Making LC Methods MS Friendly

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Columns and Supplies Technical Support

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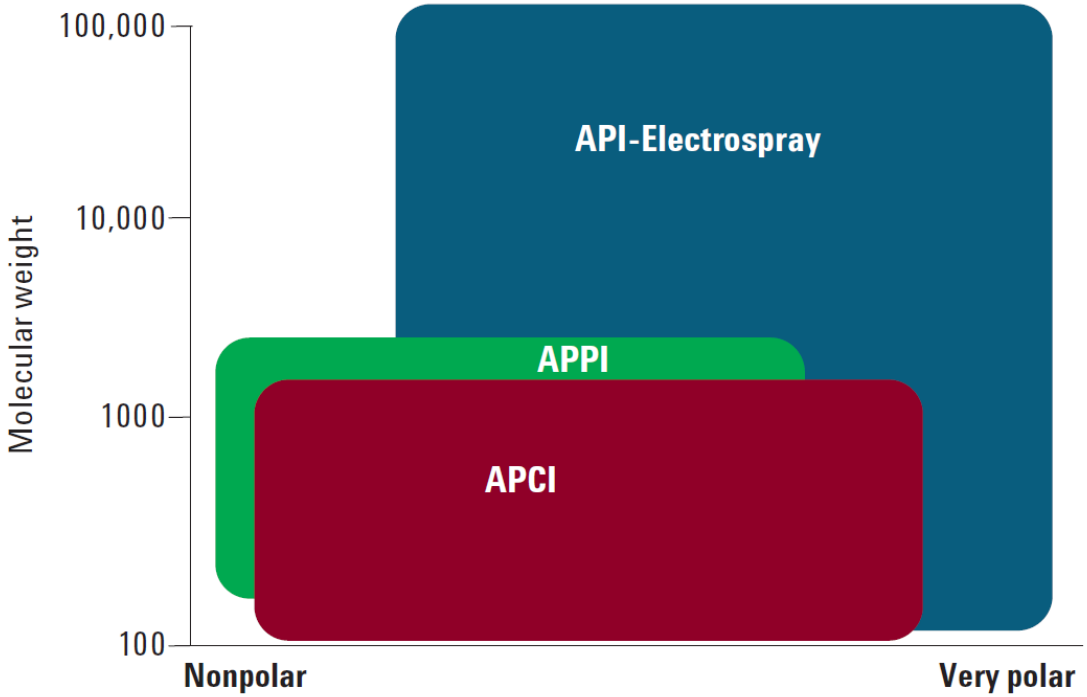
Topics

- ***LC/MS ionization techniques***
  - ESI
  - APCI
  - APPI
- ***Appropriate conditions***
  - Volatile buffers for MS
  - Ion pair chromatography
  - HILIC
- ***Appropriate columns***
  - Column diameter
  - Bonded phase
  - Particle size
- ***Adapting existing methods to LC/MS***
- ***Maximizing Sensitivity***
  - Minimize extra column volume
  - Avoiding interferences
  - Sample preparation

LC/MS Techniques and Applications

- Atmospheric pressure ionization (API)
- Three typical API methods:
  - *ESI* - electrospray ionization
  - *APCI* - atmospheric pressure chemical ionization
  - *APPI* - atmospheric pressure photoionization
- Appropriate ionization method depends largely on analyte polarity
- Positive ion mode (protonation) or negative ion mode (deprotonation)
- Masses measured as mass to charge ratio (*m*/*z*)

Applicability of Atmospheric Pressure Ionization Techniques

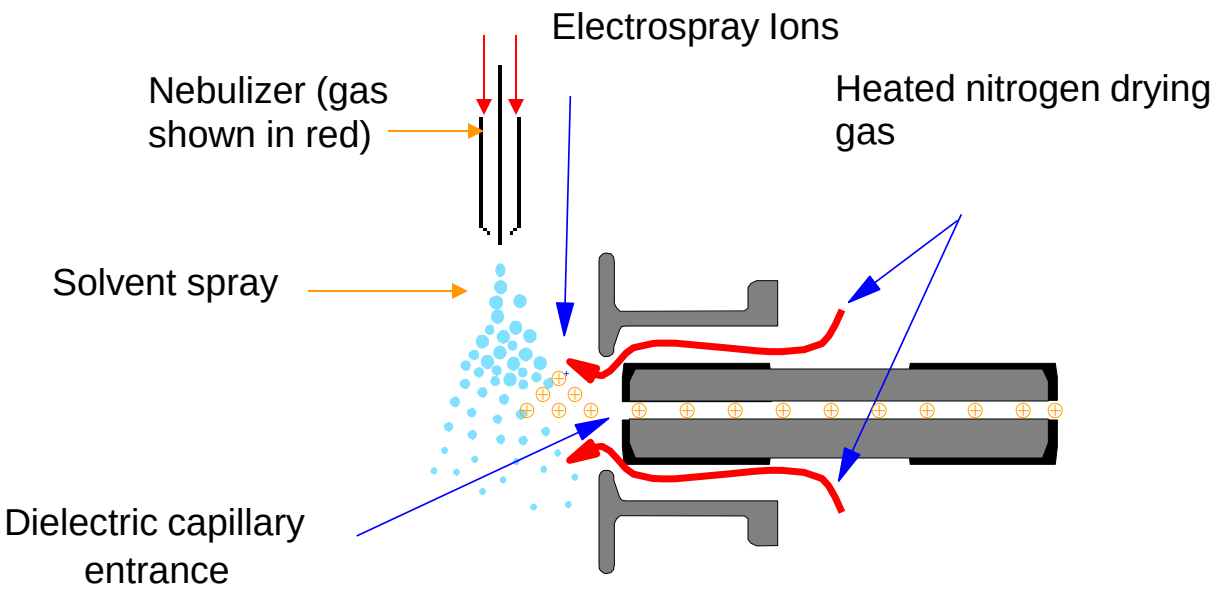


API-ESI = Atmospheric pressure electrospray ionization
APCI = Atmospheric presure chemical ionization
APPI = Atmospheric pressure photo ionization

Electrospray Ionization

- Most common ionization technique
- Used for high and low molecular weight compounds
- Ions are formed in solution and then the droplets are evaporated
- Analyte volatility not required
- Compounds containing heteroatoms such as N, S, and O typically analyze well
- Can form multiply-charged ions
- Like UV detection, ESI is concentration sensitive
- ESI is generally more sensitive for samples that are ionized in solution

Electrospray Ionization

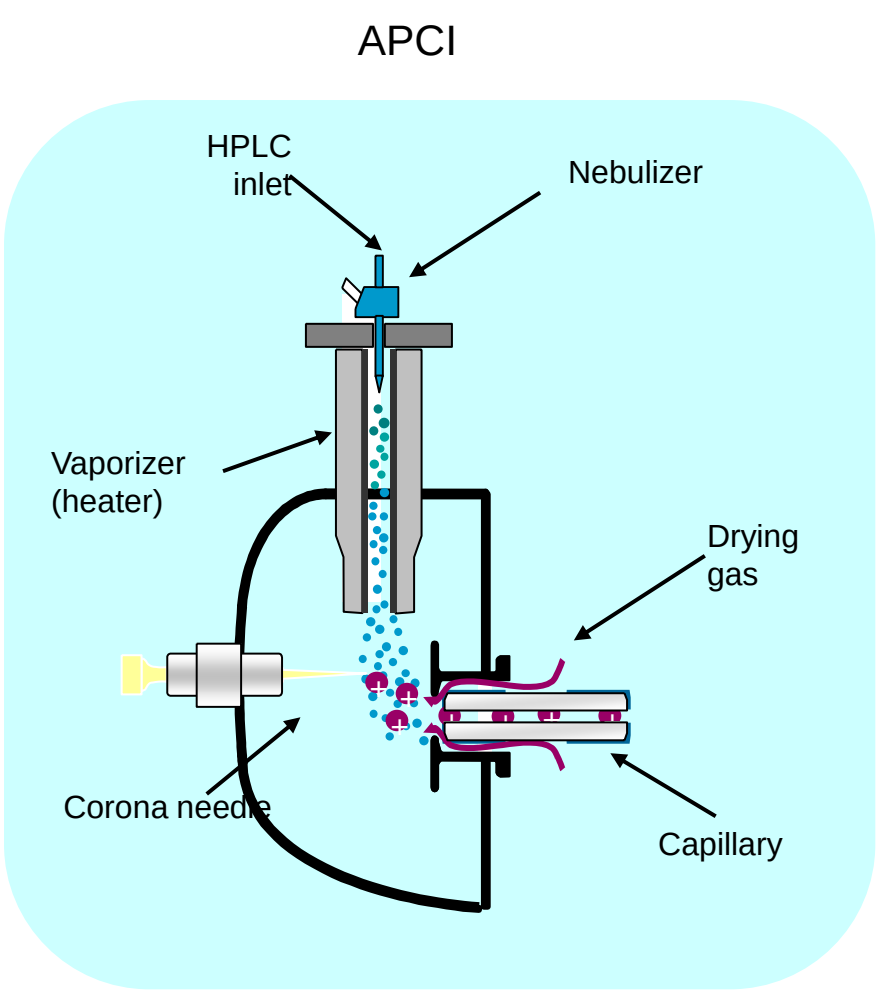


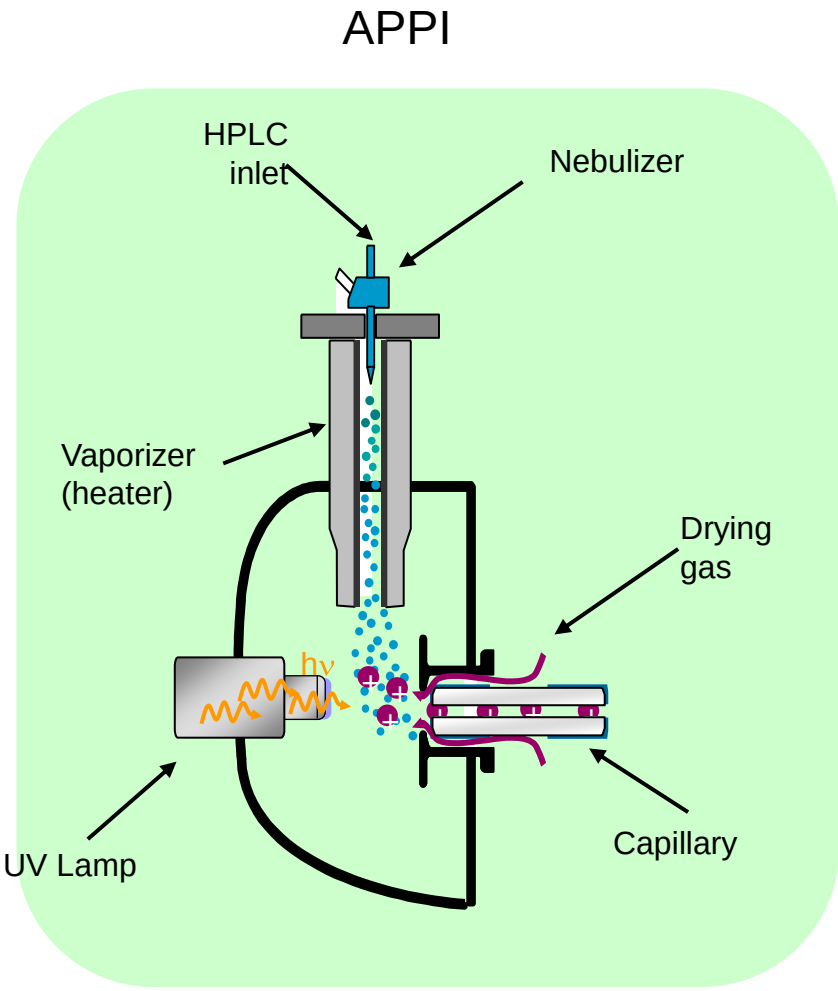
APCI and APPI Sources

- APCI
  - Analyte and mobile phase are first evaporated then ionized by corona needle
  - Good technique for low to medium polarity analytes
  - High probe temperatures desolvate and vaporize the sample
  - Could lead to sample decomposition
  - Not a good choice for thermally unstable analytes
  - Forms singly charged ions

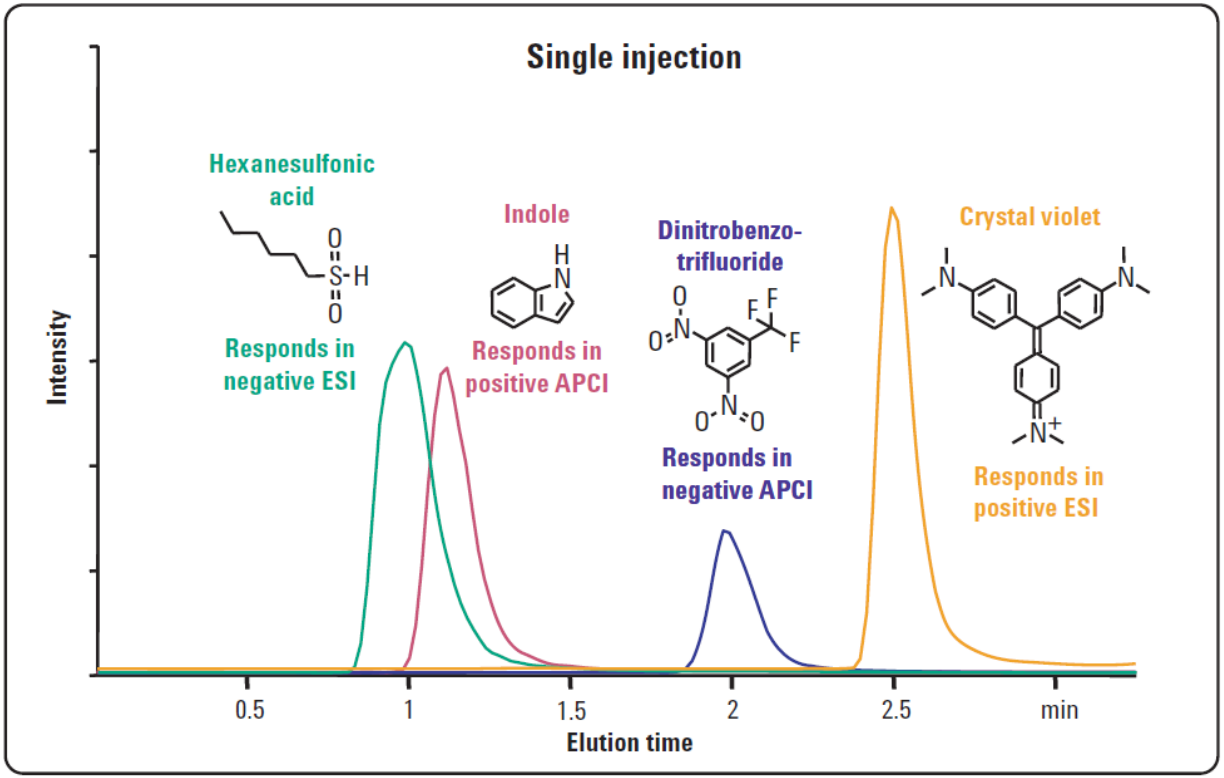
- APPI
  - Analyte and mobile phase are first evaporated then ionized with light
  - Good technique for hydrophobic conjugated ring systems
  - thermally sensitive compounds
  - May be less susceptible to ion suppression than ESI

APCI and APPI Sources for the LC/MSD



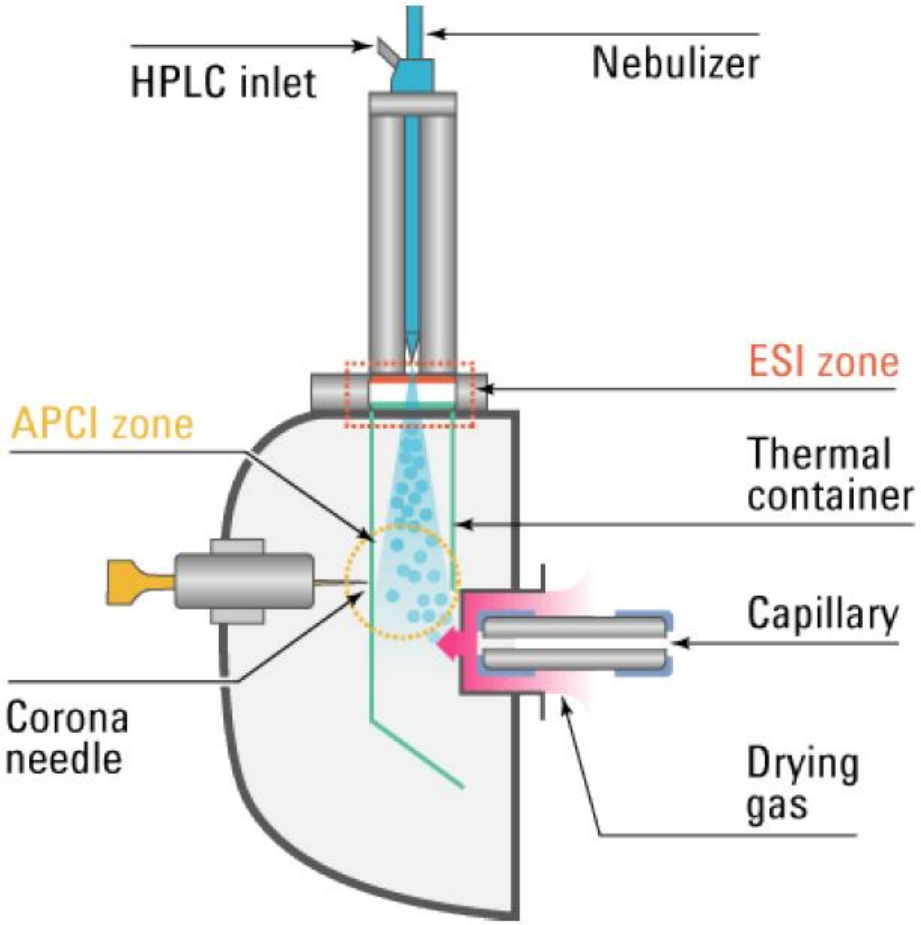


Multimode Source



- Capable of simultaneously generating ions by electrospray and APCI
- Positive ESI, negative ESI, positive APCI, and negative APCI in a single run

Multimode Source



Method Considerations for ESI

- pH of the mobile phase (and analyte pKa) affects ion formation
- Voltage applied to the electrospray probe will induce ion formation
- Choosing best mobile phase pH analytes can improve sensitivity
- Organic solvent has little effect on ionization
- Works best with buffer concentrations below 25 mM
- Works best at low flow rates (less than 0.5 mL/min)
    - 5 µL/min up to 2 mL/min (for ESI with Agilent Jet Stream thermal gradient focusing)
- Compatible with reversed phase, HILIC, normal phase

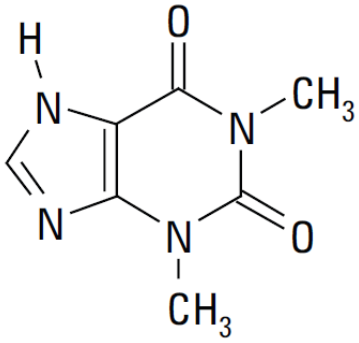
Buffer Considerations for ESI

- Buffer concentrations below 25 mM (best below 10 mM)
- Poor compatibility with non-volatile buffers
  - Deposit buildup
  - Metal ion buffers interfere with ionization
- Acidic mobile phases generally favor positive mode ionization
  - 0.1% - 1% formic acid, 0.1% - 1% acetic acid, 0.05% - 0.2% TFA
  - Ammonium salts (ammonium formate and ammonium acetate) favor formation of ammonium adducts
  - TFA causes ion suppression
  - Use TFA “fix” – post column addition of acetic or propionic acid
- Basic mobile phases generally favor negative mode ionization
  - Ammonium hydroxide, triethylamine, diethylamine, piperidine, ammonium bicarbonate
- pH 1 to 2 units away from the pKa of the analytes

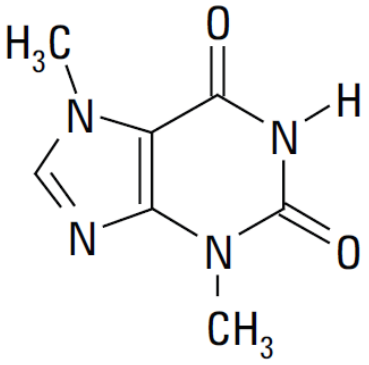
Method Considerations for APCI and APPI

- LC mobile phase solvents can interfere with ionization
- Try methanol first (acetonitrile can be a problem)
- Poor compatibility with non-volatile buffers
- Works with wider range of buffer concentrations than ESI
- Less than 100 mM
- Broader range of flow rates, up to 1.5 mL/min
- Higher sensitivity and less noise than ESI at flow rates >0.75 mL/min
- Highly flammable solvents should be avoided

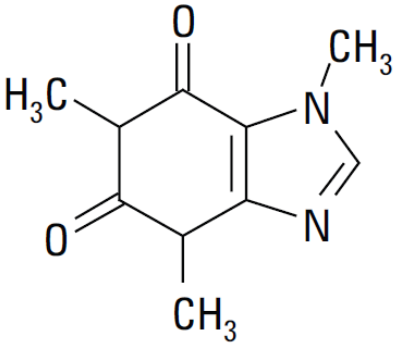
Effects of Volatile and Non-Volatile Buffers



Theophylline (TP)
M.W=180.17
pKa <1, 8.6

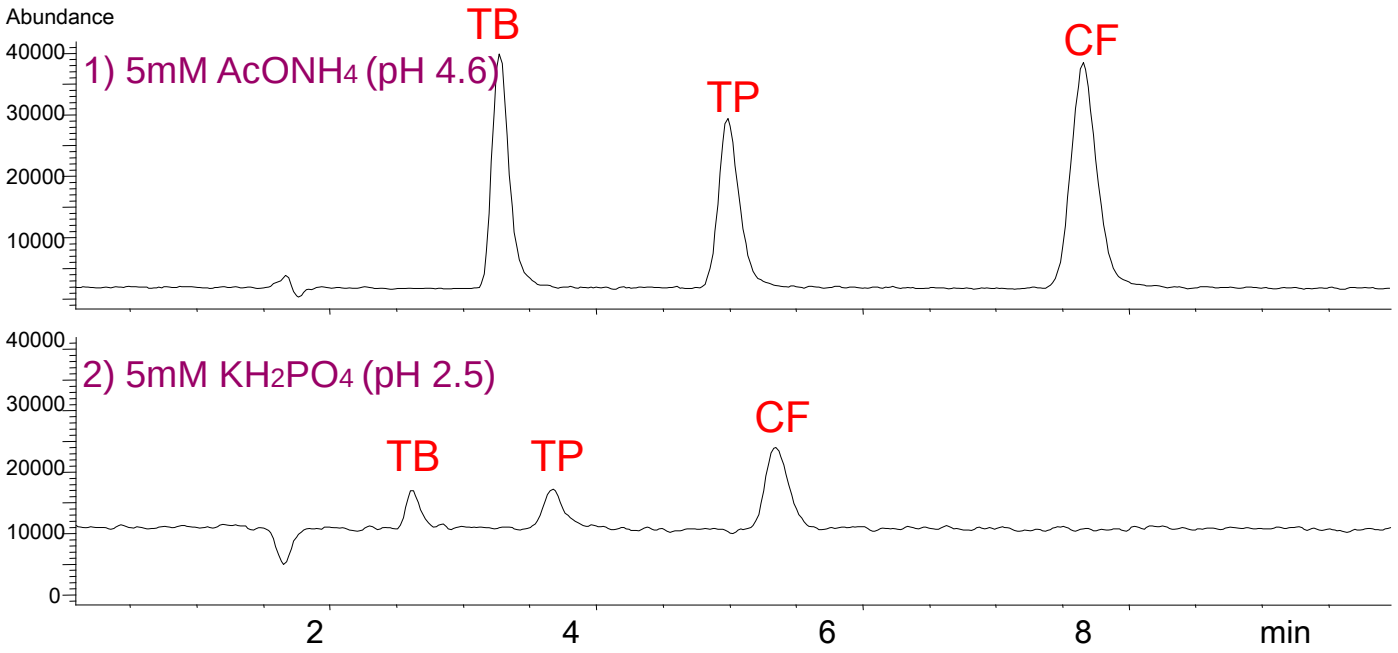


Theobromine (TB)
M.W=180.17
pKa <1, 10.0



Caffeine (CF)
M.W=194.19
pKa = 14

Volatile Buffer vs. Non Volatile Buffer



LC conditions

Column: ZORBAX Eclipse XDB-C18 2.1 x 150 mm, 5μm

Mobile Phase: 1) 5mM $AcONH_4$ (pH 4.6)/MeOH=80:20
2) 5mM KH_2PO_4 (pH 2.5)/MeOH=80:20

Flow rate: 0.2mL/min

Temp: 40ºC

Inj.volume: 5μL

MS conditions

Ionization: ESI

Mode: Positive

Mass range: m/z 100~200

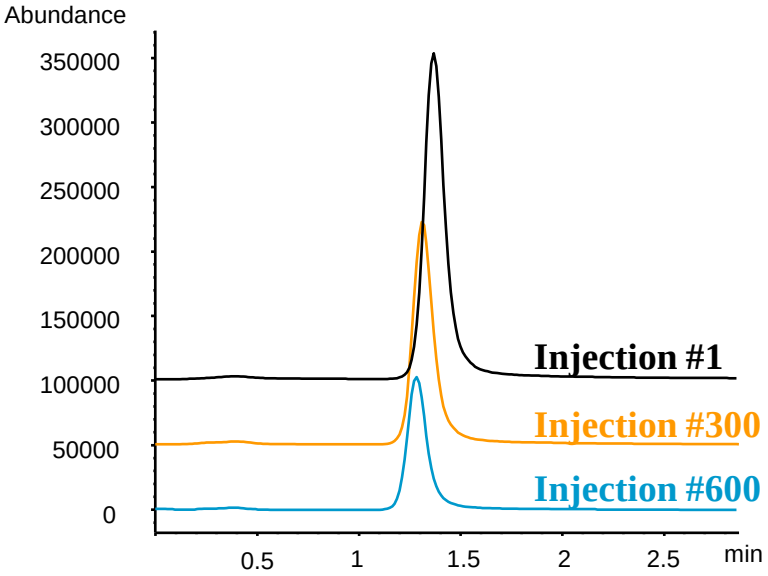
Capillary volt.: 3.5kV

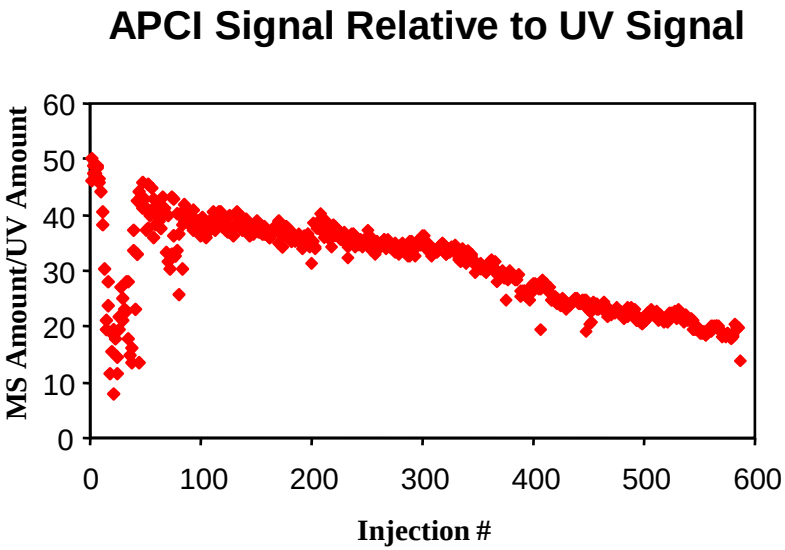
Fragmentor volt：100V

Drying gas: N_2 (12.0L/min，350℃)

Nebulizer gas: N_2 (50psi)

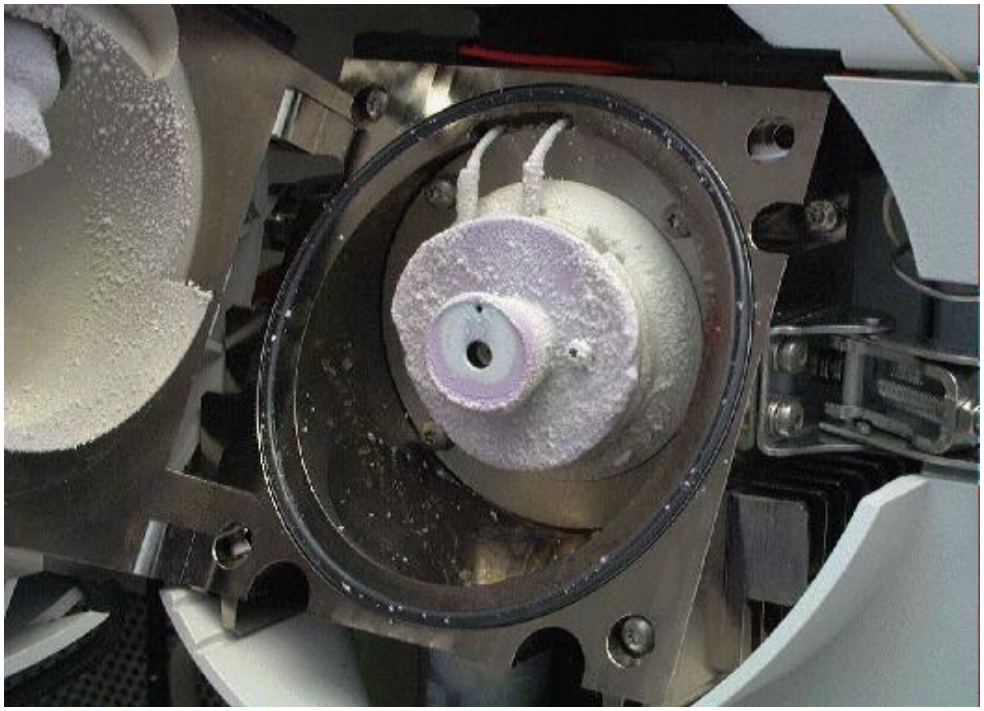
APCI Signal After 600 Injections of Salt Solution

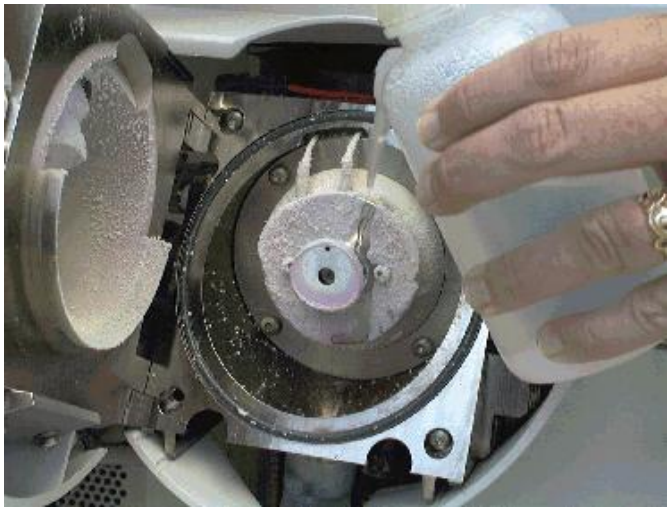




- Initial instability in the signal is probably due to changing electric fields as salt deposits in the source.

The Effects of Having Non-volatile Buffers in the Mobile Phase

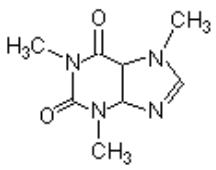




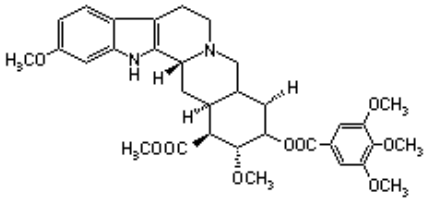
Cleaning the spray chamber

Effect of Volatile Buffer Concentration on ESI

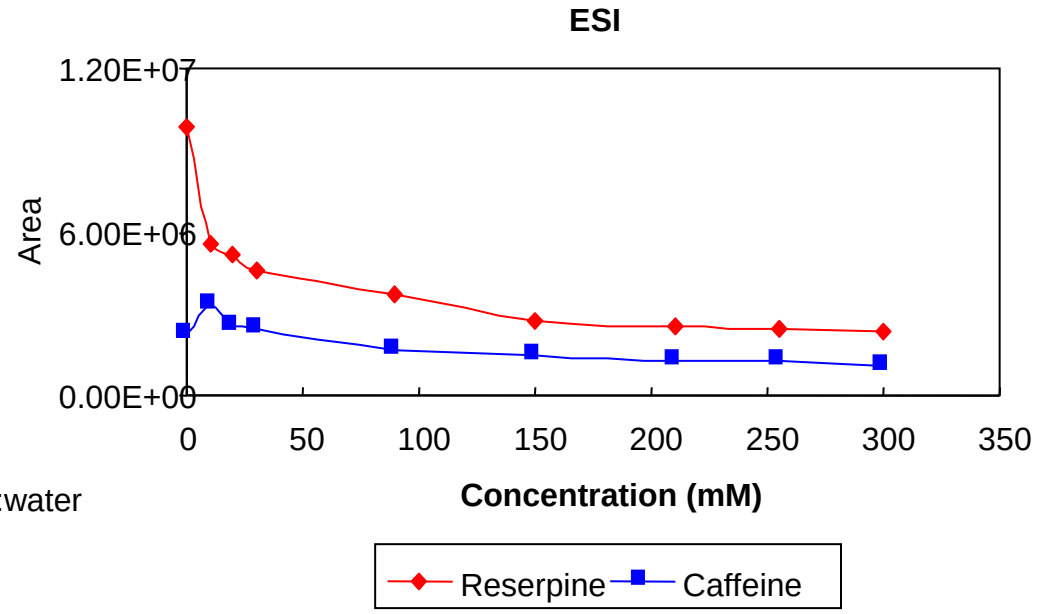
Lower buffer concentrations provide better droplet evaporation



Caffeine

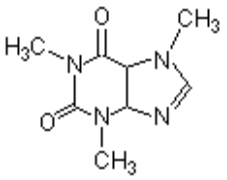


Reserpine

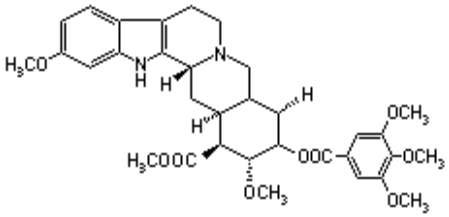


LC Conditions:
Mobile phase: ammonium acetate in 50:50 methanol:water
Flow rate 0.6 mL/min
Injection 1ul of reserpine (84 ng) or caffeine (125 ng)
FIA with 3 injections of each compound
MS Conditions:
SIM: 195.2 and 609.3
Drying gas: ESI – 350 C, 8 L/min
Nebulizer: ESI – 30 psig
Vcap: 4000V
Fragmentor: Ramped 70 V for 195.2; 120 V for 609.3
Vaporizer: 400C

Effect of Volatile Buffer Concentration on APCI



Caffeine



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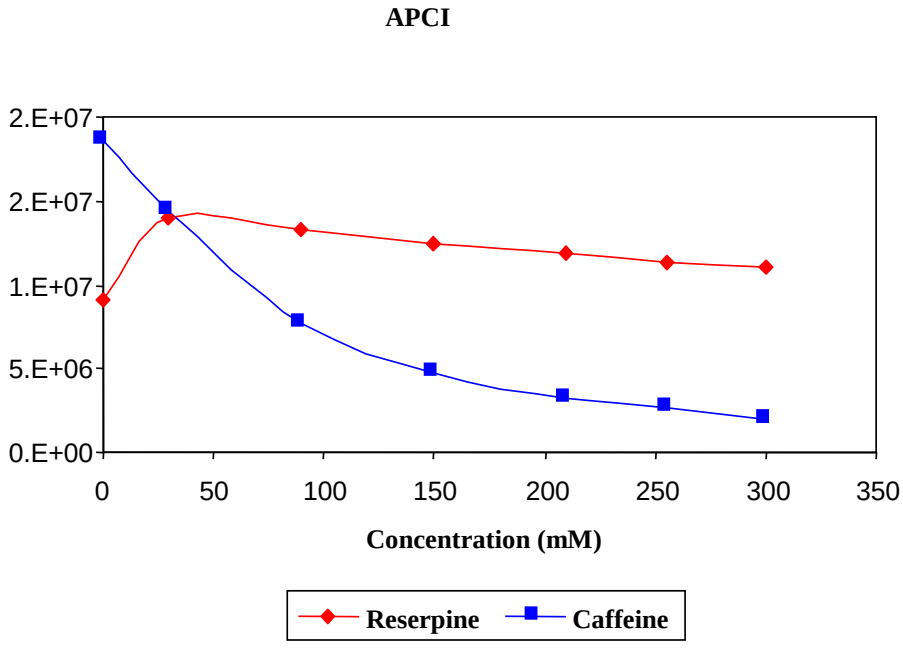

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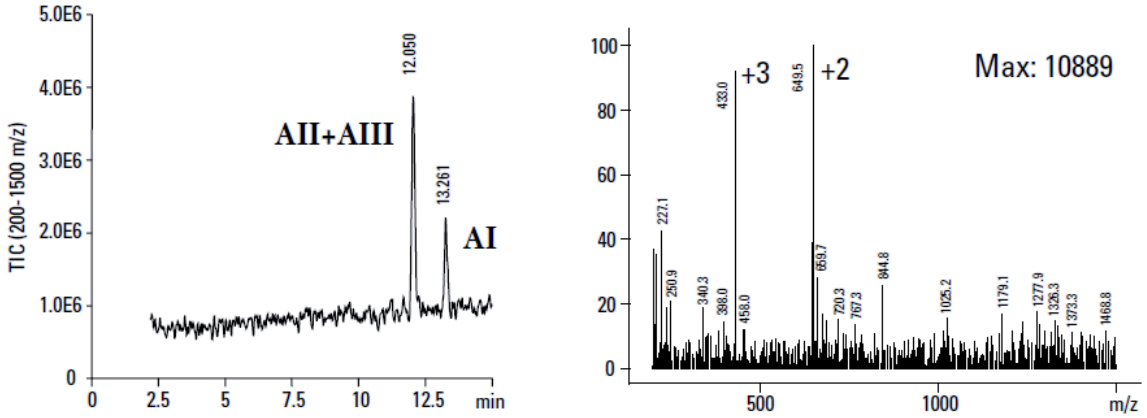
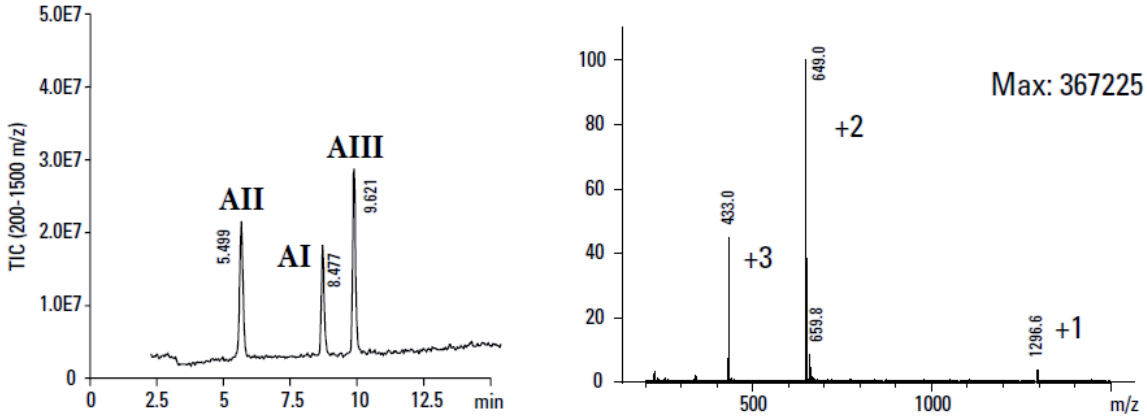
pH Effects on Selectivity and MS Sensitivity

A **Acidic Conditions:** **A: 0.1% TFA in water** **B: 0.085% TFA in 80% acetonitrile (ACN)**



Column:	**ZORBAX Extend-C18** 2.1 x 150 mm, 5 µm (HP Part No. 773700-902)
Flow Rate:	0.2 mL/min
Temperature:	35°C
Mobile Phase:	As indicated
Gradient:	15-50% B in 15 min.
LC/MS:	Pos. Ion ESI- Vf 70V, Vcap 4.5 kV, N_2- 35 psi, 12 L/min., 325°C
Sample:	Angiotensin I, II, III 2.5 µL sample (50 pmol each)

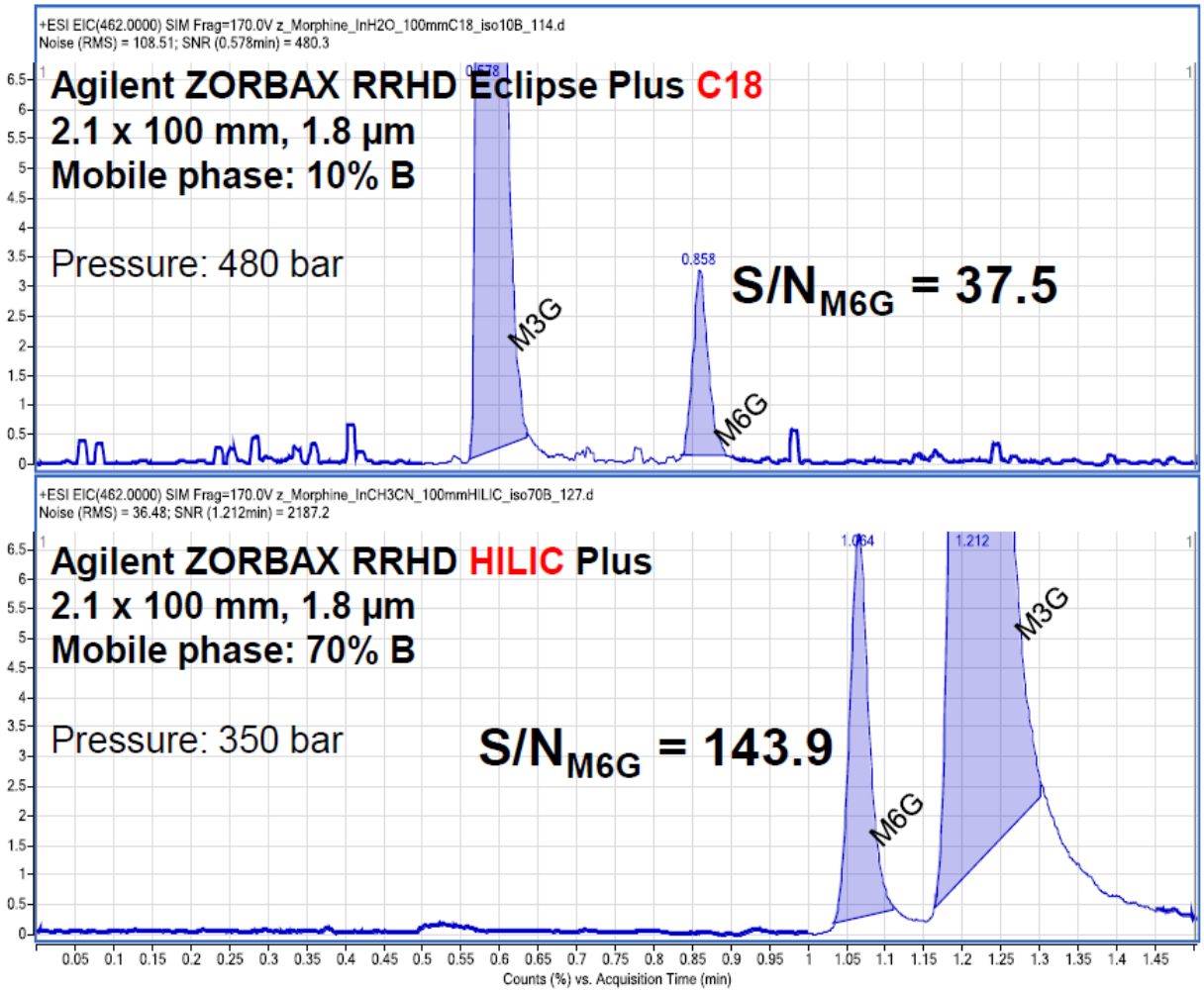
B **Basic Conditions:** **A: 10 mM NH_4OH in water** **B: 10 mM NH_4OH in 80% ACN**



Ion Pair Chromatography and LC/MS

- Mobile phase includes an ion-pair reagent
- Hydrophobic portion adsorbs to stationary phase
- Ionic portion pairs with the analyte
- Alkyl sulfonates or tetraalkyl ammonium salts
- Non-volatile
- Ion pair reagents can interfere with ionization process
- Use heptaflurobutyric acid (HFBA) and tributylamine (TBA)
- HILIC can be an alternative

HILIC vs. Reversed Phase – ESI sensitivity



Agilent 1290 Infinity LC System
Agilent 6410A LC/MS
A: 10 mM ammonium formate pH 3.2
B: acetonitrile / 100 mM ammonium formate pH 3.2 (9:1)
0.4 mL/min
Isocratic elution
Injection Volume: 2 µL
Column: 25 °C
MS: ESI+, SIM, 250 °C, 11 L/min, 30 psi, 4000 V, 200 V delta EMV, 20 ms dwell time
Sample:
Normorphine, m/z 272
Morphine, m/z 286
Morphine-6-β-D-glucuronide (M6G), m/z 462
Morphine-3-β-D-glucuronide (M3G), m/z 462

5991-1242EN

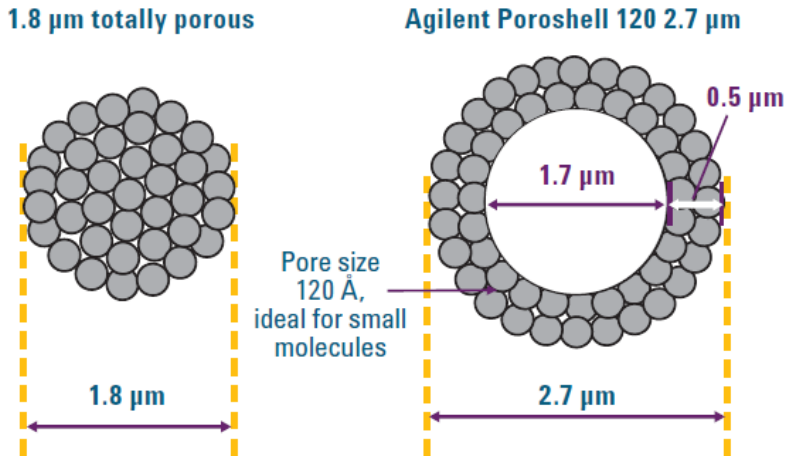
Agilent LC-MS Column Configurations

Column Type	Column I.D.	Typical Flow Rate Range
Analytical	4.6 mm	1 – 1.5 mL/min
Solvent Saver	3.0 mm	0.3 – 1 mL/min
NarrowBore	2.1 mm	0.1 – 0.5 mL/min
MicroBore	1.0 mm	0.03 – 0.2 mL/min
Capillary	0.3, 0.5 mm	2 – 40 µL/min
Nano	0.075, 0.10 mm	0.1 – 0.6 µL/min

Column Choices for LC/MS Analysis

	TFA	Formate/ Formic Acid	Acetate/ Acetic Acid	Ammonium Hydroxide
Eclipse Plus	✓	✓	✓	x
StableBond	✓	✓	✓	x
Eclipse XDB	✓	✓	✓	x
Bonus-RP	✓	✓	✓	x
Extend-C18	✓	✓	✓	✓
HILIC Plus	✓	✓	✓	x

Poroshell 120 Phases



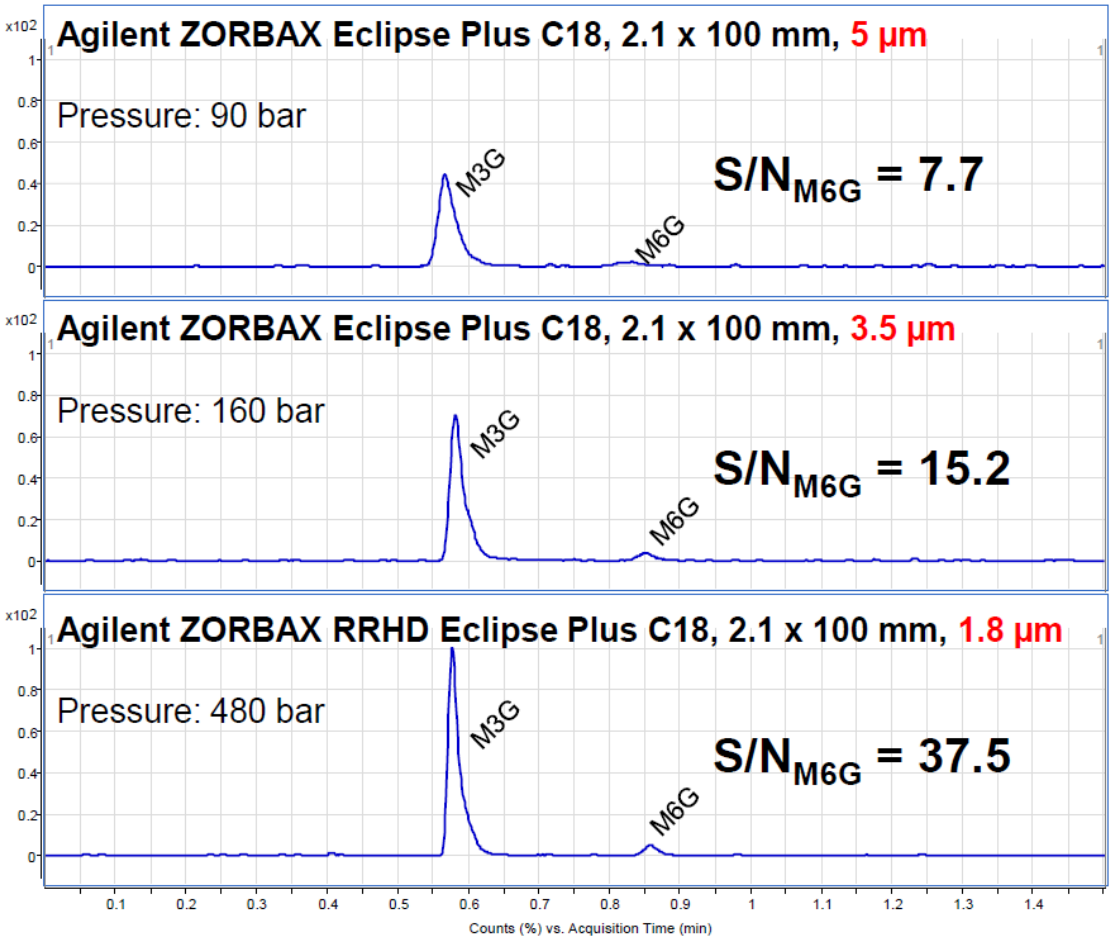
Superficially porous microparticulate column packing

Poroshell 120 particles have a 1.7 µm solid silica core with a 0.5 µm porous outer layer to make a 2.7 µm particle. This carefully selected configuration gives you all the performance advantages of sub-2 µm particles with backpressure that is comparable to a sub-3 µm particle.

- EC-C18
- EC-C8
- SB-C18
- SB-C8
- SB-Aq
- Bonus-RP
- Phenyl-Hexyl
- HILIC

5990-8795EN

Particle size and LC/MS performance



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Injection Volume: 2 µL
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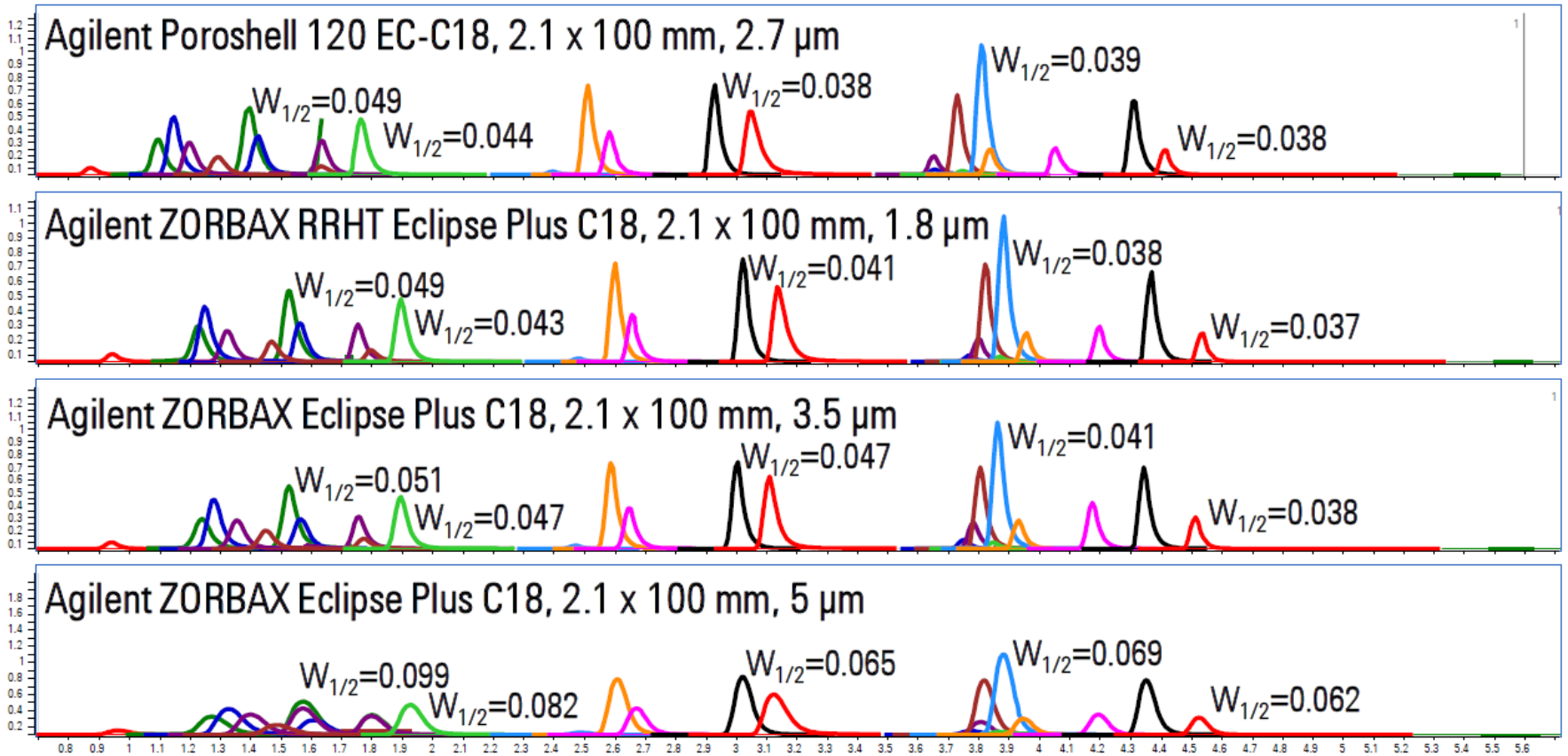
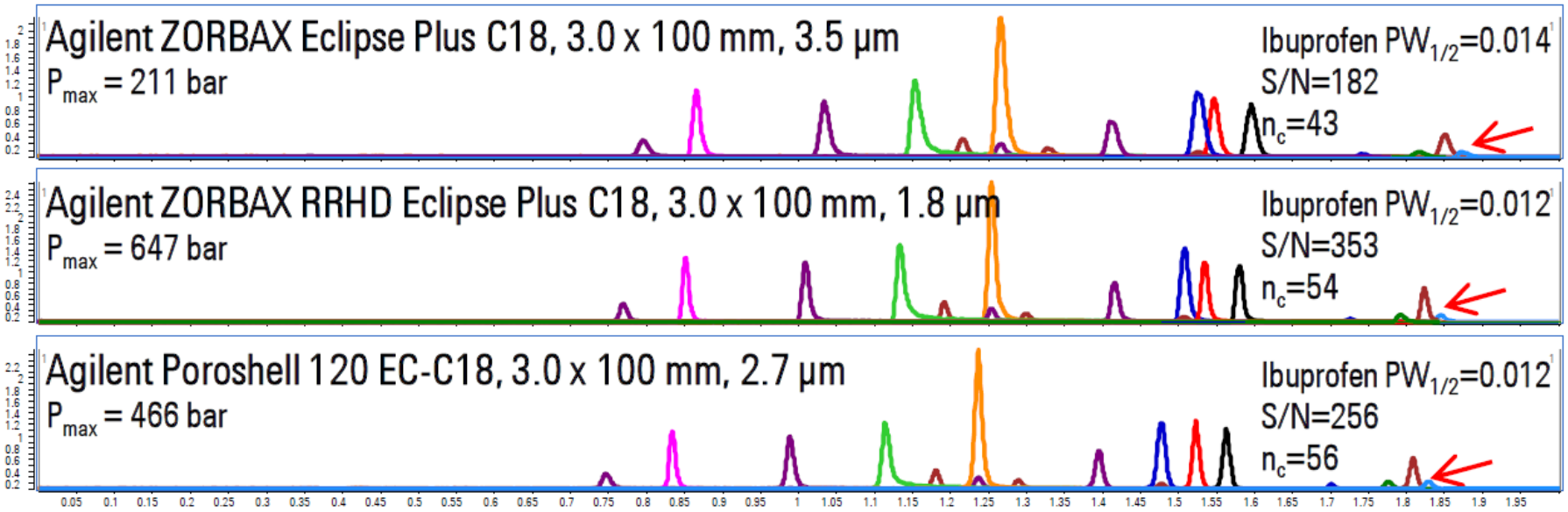
MS: ESI+, SIM, 250 °C, 11 L/min, 30 psi, 4000 V, 200 V delta EMV, 20 ms dwell time
Sample:
Morphine-6-β-D-glucuronide (M6G), m/z 462
Morphine-3-β-D-glucuronide (M3G), m/z 462

Particle Size and LC/MS performance

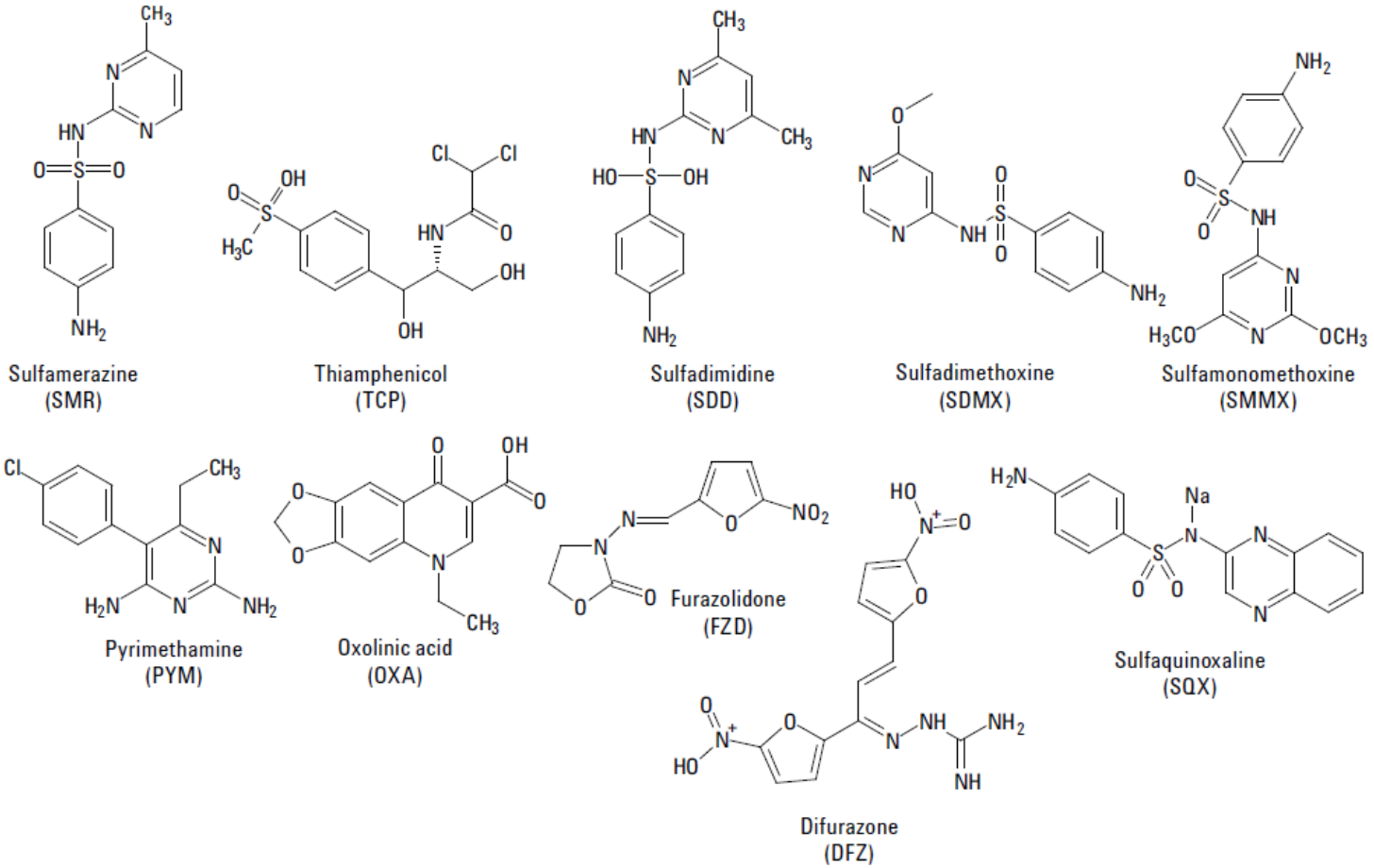


Agilent 1200 Series RRLC / 6410A Triple Quadrupole MS; Ammonium Formate pH 6.7 / Acetonitrile Gradient, 0.5 mL/min; ESI+, dMRM, 350 °C, 12 L/min, 30 psi, 2000 V; Agilent Publication 5990-6345EN

Particle Size and LC/MS performance



Transfer of Existing Method for Antibiotics



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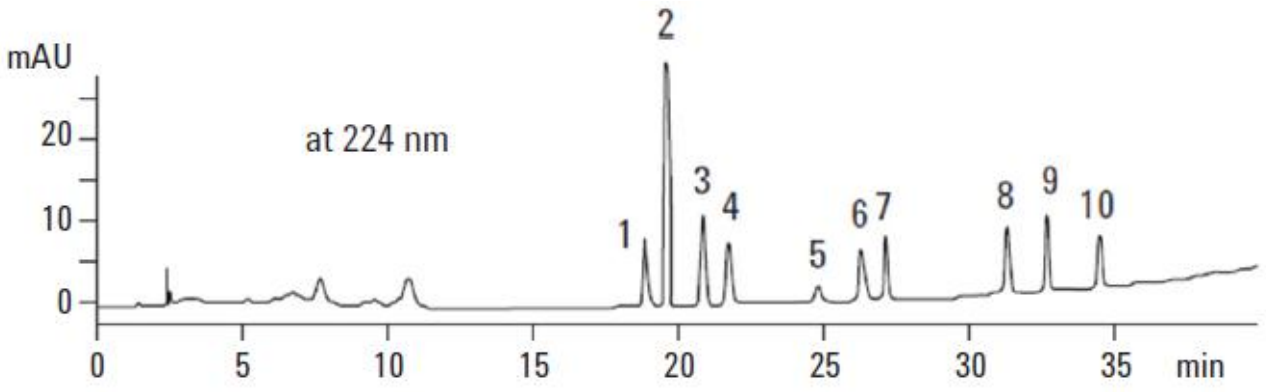
Transfer of Existing Method for Antibiotics



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

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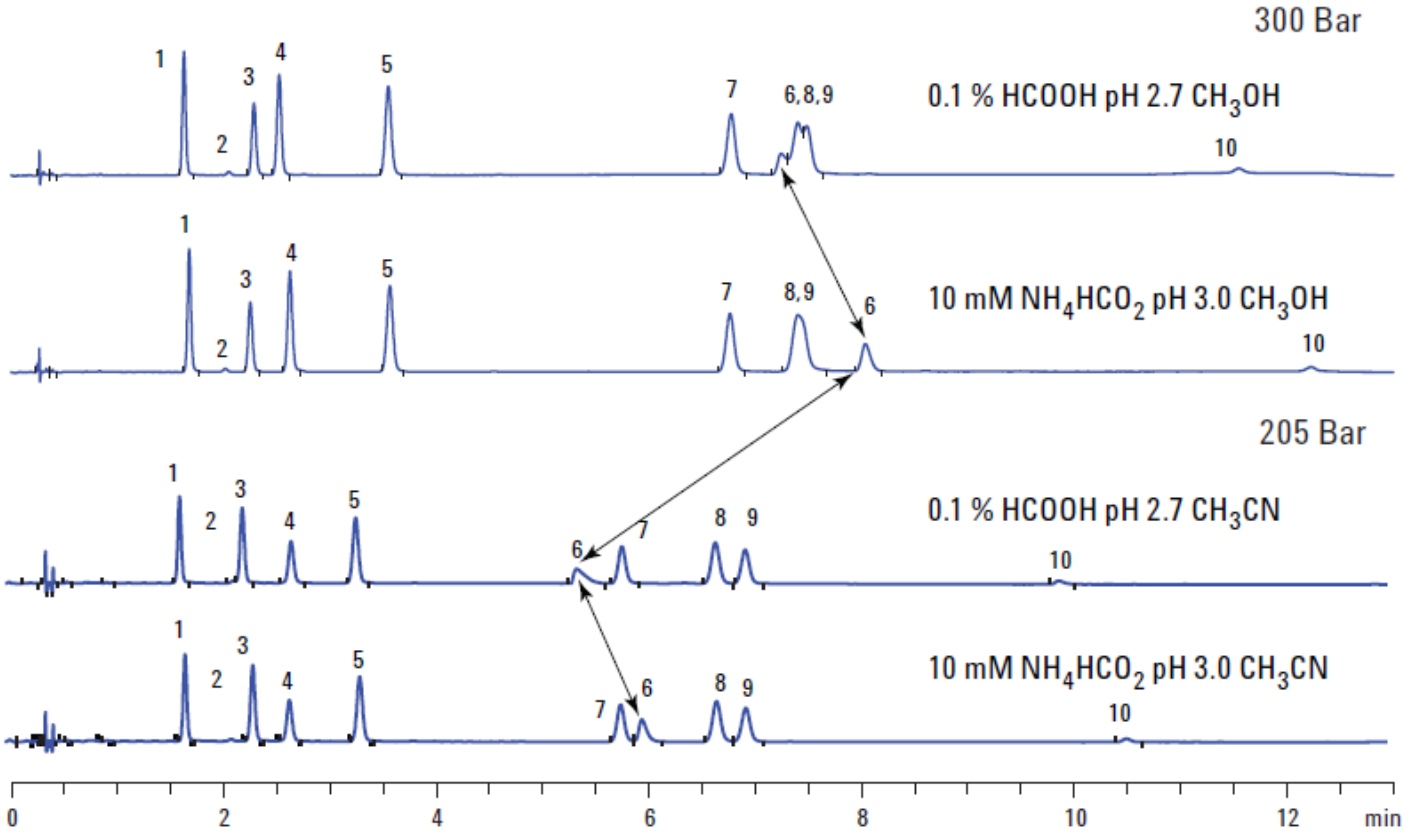
Transfer of Existing Method for Antibiotics



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

Column Temperature: 25°C; Detection: DAD 270 nm, 8 nm, ref off 3 mm, 2 uL micro flow cell; Peak width >0.05 min. (40Hz)

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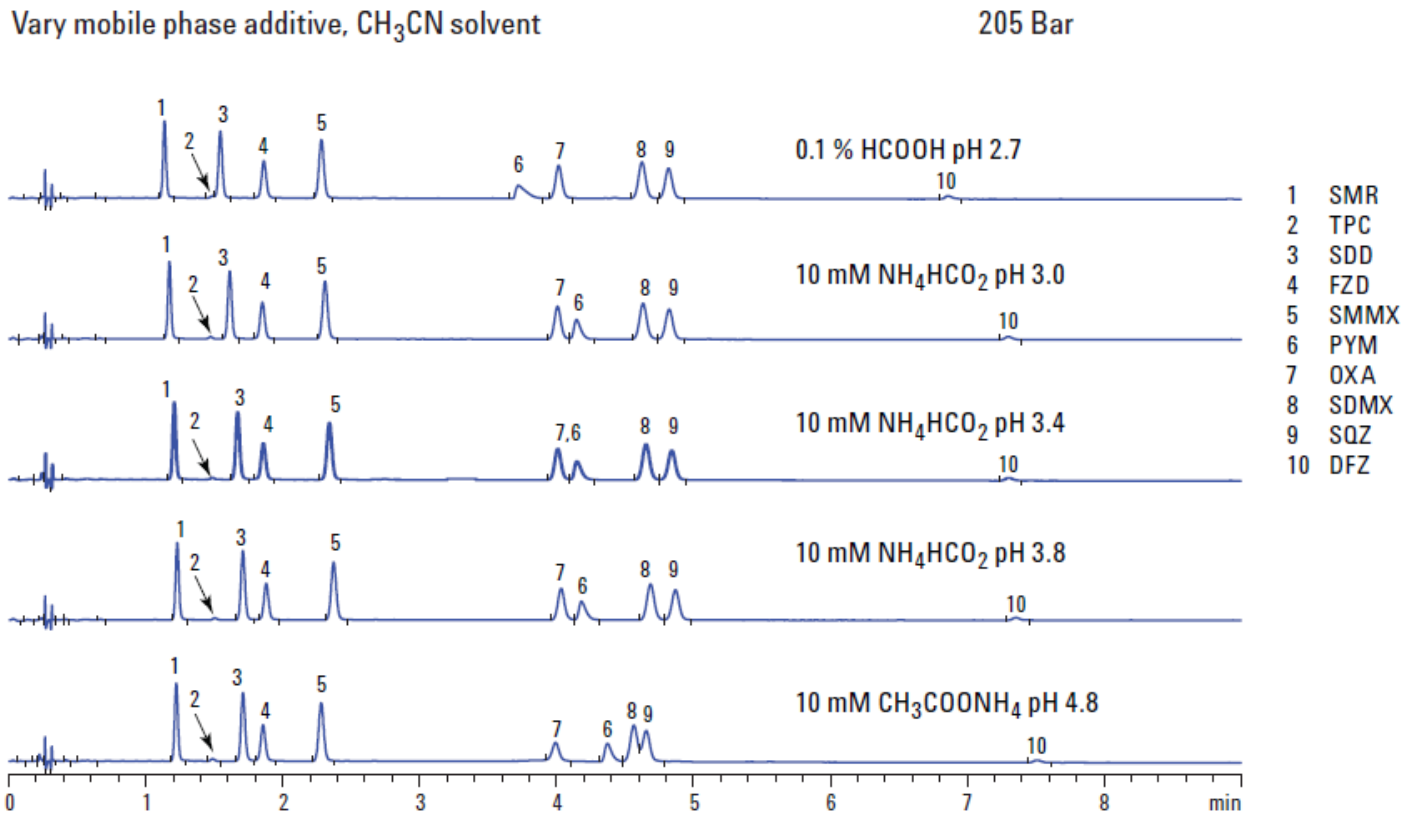
Transfer of Existing Method for Antibiotics



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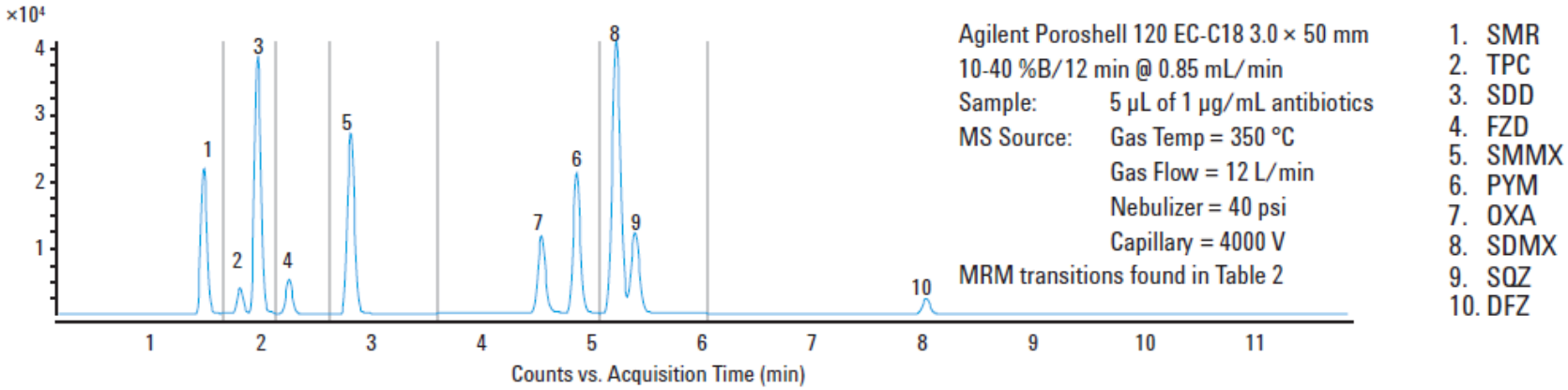
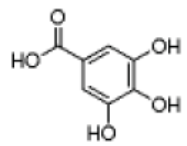


A = 10 mM NH_4HCO_2 pH 3.8
B = acetonitrile

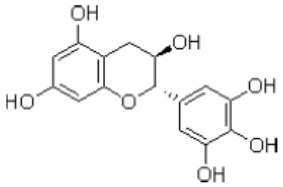
- Conditions were scaled for a 3.0 x 50 mm column
- Shows that 3.0 mm can easily be used for conventional UV and MS detection

5990-6238EN

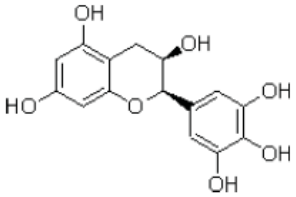
Analysis of Ten Compounds Found in Green Tea



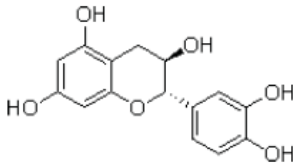
Gallic Acid
(GA)
m/z = 169



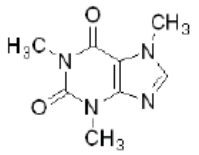
Gallocatechin
(GC)
m/z = 305



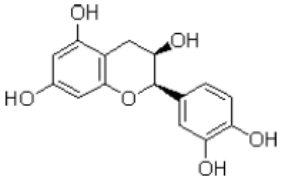
Epigallocatechin
(EGC)
m/z = 305



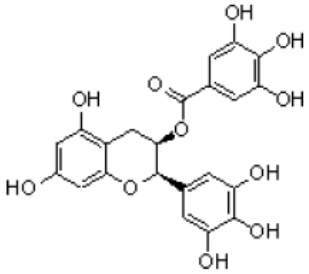
Catechin
(C)
m/z = 289



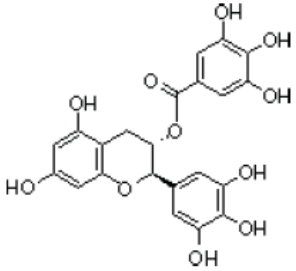
Caffeine
(Caf)
m/z = 195



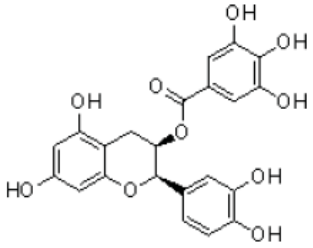
Epicatechin
(EC)
m/z = 289



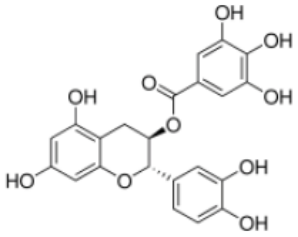
Epigallocatechin Gallate
(EGCG)
m/z = 457



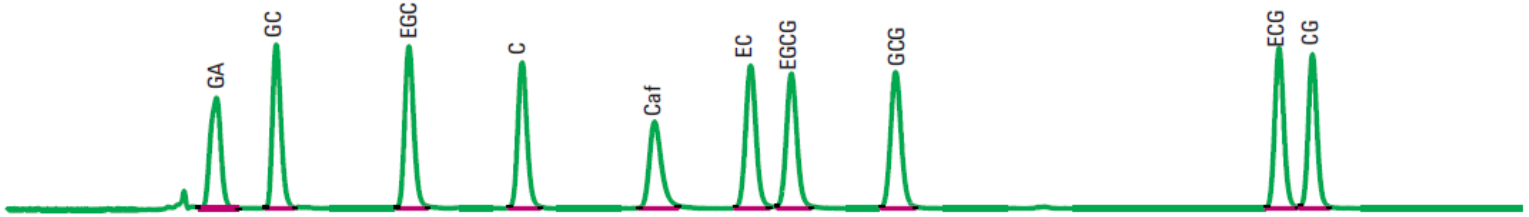
Gallocatechin Gallate
(GCG)
m/z = 457



Epicatechin Gallate
(ECG)
m/z = 441

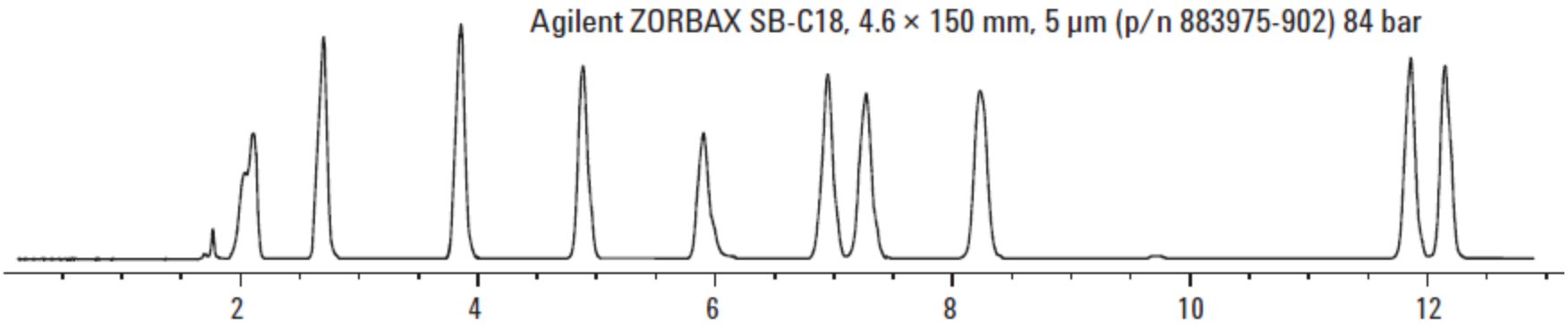


Catechin Gallate
(CG)
m/z = 441



5990-7824EN

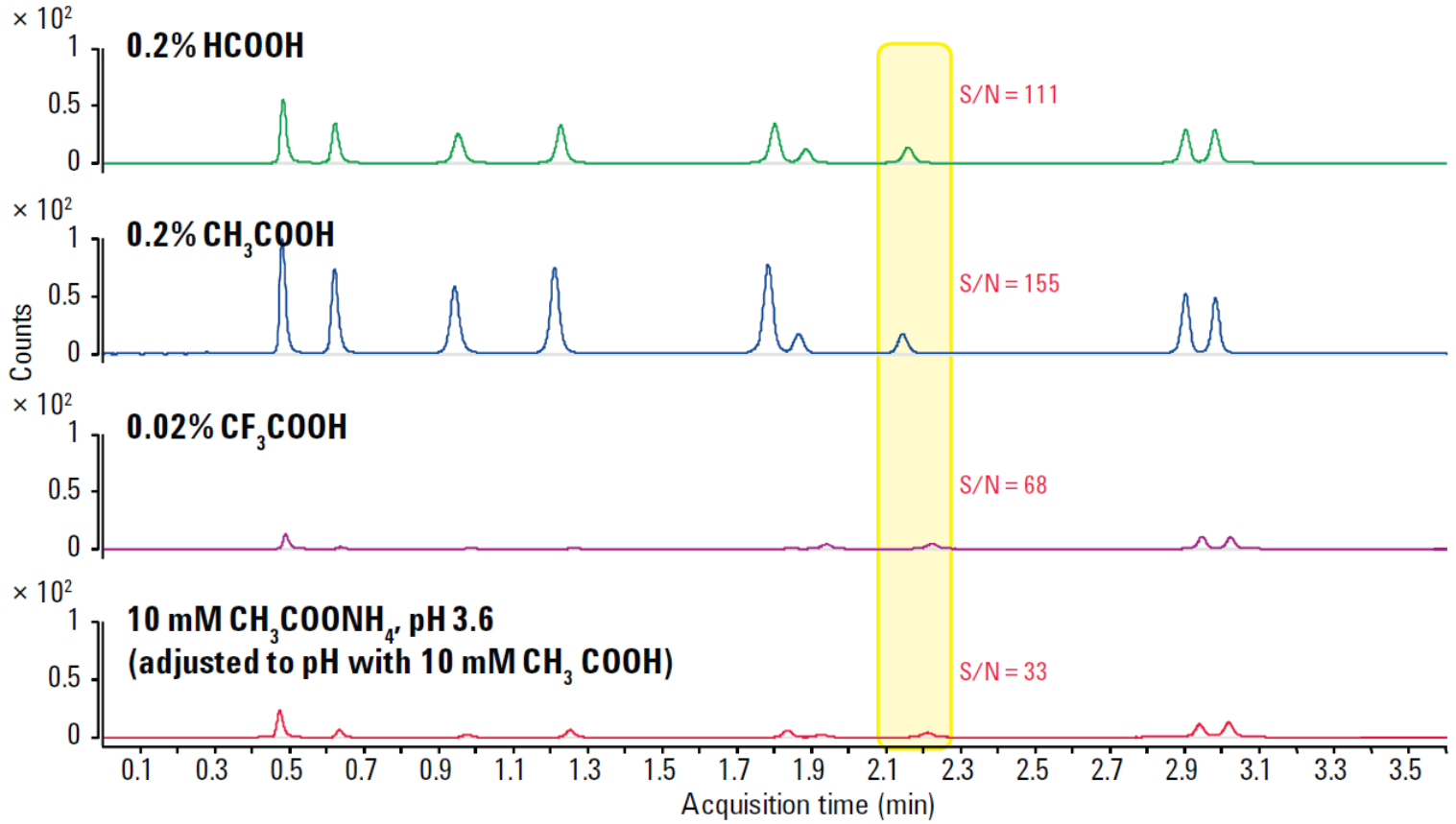
Analysis of Ten Compounds Found in Green Tea



A = 0.1% H3PO4 in H2O
B = CH3CN
1 mL/min
40 °C
Sig = 210,4 nm, Ref = Off
2-µL, 3-mm micro flow cell
Sample: 0.03 mg/mL each in H2O/CH3CN
4.6 × 150 mm Zorbax SB-C18, 5 µm
0.0 min, 10% B; 7.5 min, 15% B; 15 min, 27% B
15 µL injection

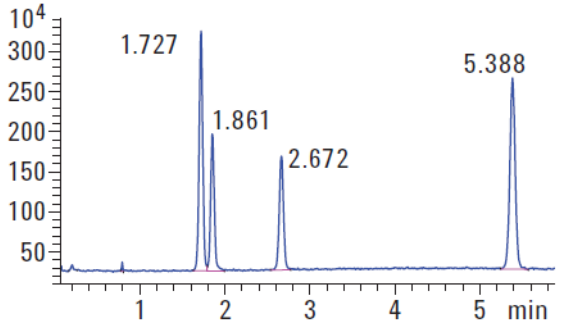
5990-7824EN

Optimizing Sensitivity with Mobile Phase Selection

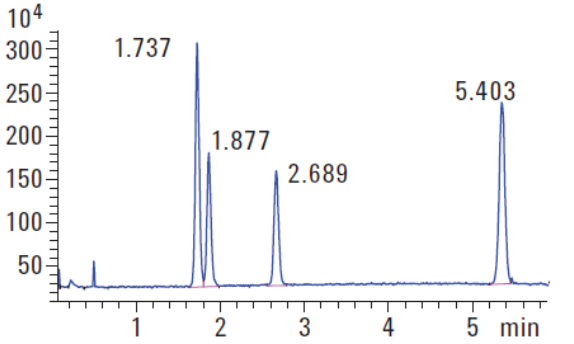


Agilent 1200 Series RRLC / 6410A Triple Quadrupole MS;
Poroshell 120 SB-C18, 2.1 x 100 mm; Acidified Water / Acetonitrile Gradient, 0.7 mL/min;
ESI-, SIM, 350 °C, 10 L/min, 50 psi, -3500 V

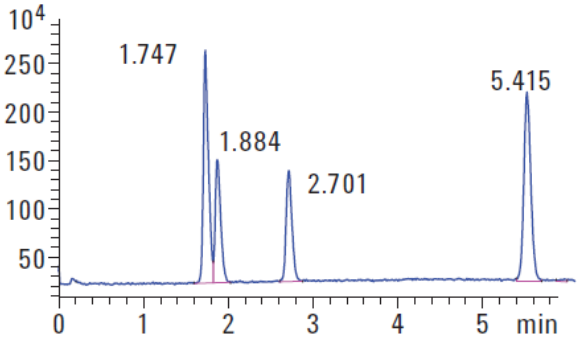
Extra Column Volume and Sensitivity



MS connection: 50 cm, 0.13 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.727	0.046	2.984	8.691
2	1.861	0.046	1.694	4.843
3	2.672	0.055	1.422	5.007
4	5.388	0.069	2.389	10.571

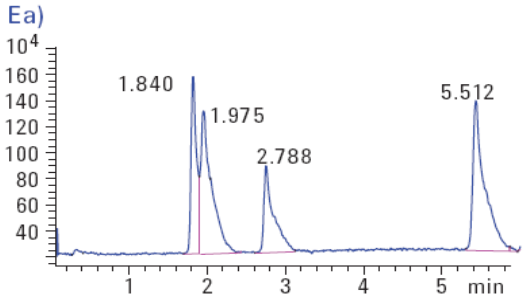


MS connection: 50 cm, 0.18 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.737	0.048	2.805	8.963
2	1.877	0.051	1.507	4.947
3	2.689	0.059	1.329	5.144
4	5.403	0.074	2.097	10.070



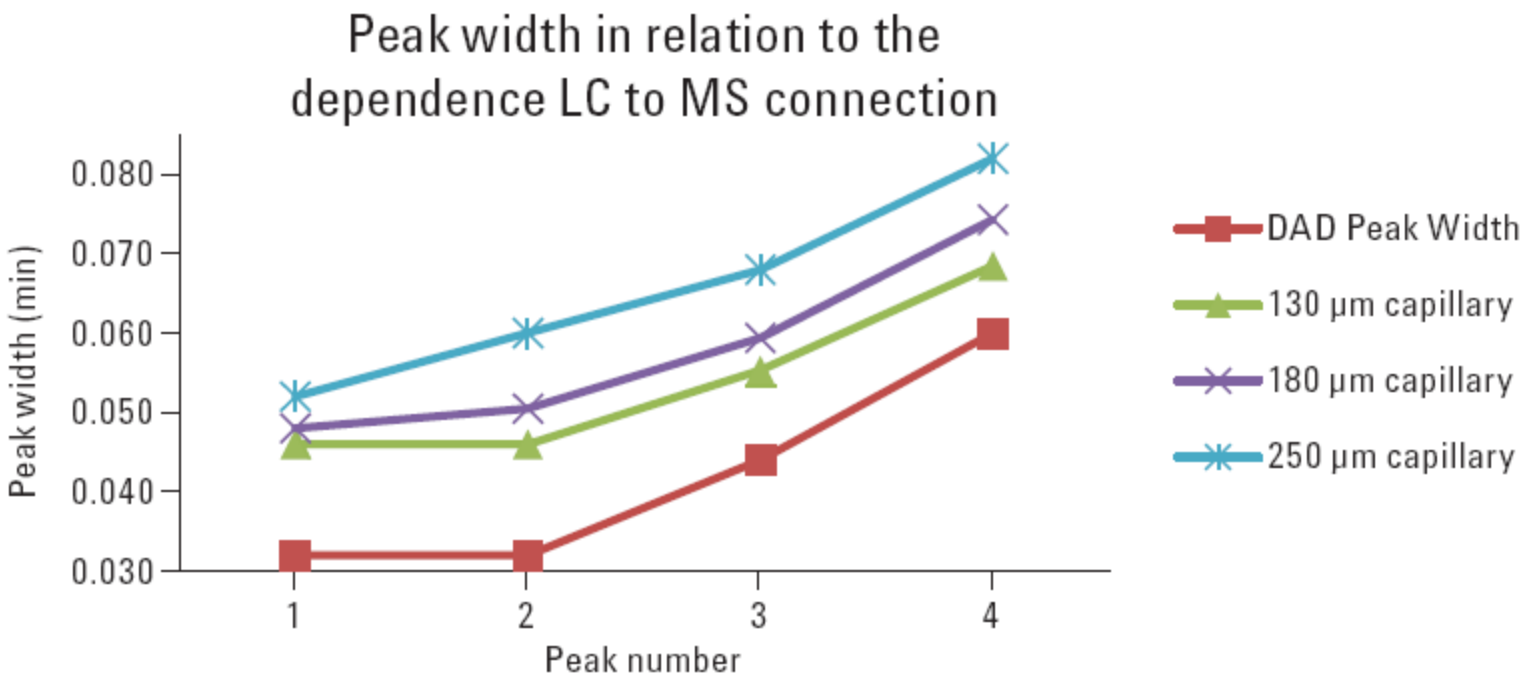
MS connection: 50 cm, 0.25 mm id - MS TIC				
Peak	RT (min)	Width (min)	Height (× 10^6)	Area (× 10^6)
1	1.747	0.051	2.310	8.539
2	1.884	0.061	1.112	4.407
3	2.701	0.068	1.149	5.295
4	5.415	0.082	1.936	10.570

Agilent 1290 Infinity Binary LC system containing 6140 Single Quad MS
Column: Agilent ZORBAX SB C18, 50 × 2.1 mm, 1.8 µm
Solvent A: Water + 0.1% formic acid; Solvent B: Acetonitrile + 0.1% formic acid; Flow rate: 0.5 mL/min
Gradient : 0 min 10% B; 5 min 20% B; 5.01 min 95% B; 6 min 95% B
Injection volume: 1 µL; Column temperature: 40 °C; Source: Gas temperature: 350 °C, nebulizer pressure: 45 psi, gas flow: 11 L/min, positive polarity, Scan: 100 – 1000 *m/z*
Sample: Solution of Sulfamethizole (first peak, *m/z 271.0), Sulfamethazine* (second peak, *m/z 279.0), Sulfachloropyridazine (third peak, m/z 285.0), Sulfadimethoxine (fourth peak, m/z 311.0) each at a* concentration of 100 ng/µL.

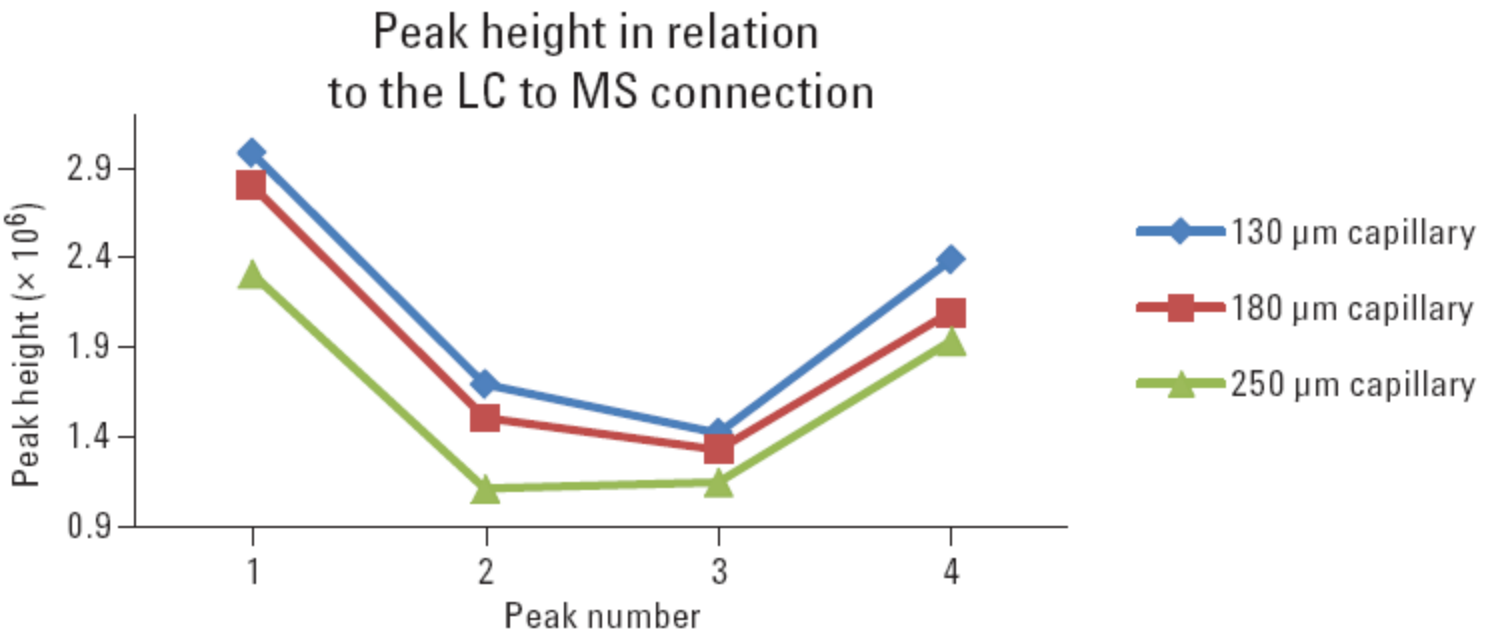


50 cm x 0.5 mm id

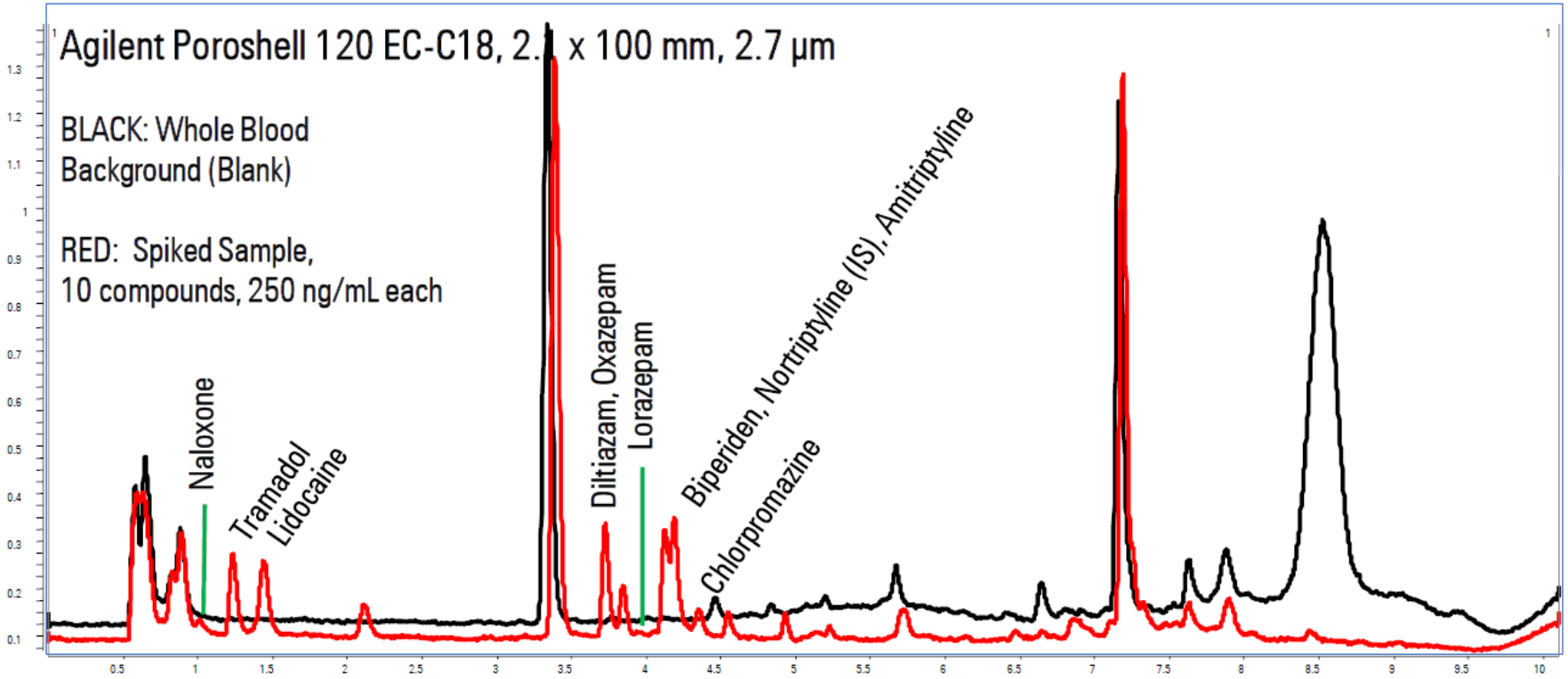
Extra Column Volume and Sensitivity



Extra Column Volume and Sensitivity



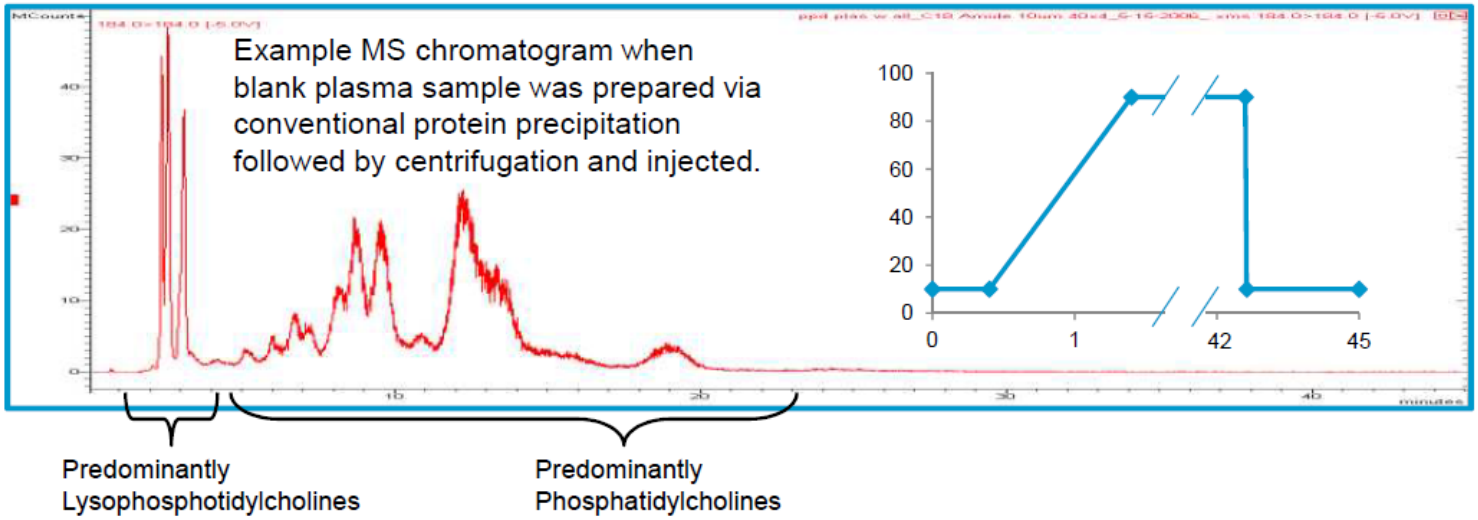
Avoiding Interferences and Ion Suppression

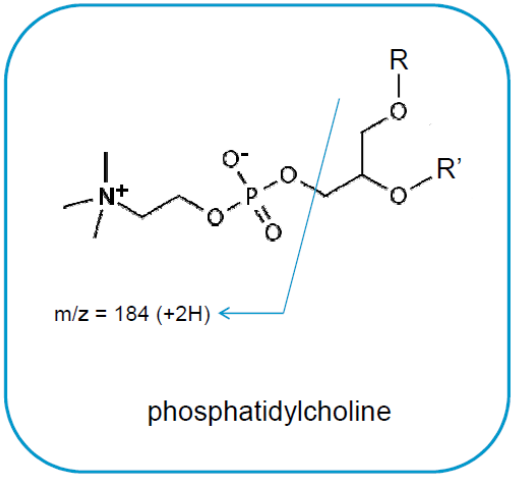


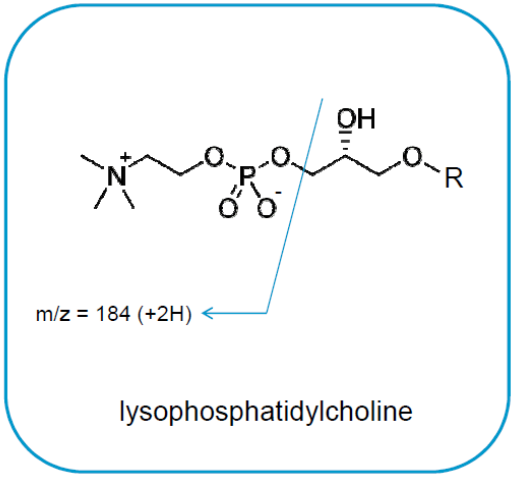
Agilent 1200 Series RRLC / 6460A Triple Quadrupole MS; Ammonium Acetate pH 5 / Acetonitrile Gradient, 0.4 mL/min; ESI+, Scan 100-800, 400 °C, 12 L/min, 40 psi, 3500 V;

5990-8623EN

Interferences and Sample Prep

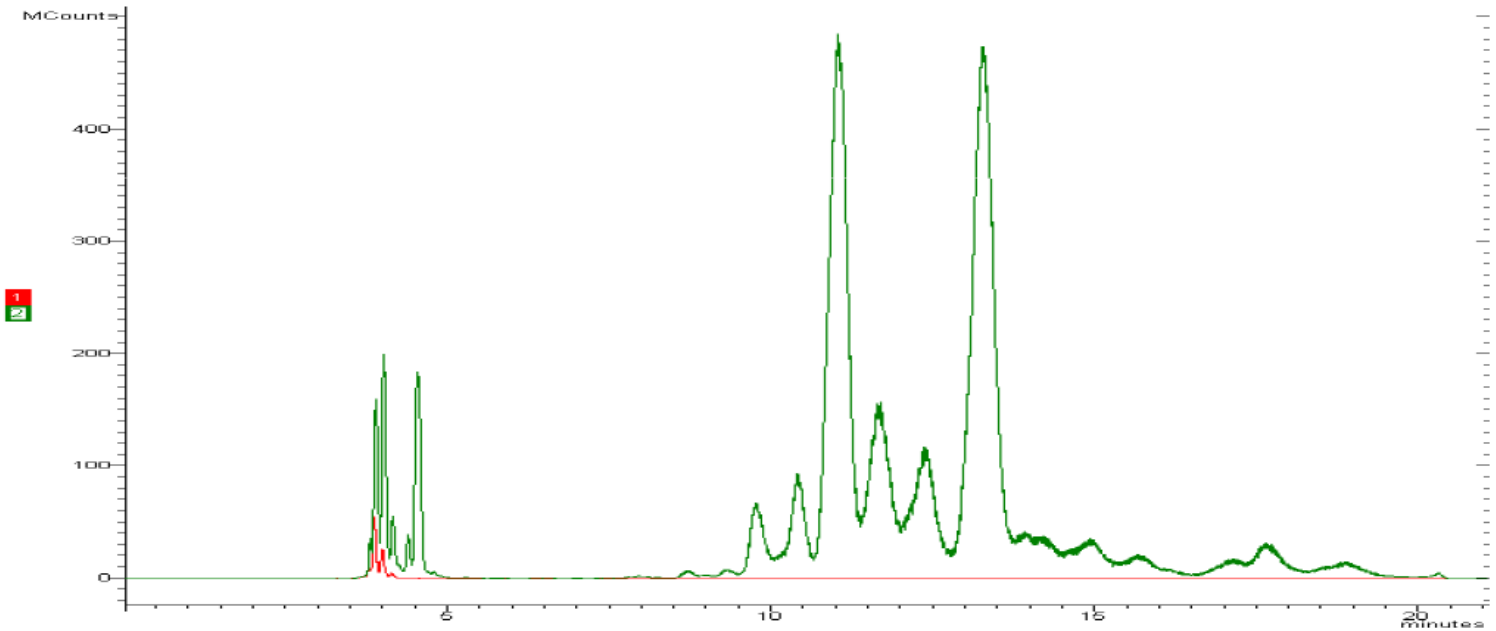






m/z 184

Interferences and Sample Prep



MS Transition 184 → 184

- Green = precipitate only
- Red = precipitate that has been lipid-stripped with CaptivaND*Lipids*

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Preventing Instrument Down Time

Clean Source

After SPE

After PPT

Summary

- ESI is most common atmospheric pressure ionization technique
- APCI and APPI for less polar molecules that do not ionize well by ESI
- Flow rate and mobile phase buffer selection are important for best LC/MS performance
- Importance of choosing column ID and phase for the best results
- Column particle size and efficiency can avoid interferences and maximize sensitivity
- Removing interferences when avoiding them is not enough

Contact Tech Support

1-800-227-9770

lc-column-support@agilent.com