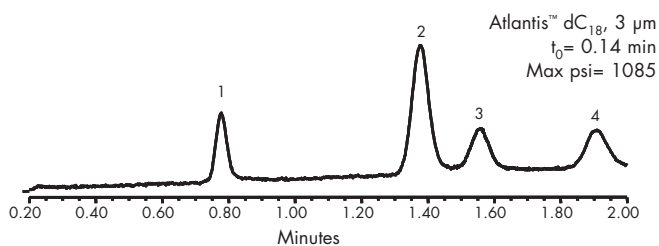
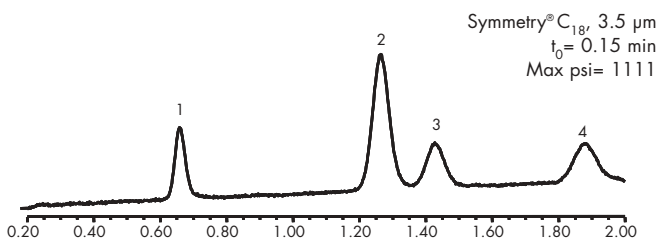
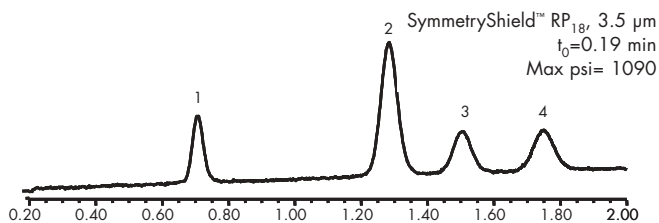
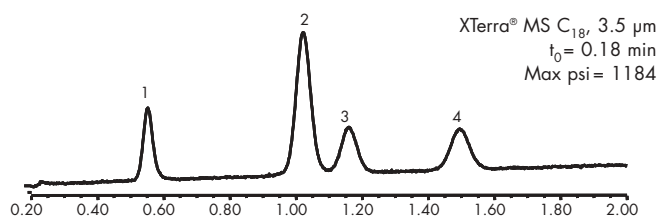
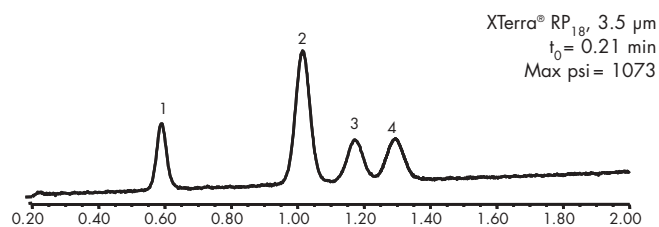
Fenoprofen



Flurbiprofen



Ibuprofen



PROFENS, ISOCRATIC

CONDITIONS

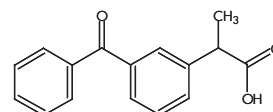
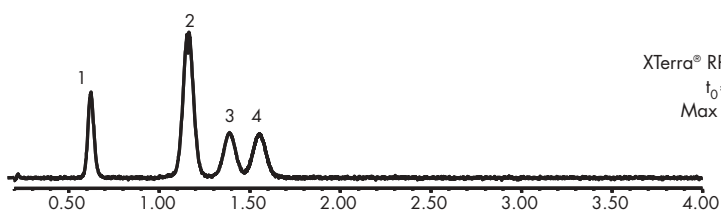
Columns: XTerra® RP₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186001893)
 XTerra® MS C₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186001891)
 Atlantis™ dC₁₈, 4.6 x 20 mm IS™ 3 µm, (P/N:186002062)
 SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186002092)
 Symmetry® C₁₈, 4.6 x 20 mm IS™, 3.5 µm, (P/N:186002090)

Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Acetonitrile
 Flow Rate: 3.0 mL/min

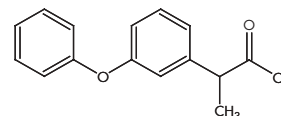
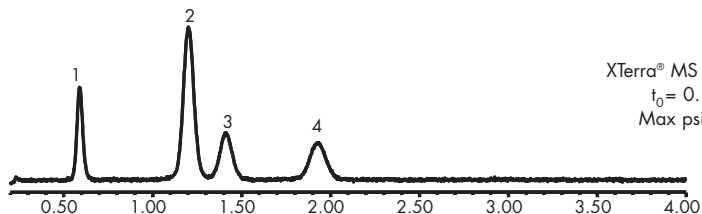
Isocratic Mobile Phase Conditions: 65% A, 35% B
 Injection Volume: 10 µL
 Sample concentration: 20 µg/mL
 Temperature: 30°C
 Detection: UV @ 220 nm
 Instrument: Alliance® 2695 with 996 PDA

Compounds

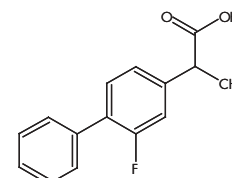
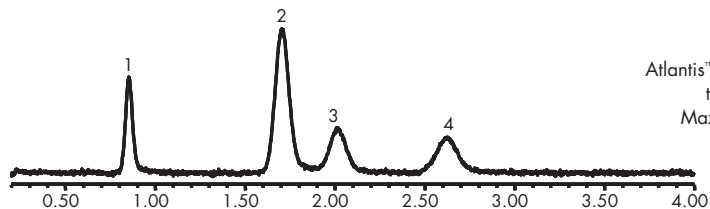
1. Ketoprofen
2. Fenoprofen
3. Flurbiprofen
4. Ibuprofen



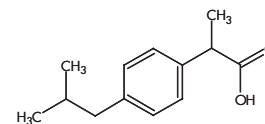
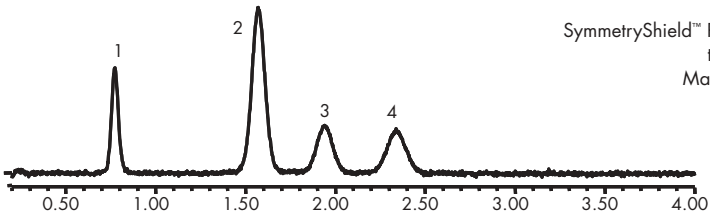
Ketoprofen



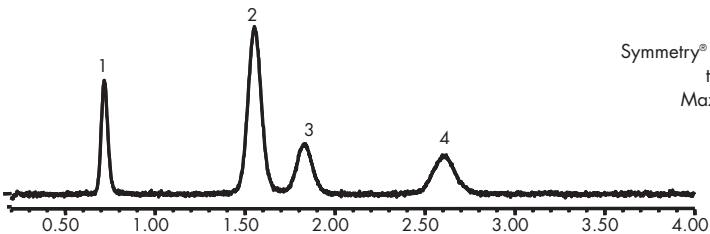
Fenoprofen



Flurbiprofen



Ibuprofen



Minutes

STERIODS

CONDITIONS

Columns: Atlantis™ dC₁₈, 4.6 x 20 mm IS™ 3 μm, (P/N:186002062)
 Symmetry® C₁₈, 4.6 x 20 mm IS™ 3.5 μm, (P/N:186002090)
 SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™ 3.5 μm, (P/N:186002092)
 XTerra® MS C₁₈, 4.6 x 20 mm IS™ 3.5 μm, (P/N:186001891)

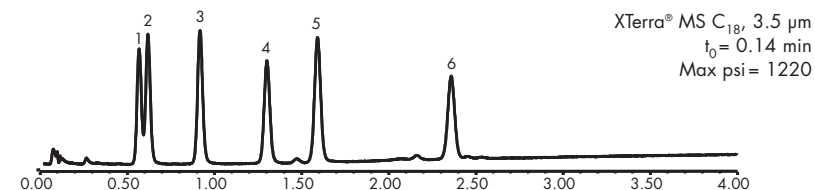
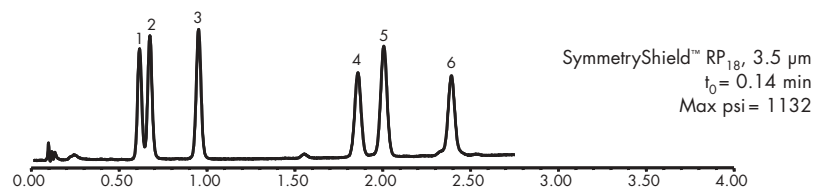
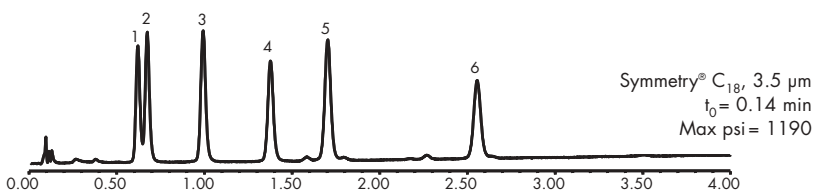
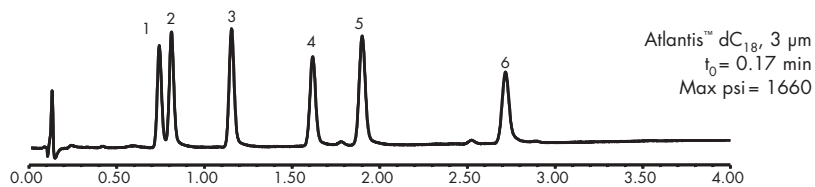
Mobile Phase A: Water
 Mobile Phase B: Acetonitrile
 Flow Rate: 3.0 mL/min

Gradient: Time Profile
 (min) %A %B
 0.0 80 20
 4.0 36 65

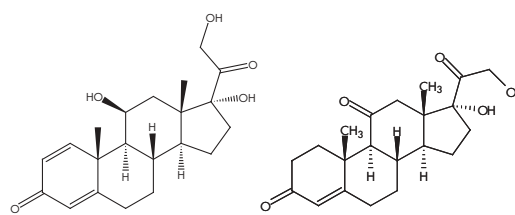
Injection Volume: 10 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 220 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

1. Prednisolone
2. Cortisone
3. Corticosterone
4. Estradiol
5. Estrone
6. Progesterone

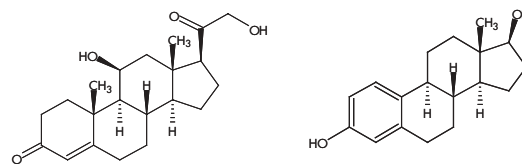


Minutes



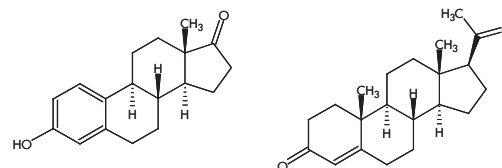
Prednisolone

Cortisone



Corticosterone

Estradiol



Estrone

Progesterone

STEROIDS

CONDITIONS

Columns: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3 μm, (P/N:186002058)
 SymmetryShield™ RP₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N:186002068)
 Symmetry® C₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N:186002066)
 XTerra® MS C₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N:186001923)

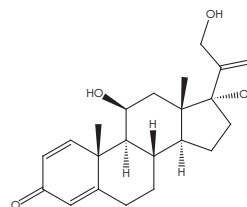
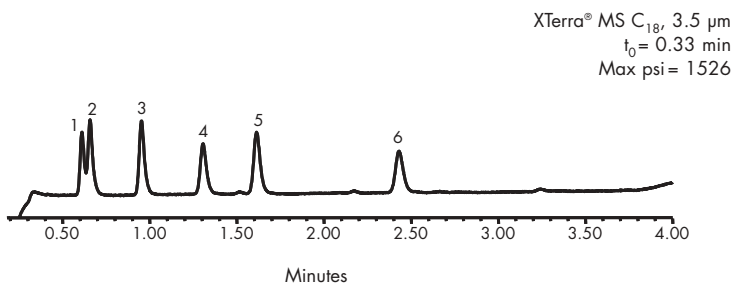
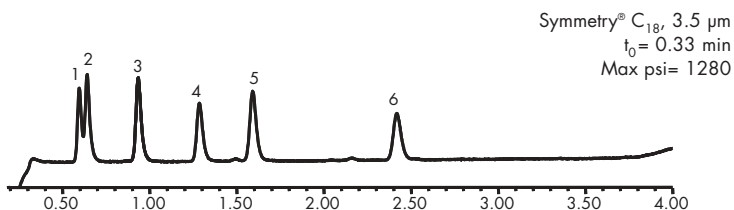
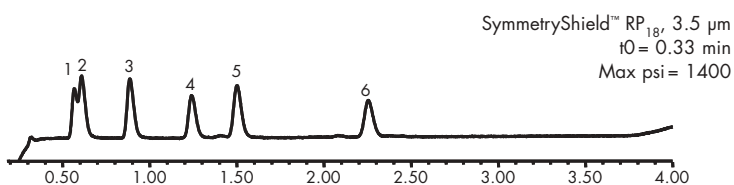
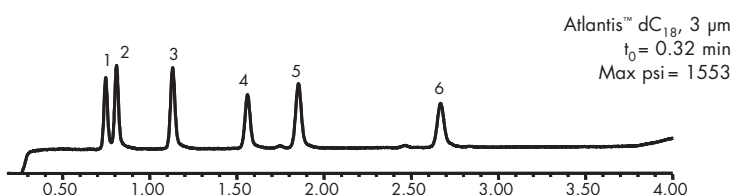
Mobile Phase A: Water
 Mobile Phase B: Acetonitrile
 Flow Rate: 0.6 mL/min

Gradient: Time Profile
 (min) %A %B
 0.0 80 20
 4.0 36 64

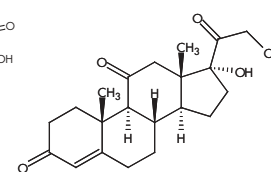
Injection Volume: 5 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 220 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

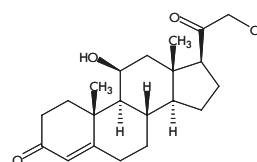
1. Prednisolone
2. Cortisone
3. Corticosterone
4. Estradiol
5. Estrone
6. Progesterone



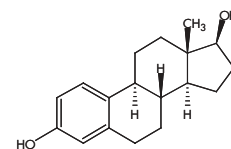
Prednisolone



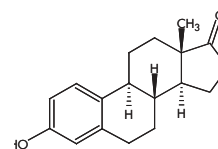
Cortisone



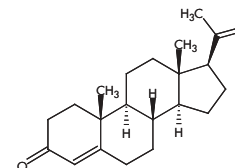
Corticosterone



Estradiol



Estrone



Progesterone

SULFONAMIDES

CONDITIONS

Columns: Atlantis™ dC₁₈, 4.6 x 20 mm IS™, 3 μm, (P/N:186002062)
 Symmetry® C₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N:186002090)
 SymmetryShield™ RP₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N:186002092)
 XTerra® MS C₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N:186001891)
 XTerra® RP₁₈, 4.6 x 20 mm IS™, 3.5 μm, (P/N:186001893)

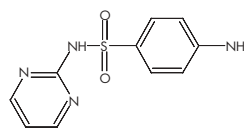
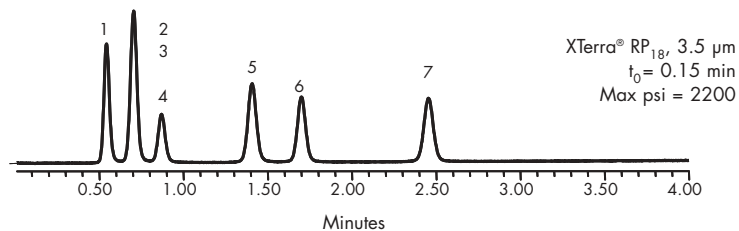
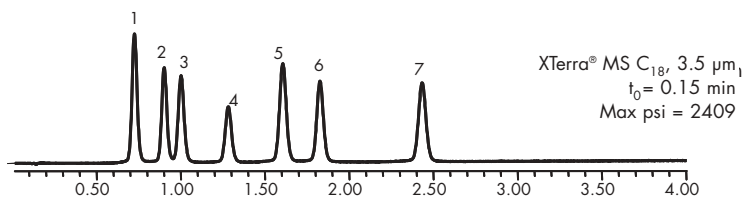
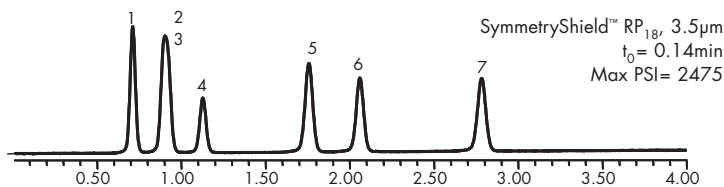
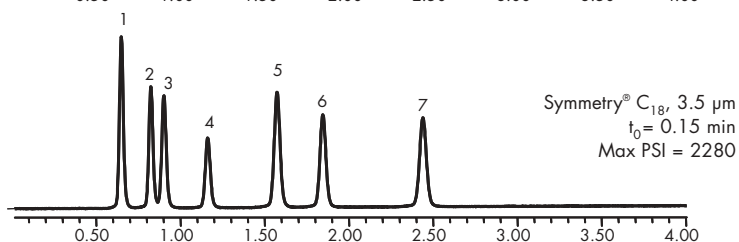
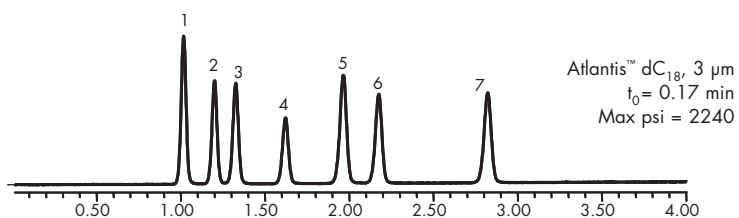
Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Methanol
 Flow Rate: 3.0 mL/min

Gradient: Time Profile
 (min) %A %B
 0.0 100 0
 4.0 50 50

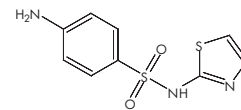
Injection Volume: 10 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 270 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

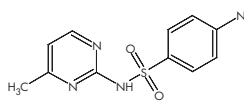
1. Sulfadiazine
2. Sulfathiazole
3. Sulfamerazine
4. Sulfadimidine
5. Sulfamethoxazole
6. Sulfisoxazole
7. Sulfadimethoxine



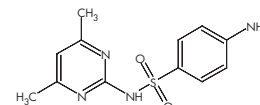
Sulfadiazine



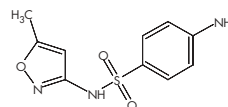
Sulfathiazole



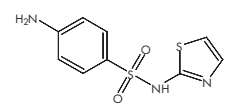
Sulfamerazine



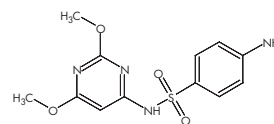
Sulfadimidine



Sulfamethoxazole



Sulfisoxazole



Sulfadimethoxine

SULFONAMIDES

CONDITIONS

Columns: Atlantis™ dC₁₈, 2.1 x 20 mm IS™, 3 μm, (P/N:186002058)
 SymmetryShield™ RP₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N:186002068)
 Symmetry® C₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N:186002066)
 XTerra® MS C₁₈, 2.1 x 20 mm IS™, 3.5 μm, (P/N:186001923)

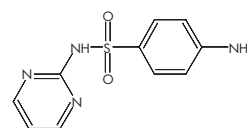
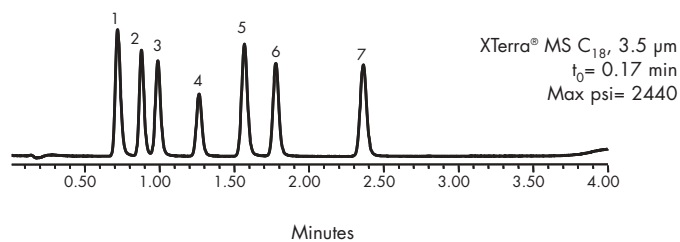
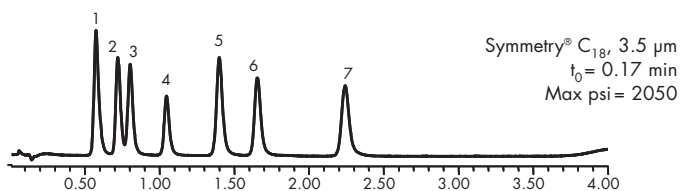
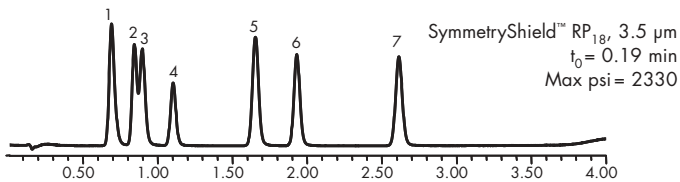
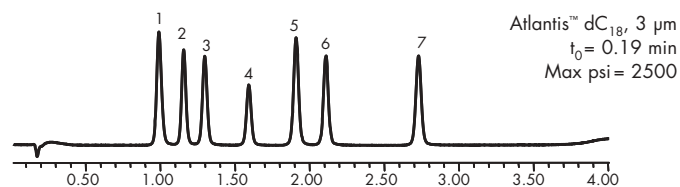
Mobile Phase A: 0.1% HCOOH in Water
 Mobile Phase B: 0.1% HCOOH in Methanol
 Flow Rate: 0.6 mL/min

Gradient: Time Profile
 (min) %A %B
 0.0 100 0
 4.0 50 50

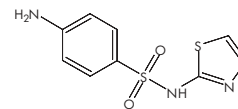
Injection Volume: 10 μL
 Sample concentration: 20 μg/mL
 Temperature: 30°C
 Detection: UV @ 270 nm
 Instrument: Alliance® 2795 with 996 PDA

Compounds

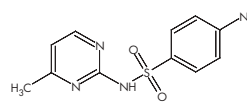
1. Sulfadiazine
2. Sulfathiazole
3. Sulfamerazine
4. Sulfadimidine
5. Sulfamethoxazole
6. Sulfisoxazole
7. Sulfadimethoxine



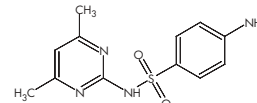
Sulfadiazine



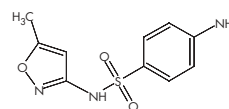
Sulfathiazole



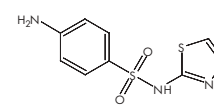
Sulfamerazine



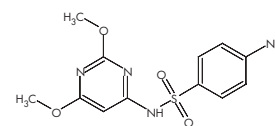
Sulfadimidine



Sulfamethoxazole



Sulfisoxazole



Sulfadimethoxine

APPENDIX A

BANDSPREAD TEST

When the HPLC Instrument is set up for *IS*[™] columns, it is recommended that a bandspread test be conducted.

PROCEDURE

- Disconnect column from system
- Connect injector directly to detector
- Flow rate 1 mL/min, detector sensitivity 0.5-1.0 AUFS, time constant 0.2 seconds or less
- Dilute test mix 1:10 in mobile phase
- Inject 2 to 5 μ L of this solution
- Using 5 sigma method measure at 4.4% of peak height
- Bandspread calculation (μ L)= Peak Width (cm) x 50

COMPARE RESULTS

- Typical LC System should be $100\mu\text{L} \pm 30\mu\text{L}$
- Narrowbore HPLC System should be no greater than 20 μ L

APPENDIX B

ALLIANCE® 2695 SEPARATIONS MODULE: OPTIMIZATION AND PERFORMANCE WITH 4.6 mm I.D. INTELLIGENT SPEED (IS™) COLUMNS

INTRODUCTION

The Waters Intelligent Speed (IS™) line of columns have a 20 mm packed bed and optimized hardware that can be run at higher flow rates and lower backpressures without sacrificing resolution, reducing run times by up to 90 %. This technical note focuses on optimization and performance of the Alliance® 2695 Separations Module for use with IS™ columns. A series of fast gradients were run at different flow rates to determine the effect on the reproducibility of retention times and peak areas. Peak capacities for the columns at the different flow rates were also calculated.

Additional information on Waters instruments with IS™ columns can be found in Technical Note 720000722EN titled: Waters 2996 Photodiode Array Detector: Optimization for Intelligent Speed Columns.

Excellent run to run reproducibility achieved for fast gradients with no modifications to instrument configuration

A series of gradients varying from 0.6 to 4.0 minutes going from 0 to 50 % ACN at flow rates varying from 1.0 to 4.0 mL/minute were run. Figure 1 shows the retention time reproducibility that was achieved for six replicate injections under each gradient condition. Excellent retention time reproducibility was achieved for a wide range of fast, steep gradients. Only at 4 mL/min with 1 minute and faster gradients did the retention time reproducibility exceed 0.5 % RSD. Figure 2 shows the peak area reproducibility for the same conditions. All of the gradients resulted in %RSD values for the peak area that were well below instrument specification (<0.5 % RSD), indicating no compromise in system performance when using fast, steep gradients.

System: Waters Alliance® 2695 Separations Module
 Waters 2996 Photodiode Array Detector
 Waters Empower™ Software
 Column: XTerra® MS C₁₈ 4.6 x 20 mm, IS™ 2.5 µm at 25°C
 Detection: Wavelength range 195 – 285 nm
 Extracted channel at 220 nm
 Sampling rate 10 pts/s
 No digital filtering
 Mobile Phase: A = 0.1% TFA in water
 B = acetonitrile
 Sample: 10 µL injections of beta blockers
 (0.1 µg/mL atenolol,
 0.1 µg/mL metoprolol,
 0.05 µg/mL pindolol)

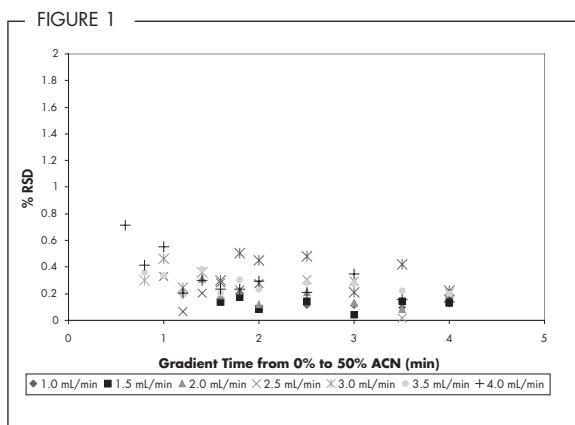


Figure 1: Retention time %RSD as a function of gradient time and flow rate

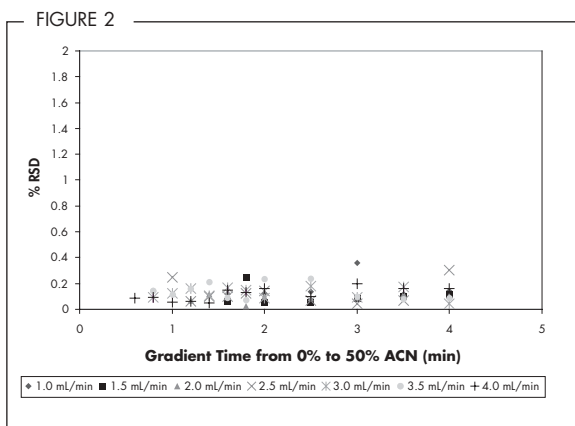


Figure 2: Peak area %RSD as a function of gradient time and flow rate

TABLE 1

Flow Rate (mL/min)	Gradient Time (min)			
	1.0	2.0	3.0	4.0
1.0	–	–	17	24
1.5	–	16	21	28
2.0	–	20	26	34
2.5	14	22	28	38
3.0	16	29	37	42
3.5	18	28	38	45
4.0	19	28	38	46

Table 1: Peak capacities as a function of gradient time and flow rate

APPENDIX B

HIGHER FLOW RATES PRODUCE THE BEST PEAK CAPACITIES

Peak capacity is a function of the peak width, which is in turn a function of the analyte's linear velocity, and band broadening effects. According to the van Deemter equation, as the flow rate increases, there will reach a point where band broadening effects are minimized and the narrowest peaks will be achieved. For *IS*[™] 4.6 mm I.D. columns, this point is between 3 and 4 mL/min, under most conditions. Table 1 lists the peak capacities achieved for various gradients. The optimal peak capacity for each gradient is listed in bold.

SHORTER RUN AND RE-EQUILIBRATION TIMES

IS[™] columns allow for much faster separations, Figure 3, increasing sample throughput and decreasing solvent consumption per analysis. Additionally, the higher flow rates and shorter columns allow for much faster post gradient re-equilibration times. Recommended re-equilibration times are 3 times system volume and 5 times column volume. For a standard 4.6 x 100 mm column on the Alliance® 2695 Separations Module at 1 mL/min, re-equilibration time is 10.3 minutes. For an *IS*[™] column, 4.6 x 20 mm at 3 mL/min, re-equilibration time is only 1.2 minutes, resulting in much faster injection to injection cycle times.

SUMMARY

The Alliance® 2695 Separations Module delivers excellent retention time and area reproducibility for fast gradient separations (0 to 50% ACN over 0.6 to 4.0 minutes) without instrument modifications. The best peak capacities for the 4.6 mm I.D. *IS*[™] columns are achieved between 3 and 4 mL/minute. Run times and the re-equilibration times are greatly reduced with *IS*[™] columns (re-equilibration times reduced 8.6 times as compared to 100 mm columns), allowing for increased sample throughput and increased productivity.

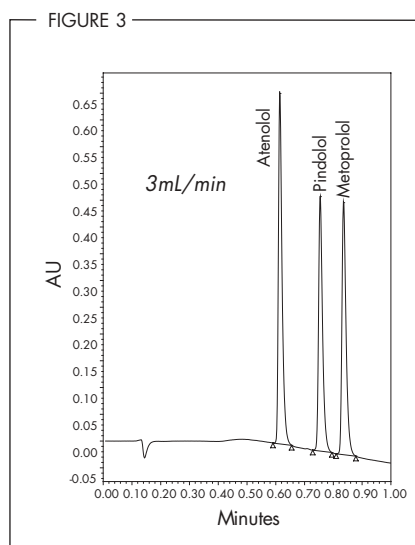


Figure 3: Fast gradient separation of 3 beta blockers

2996 PHOTODIODE ARRAY DETECTOR: OPTIMIZATION FOR INTELLIGENT SPEED (IS™) COLUMNS

INTRODUCTION

The Waters® Intelligent Speed (IS™) line of columns have a 20 mm packed bed and optimized hardware that allow for fast, reproducible separations which generate peaks that are often only 2 to 4 seconds wide. This technical note will focus on optimizing the 2996 PDA detector settings for use with the IS™ columns. The effects of sampling rate on reproducibility, peak shape, and resolution are examined along with the effects of digital filtering on signal to noise, resolution, and peak capacity. Additional information on Waters Instruments with IS™ columns can be found in technical note 720000723EN titled: *Alliance® 2695 Separations Module: Optimization and Performance with 4.6 mm I.D. Intelligent Speed (IS™) Column*

NARROW PEAKS REQUIRE HIGHER SAMPLING RATES

Figure 1 shows the same one minute gradient separation performed at different sampling rates. At sampling rates below 5 pts/s, there is a visible effect on peak shape. Table 1 lists the reproducibility of the peak area and retention times for 10 injections, along with the points across the peaks at the different sampling rates. The reproducibility of the peak area is above 0.5 % RSD (Alliance® system specification for injection precision) at sampling rates below 5 pts/s. In addition, when working with peaks which are only 3 seconds wide, the number of points across the peak fall below the minimum number needed for reproducible quantitation (15) at sampling rates below 5 pts/s.

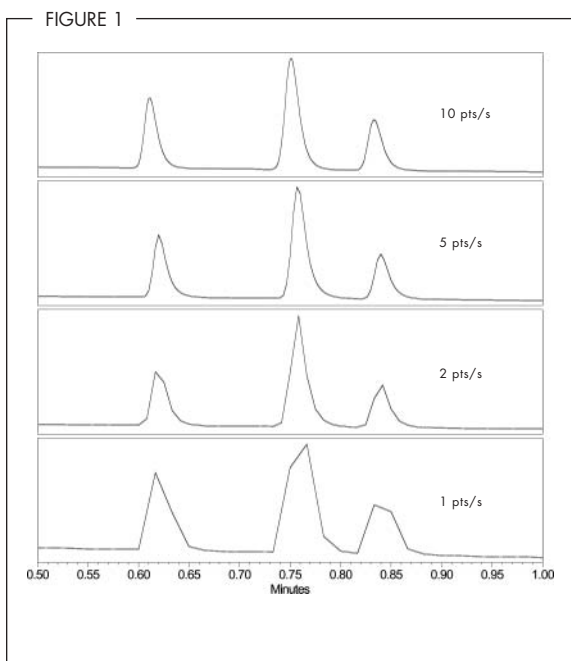


Figure 1: Effect of decreasing the sampling rate

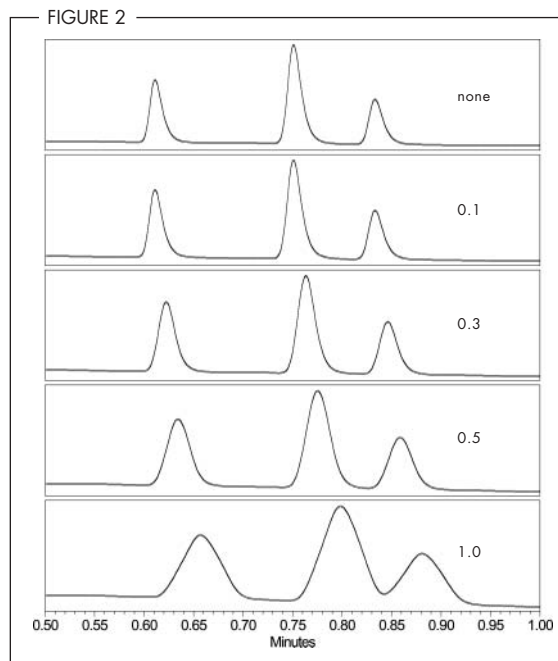


Figure 2: Effect of increasing the filter response of the digital filter

CONDITIONS

System:	Waters Alliance® 2695 Separations Module Waters 2996 PDA Detector Waters Empower™ Software	Mobile Phase:	A = 0.1 % TFA in water B = acetonitrile
Column:	Xterra® MS C ₁₈ , 4.6 x 20 mm, IS™ 2.5 µm at 25 °C	Flow Rate:	3.0 mL/min
Detection:	Wavelength range 195 – 285 nm Extracted channel at 220 nm	Gradient:	0 % to 50 % over 1 minute
		Sample:	10 µL injection of beta blockers (0.1 µg/mL atenolol, 0.1 µg/mL metoprolol, 0.05 µg/mL pindolol)

APPENDIX C

DIGITAL FILTERING AFFECTS RESOLUTION AND PEAK CAPACITY

Digital filtering is performed on data to improve the signal-to-noise ratios. The filter calculates a data point that is a modified rolling average for a wavelength over a number of readings. Figure 2 shows that as the filter response for the digital filter increases (more averaging) the peak width increases. At a filter response of 1.0, baseline resolution is lost. This increased peak width results in reduced peak capacity. Table 2 lists the peak capacities obtained at the different filter response levels along with the signal-to-noise ratios. The data show that there is a minimal increase in the signal to noise for a 2 fold reduction in resolution. At a filter response of 1.0 there is a significant increase in the signal to noise, however at the cost of a loss in resolution and diminished peak capacities.

SUMMARY

Slower sampling rates can dramatically affect peak shape, resulting in poor area and retention time reproducibility. For most fast gradients 5-10 pts/s will be required. For very narrow peaks, such as those resulting from separations on the IS™ line of columns, use digital filtering with caution. Digital filtering does not need to be used except in separations where increased sensitivity is much more important than resolution and high peak capacity.

TABLE 1

Sample Rate	%RSD of Retention Time	% RSD of Area	Point Across Peak
10 pts/s	0.440	0.09	39
5 pts/s	0.562	0.20	21
2pts/s	0.597	0.68	9
1pt/s	1.034	3.85	5

Table 1: Reproducibility and points across the peak for metoprolol as a function of sampling rate

TABLE 2

Filter Response	Signal to Noise Increase	Resolution	Peak Width	Peak Capacity
None	–	3.16	3.82s	16
0.1	1.04x	2.99	4.02s	15
0.3	0.94x	2.45	4.56s	14
0.5	1.04x	1.82	5.27s	12
1.0	1.57x	1.08	7.01s	9

Table 2: Effect of filter response level on chromatographic parameters

ORDERING INFORMATION

Waters IS™ columns are available in a full range of proprietary, industry-leading chemistries enabling total flexibility in developing fast separations using simple mobile phases.

ATLANTIS™ dC₁₈ IS™ COLUMNS

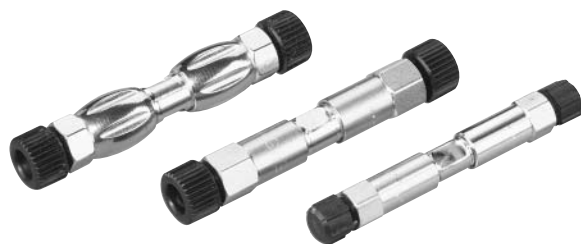
ID (mm)	Length (mm)	Particle Size	dC ₁₈
2.1 mm	20 mm	3 µm	186002058
2.1 mm	20 mm	5 µm	186002059
3.0 mm	20 mm	3 µm	186002060
3.0 mm	20 mm	5 µm	186002061
4.6 mm	20 mm	3 µm	186002062
4.6 mm	20 mm	5 µm	186002063

SYMMETRY® IS™ COLUMNS

ID (mm)	Length (mm)	Particle Size	Symmetry®		SymmetryShield™	
			C ₁₈	C ₈	RP ₁₈	RP ₈
2.1 mm	20 mm	3.5 µm	186002066	186002067	186002068	186002069
2.1 mm	20 mm	5 µm	186002070	186002071	186002072	186002073
3.0 mm	20 mm	3.5 µm	186002074	186002075	186002076	186002077
3.0 mm	20 mm	5 µm	186002078	186002079	186002080	186002081
3.9 mm	20 mm	3.5 µm	186002082	186002083	186002084	186002085
3.9 mm	20 mm	5 µm	186002086	186002087	186002088	186002089
4.6 mm	20 mm	3.5 µm	186002090	186002091	186002092	186002093
4.6 mm	20 mm	5 µm	186002094	186002095	186002096	186002097

XTERRA® IS™ COLUMNS

ID (mm)	Length (mm)	Particle Size	MS C ₁₈	MS C ₈	RP ₁₈	RP ₈
2.1 mm	20 mm	2.5 µm	186001921	186001922	—	—
2.1 mm	20 mm	3.5 µm	186001923	186001924	186001925	186001926
2.1 mm	20 mm	5 µm	186001979	186001980	186001982	186001983
3.0 mm	20 mm	2.5 µm	186001972	186001973	—	—
3.0 mm	20 mm	3.5 µm	186001974	186001975	186001976	186001977
3.0 mm	20 mm	5 µm	186001984	186001985	186001986	186001987
3.9 mm	20 mm	2.5 µm	186001899	186001897	—	—
3.9 mm	20 mm	3.5 µm	186001900	186001898	186001902	186001901
3.9 mm	20 mm	5 µm	186001988	186001989	186001990	186001991
4.6 mm	20 mm	2.5 µm	186001889	186001890	—	—
4.6 mm	20 mm	3.5 µm	186001891	186001892	186001893	186001894
4.6 mm	20 mm	5 µm	186001992	186001993	186001994	186001995



Visit us on the internet: <http://www.waters.com/intelligentspeed>

Australia:

Waters Australia Pty. Limited

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