Fully Using Agilent High Efficiency Columns with LC/MS

Technical Overview

Abstract

Advancements in liquid chromatography, such as UHPLC and LC/MS, require that special care is taken when optimizing method parameters. Various applications will show the effects of different mobile phases using MS detection with respect to ion suppression, and how particle size and data collection rates affect MS performance, with consideration to both sensitivity and peak capacity.

Introduction

New columns with smaller particles sizes, including both sub 2-µm and superficially porous 2–3 µm columns, improve LC productivity by providing more resolution and more analysis speed. The same types of columns can be used for LC/MS applications. Many questions arise on the compatibility of these columns with LC/MS and the options for increasing productivity. The first questions focus on adequate detector speed with the MS, as well as how fast and how many compounds can be adequately resolved for the MS. Additional questions focus on obtaining good peak shape with LC/MS compatible mobile phases. Various application examples will be used to compare columns with different particle sizes and demonstrate that adequate detector speed is available to take advantage of the high efficiency possible with these columns. Additional considerations around mobile phase and peak shape for optimal LC/MS results will be shown.

Agilent Technologies

Experimental

An Agilent 1200 Rapid Resolution LC (RRLC) System, an Agilent 1290 Infinity LC System, and an Agilent G6410A Triple Quadrupole Mass Spectrometer were used in this experiment. The MS was used in a variety of modes (including Scan, SIM, MRM) to illustrate the detection improvements possible over a range of analytical methods utilizing MS detection. All connecting capillaries had a small 0.12 mm id in the shortest possible lengths to ensure minimal loss of efficiency through extra column volume and sample band broadening. Detailed method parameters are shown with their respective chromatograms.

Conditional peak capacity will be used to evaluate the different method parameters. Peak capacity is the number of peaks that can be theoretically separated over a gradient time, see Equation 1.

Equation 1. Conditional Peak Capacity

$$\text{Conditional peak capacity} = n_c = \frac{t_{R,n} - t_{R,1}}{W}$$

$t_{R,n}$ and $t_{R,1}$: Retention times of the last and first eluting peaks

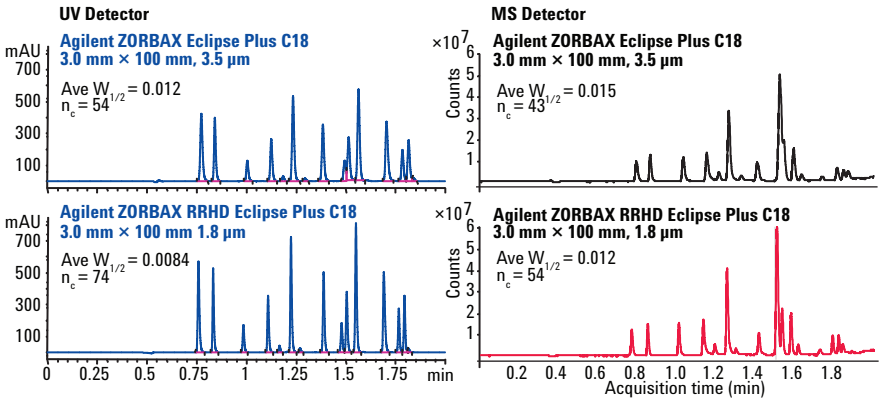
W: $\frac{W_{1/2}}{2.35} \times 4$ (Average 4σ peak width)

$W_{1/2}$: is the average peak width at half height.

Results and Discussion

Different particle sizes with MS detection

A common misconception regarding LC/MS is that small particle columns lose their advantages over slightly larger particle columns because of sample band broadening due to increased extra-column volume with the MS detector. Figure 1 shows that this is not the case. While it is true that some of the small particle's advantages are muted, they still exist. This example shows a separation of 15 analgesic compounds analyzed independently with either UV or MS detection. The detectors are not connected in series, as this would immediately put the MS at a disadvantage due to band broadening that would occur in the diode array detector's flow cell as the sample travels through to the MS source. It is shown that when upgrading from a 3.5 µm column to a 1.8 µm column, with UV detection, a 37% increase in peak capacity is realized, however, only a 26% increase in peak capacity is seen with MS detection. Even though the extra volume in the mass spectrometer neutralizes some of the benefits of smaller particles, there is still a noticeable improvement with the high efficiency column.



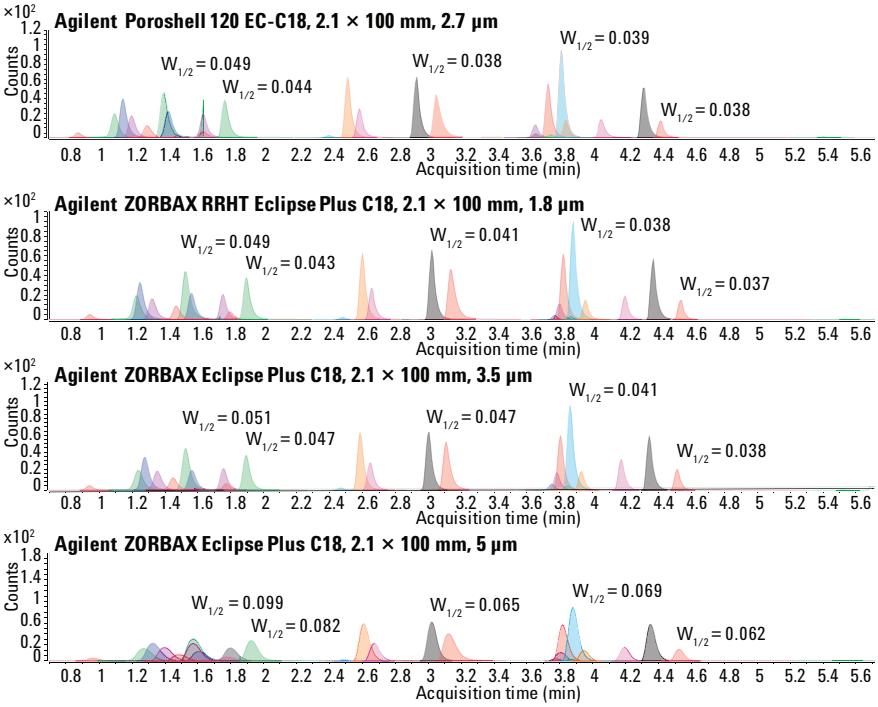
Instrument	Agilent 1290/6410 LC/MS/MS
A	0.2% formic acid in water
B	acetonitrile
Flow rate	1 mL/min
T	0 1.5
%B	15 95
Stop time	2 min
Post run time	1.5 min
Sample	20 µL injection of 1 µg/mL standard in water
TCC	Ambient, no temperature control (approx 24 °C)
DAD	Sig = 260, 4 nm; Ref = Off
Source	ESI+, 350 °C, 12 L/min, 50 psi, 3500 V
Fragmentor	135 V
Scan	100–400, 5 ms scan time 0.2 amu step, 28.36 cycles/s 35.3 ms/cycle

Compounds (in elution order) with identifying mass:

acetaminophen: *m/z* 109	phenacetin: *m/z* 179	ketoprofen: *m/z* 254
caffeine: *m/z* 194	salicylic acid: *m/z* 120	diflunisal: *m/z* 332
2-acetamidophenol: *m/z* 109	sulindac: *m/z* 356	diclofenac: *m/z* 295
acetanilide: *m/z* 135	piroxicam: *m/z* 332	celecoxib: *m/z* 381
acetylsalicylic acid: *m/z* 120	tolmetin: *m/z* 257	ibuprofen: *m/z* 160

Figure 1. Comparison of 3.5 versus 1.8 µm performance with UV and MS scan detection. Refer to Agilent Publication 5990-8428EN for more information regarding this specific application.

Knowing that the advantages of smaller particles can be seen with MS detection, four different particle sizes are compared in Figure 2 with a complex, 25 compound toxicology analysis using LC/MS/MS. The top chromatogram shows a superficially porous 2.7 µm, while the following three chromatograms show traditional totally porous particles that are 1.8, 3.5, and 5 µm respectively. The three totally porous particles all share the same bonding chemistry, while the superficially porous particles have very similar chemistry; the result is nearly identical selectivity across the four analyses. Immediately, the disadvantages of the 5 µm column with such a complex analysis are evident. With the 5 µm column, all peaks are much more broad than with the three smaller particle sizes, and there is abundant coelution of peaks, particularly with the early eluting compounds. In this toxicology example, the differences among the 2.7, 1.8, and 3.5 µm columns appear more subtle. The 3.5 µm column produces slightly wider peaks across the gradient, while the 2.7 and 1.8 µm columns are more comparable. The larger 2.7 µm superficially porous columns are capable of similar performance to the smaller 1.8 µm totally porous particles due to their narrower particle size distribution and thin porous shells with a short mass transfer distance.



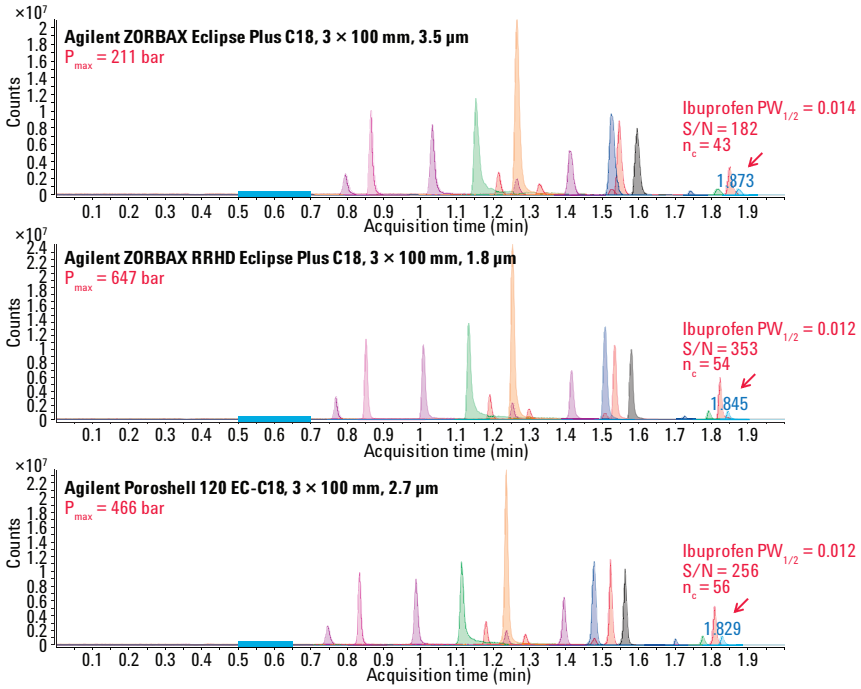
Instrument	Agilent 1200/6410 LC/MS/MS				
A	5 mM ammonium formate w/ 0.01% formic acid				
B	acetonitrile w/ 0.01% formic acid				
Flow rate	0.5 mL/min				
T	0	0.5	3.0	4.0	6.0
%B	10	15	50	95	95
Stop time	6 min				
Post run time	2 min				
Sample	5 µL injection of Agilent LC/MS Toxicology Test Mixture (p/n 5190-0470), diluted 1:10 in water				
TCC	60 °C				
MS	350 °C, 12 L/min, 30 psi, 2000 V, AP-ESI+, dMRM				

Compounds in elution order:

codeine	MDEA	lorazepam
oxycodone	heroin	methadone
amphetamine	cocaine	alprazolam
MDA	meperidine	temazepam
hydrocodone	trazodone	proadifen
methamphetamine	PCP	diazepam
MDMA	oxazepam	THC
strychnine	nitrazepam	
phentermine	verapamil	

Figure 2. Comparison of superficially porous 2.7 µm and totally porous 5, 3.5, and 1.8 µm performance with MS/MS detection. Refer to Agilent Publication 5990-6345EN for more information regarding this specific application.

Figure 3 shows a closer comparison of 2.7, 1.8, and 3.5 µm columns, with a more simplified analysis of 15 common analgesic compounds. Again, the very similar selectivity across the three columns is apparent. This feature of Agilent's LC column family is advantageous for laboratories that require transferability among their methods. As shown by the pressures on the left side of the chromatograms, the 3.5 µm column would be ideal for performing this analysis on a 400 bar HPLC, while the Agilent Poroshell 120 column would work best on a 600 bar instrument, and the 1.8 µm column is beneficial for a > 600 bar UHPLC. Conditional peak capacity on the smaller particle columns is > 20% higher than the 3.5 µm column. Also, sensitivity (signal-to-noise) is greatly improved by more than 40% with the taller sharper peaks on the 1.8 and 2.7 µm columns, as compared to the 3.5 µm.



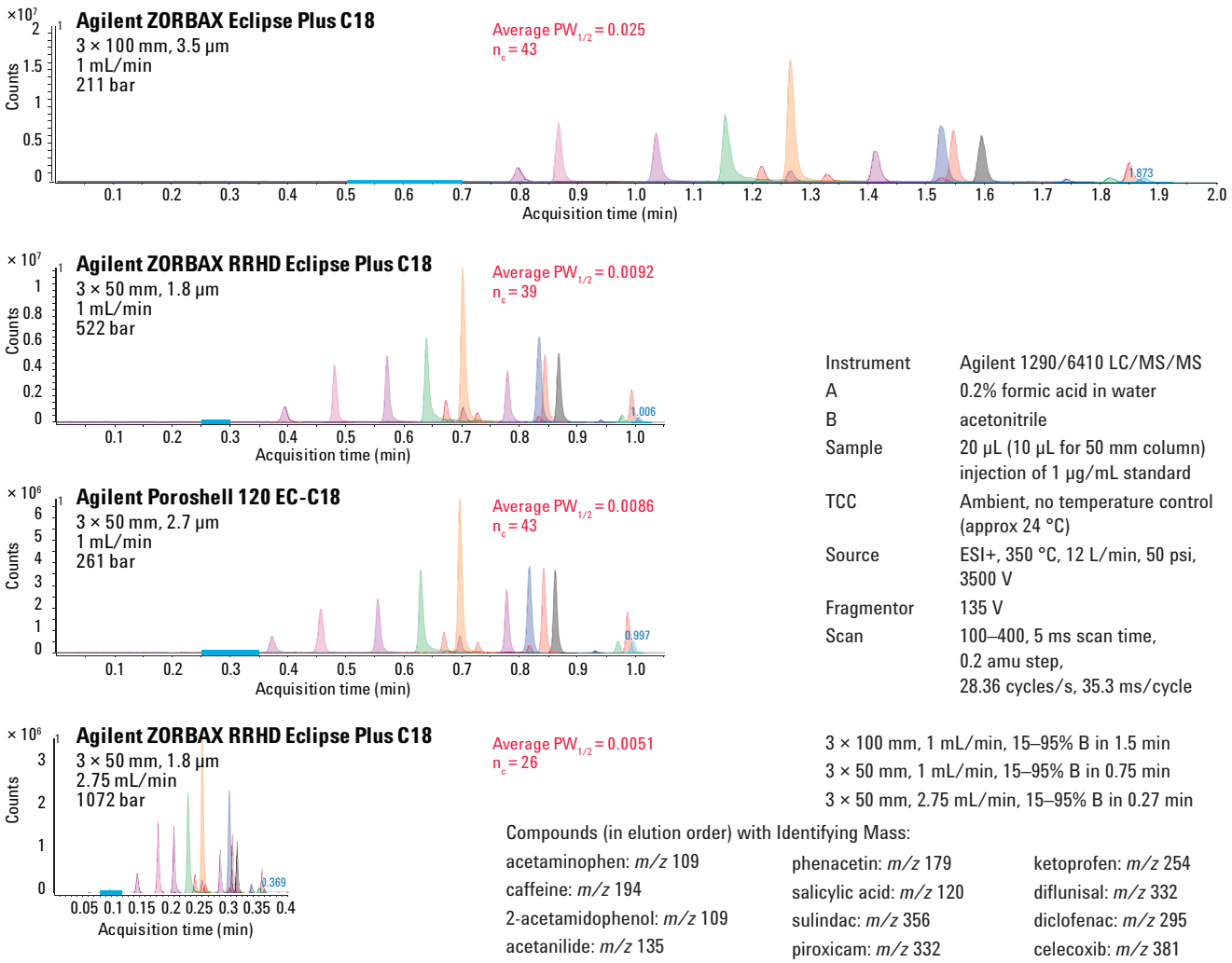
Instrument	Agilent 1290/6410 LC/MS/MS	
A	0.2% formic acid in water	
B	acetonitrile	
Flow rate	1 mL/min	
T	0	1.5
%B	15	95
Stop time	2 min	
Post run time	1.5 min	
Sample	20 µL injection of 1 µg/mL standard in water	
TCC	Ambient, no temperature control (approx 24 °C)	
Source	ESI+, 350 °C, 12 L/min, 50 psi, 3500 V	
Fragmentor	135 V	
Scan	100–400, 5 ms scan time, 0.2 amu step, 28.36 cycles/s, 35.3 ms/cycle	

Compounds (in elution order) with Identifying Mass:

acetaminophen: *m/z* 109	phenacetin: *m/z* 179	ketoprofen: *m/z* 254
caffeine: *m/z* 194	salicylic acid: *m/z* 120	diflunisal: *m/z* 332
2-acetamidophenol: *m/z* 109	sulindac: *m/z* 356	diclofenac: *m/z* 295
acetanilide: *m/z* 135	piroxicam: *m/z* 332	celecoxib: *m/z* 381
acetylsalicylic acid: *m/z* 120	tolmetin: *m/z* 257	ibuprofen: *m/z* 160

Figure 3. Comparison of superficially porous 2.7 µm and totally porous 3.5 and 1.8 µm performance with MS scan detection. Refer to Agilent Publication 5990-8428EN for more information regarding this specific application.

Because the highly efficient 1.8 and 2.7 µm columns outperform the 3.5 µm column by so much, shorter columns may be used, as seen in Figure 4. Comparing 50 mm, 1.8 and 2.7 µm columns to a 100 mm, 3.5 µm column at the same flow rate, results in half the analysis time for the 1.8 and 2.7 µm columns. The result is similar peak capacity for all three analyses. Therefore, the 1.8 and 2.7 µm columns can theoretically separate the same number of peaks in half the time of the 3.5 µm column. Pressure is also noteworthy in this example, as the Poroshell 120, 50 mm column generates similar pressure to the 100 mm, 3.5 µm column, each well under 400 bar, so these analyses could be run on any LC system. When pressure is not an issue, the Agilent ZORBAX RRHD Eclipse Plus C18 50 mm, 1.8 µm column can be pushed to over 1000 bar, resulting in an 80% reduction in analysis time, as compared to the original 100 mm, 3.5 µm analysis. While some peak capacity is lost, and coelution occurs for such a fast analysis, the highly selective MS detector can still manage ample resolving power for these 15 analgesics in 0.4 min. It should be noted that using this G6410A MS with an ESI source at 2.75 mL/min is not considered a good practice. In this example flow rate was increased to demonstrate ample scan rate by the MS under extreme conditions, however, this is not a practice that should be used with this MS. Newer technologies like the Agilent Jet Stream Thermal Gradient Focusing Technology with ESI source can be used to extend the flow rate range beyond 2 mL/min without deleterious effects on the analysis. For more information refer to Agilent Publication 5990-3494EN.



Instrument	Agilent 1290/6410 LC/MS/MS
A	0.2% formic acid in water
B	acetonitrile
Sample	20 µL (10 µL for 50 mm column) injection of 1 µg/mL standard
TCC	Ambient, no temperature control (approx 24 °C)
Source	ESI+, 350 °C, 12 L/min, 50 psi, 3500 V
Fragmentor	135 V
Scan	100–400, 5 ms scan time, 0.2 amu step, 28.36 cycles/s, 35.3 ms/cycle

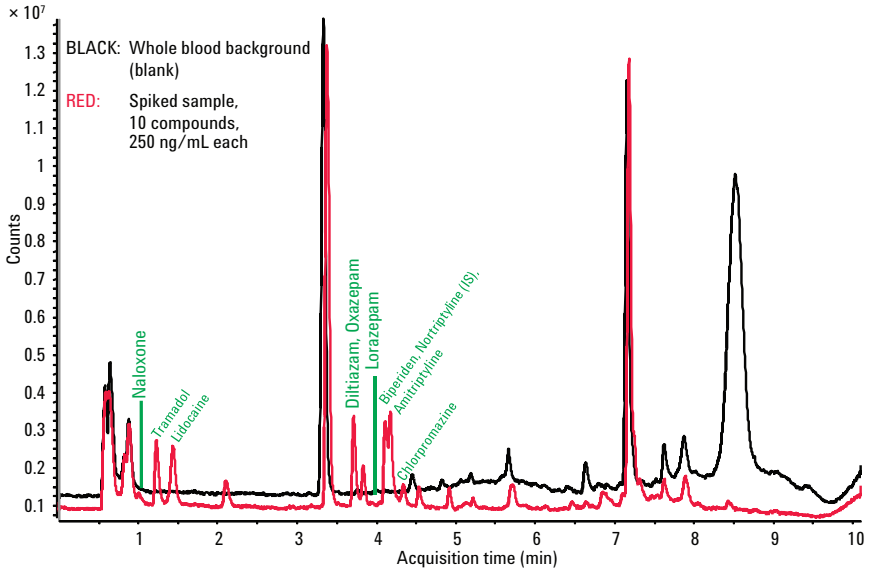
3 × 100 mm, 1 mL/min, 15–95% B in 1.5 min
3 × 50 mm, 1 mL/min, 15–95% B in 0.75 min
3 × 50 mm, 2.75 mL/min, 15–95% B in 0.27 min

Compounds (in elution order) with Identifying Mass:

acetaminophen: *m/z* 109	phenacetin: *m/z* 179	ketoprofen: *m/z* 254
caffeine: *m/z* 194	salicylic acid: *m/z* 120	diflunisal: *m/z* 332
2-acetamidophenol: *m/z* 109	sulindac: *m/z* 356	diclofenac: *m/z* 295
acetanilide: *m/z* 135	piroxicam: *m/z* 332	celecoxib: *m/z* 381
acetylsalicylic acid: *m/z* 120	tolmetin: *m/z* 257	ibuprofen: *m/z* 160

Figure 4. Example of high speed analysis possible with superficially porous 2.7 µm and totally porous 1.8 µm versus 3.5 µm with MS detection. Refer to Agilent Publication 5990-8428EN for more information regarding this specific application.

A practical example of what better peak capacity can do for an analysis is illustrated in Figure 5. Here is an overlay of a blank whole blood extract with a spiked whole blood extract. Because the Agilent Poroshell 120 column produces such sharp peaks and consequently high peak capacity, it can separate the 10 analytes from the background peaks in the sample matrix. Occasionally, coeluting peaks can suppress the signal of one or both of the compounds, therefore the best protection against this ion suppression is to separate all peaks chromatographically.



Instrument:	Agilent 1200/6460 LC/MS/MS		
A	5 mM Ammonium acetate, pH 5; 20:80 Methanol: Water		
B	5 mM Ammonium Acetate in Acetonitrile		
Flow rate	0.4 mL/min		
T	0	5.5	7.5
%B	20	75	75
Sample	10 µL injection of whole blood extract		
Column	Agilent Poroshell 120 EC-C18 2.1 × 100 mm, 2.7 µm		
Source	400 °C, 12 L/min, 40 psi, 3500 V		
Fragmentor	80 V		
MS Scan mass range	100–800		
Scan time	20 sec		
ESI	Positive polarity		

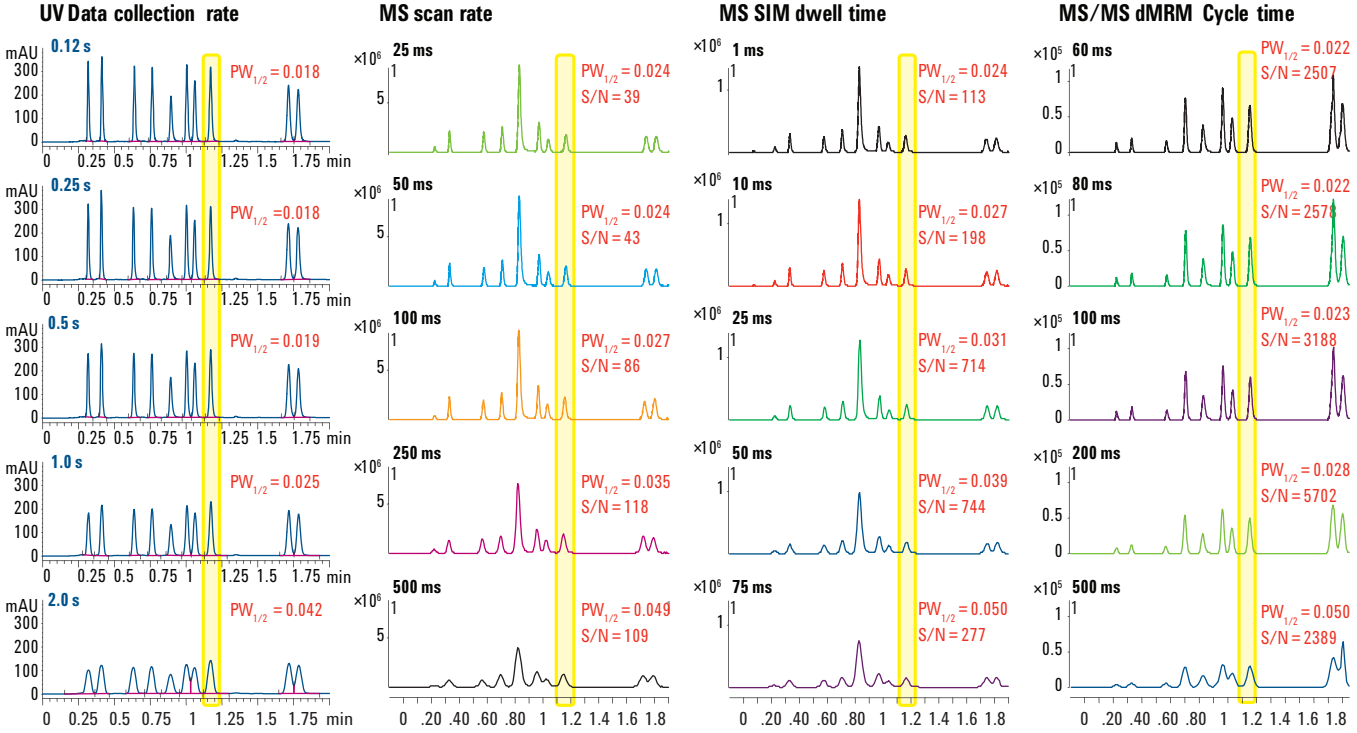
QuEChERS extraction of whole blood:

A 1-mL aliquot of whole blood was added to a centrifuge tube and spiked with appropriate volume from a concentrated stock mixture to yield 250 ng/mL of the component mix, added 20 µL of IS stock solution (nortriptyline), two ceramic homogenizers, then vortexed. Then 2 mL of acetonitrile solutions (with or without acid) was added and vortexed. A premixed amount of the extraction salts was added and vigorously shaken, centrifuged at 5000 rpm for 5 min. One milliliter of the extract was transferred into a d-SPE tube (2 mL centrifuge tube) containing 50 mg of PSA and 100 mg of $MgSO_4$ for matrix cleanup; vortexed for 1 min and centrifuged at 18,000 rpm for 3 min. A 200 µL aliquot of the extract was transferred into a LC vial containing 800 µL of water, vortexed and analyzed.

Figure 5. Separation of analytes from whole blood sample matrix background with a highly efficient superficially porous 2.7 µm column and MS scan detection.

Optimizing data collection rates with MS to balance sensitivity and peak capacity

The tea analysis in Figure 6, shown on an Agilent ZORBAX RRHD SB-C18 column illustrates the effects of various data collection rates on chromatographic quality, including UV, MS scan, MS SIM, and MS/MS detection modes. Using Equation 1, the conditional peak capacity of this analysis is compared alongside signal-to-noise calculations for each chromatogram. In all cases, the same pattern can be seen. The mass spectrometer has more than enough speed for data collection than it needs for this analysis, to the point where negative effects are shown with faster data collection rates when too many data points are collected. The fastest data collection rates generate the narrowest peaks, resulting in the highest peak capacity; however, the fastest data collection rates also generate the most baseline noise, which decreases the signal-to-noise ratio. When sensitivity is the most important factor of an analysis, a slightly slower data collection rate should be used; though this will decrease the conditional peak capacity. Additionally regarding sensitivity is the 50X improvement in signal-to-noise when comparing MS scan to MS/MS detection.



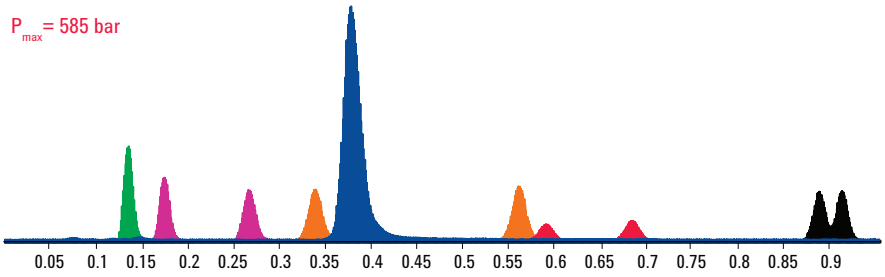
Instrument	Agilent 1290/6410 LC/MS/MS		
A	0.2% acetic acid in water		
B	acetonitrile		
Flow rate	1 mL/min		
T	0	0.5	2
%B	10	15	27
Stop time	2 min		
Post run time	2 min		

Sample	1 µL injection of 6 µg/mL standard in water
TCC	40 °C
Column	Agilent ZORBAX RRHD SB-C18 2.1 × 100 mm, 1.8 µm
DAD	Sig = 210, 4nm; Ref = Off
Source	ESI+, 350 °C, 10 L/min, 50 psi, 3500 V
MS, Pos SIM	171, 307, 195, 291, 459, 443

dMRM transitions (Fragmentor = 135 V)	
Gallic acid	171 → 109, 127 (CE=5)
(Epi)gallocatechin	307 → 139, 121 (CE=50)
(Epi)catechin	291 → 139,123 (CE=15)
Caffeine	195 → 138, 110 (CE=30)
(Epi)gallocatechin gallate	459 → 139, 289 (CE=5)
(Epi)catechin gallate	443 → 139, 123 (CE=40)

Figure 6. Comparison of detector data collection rates with UV, MS scan, MS SIM and MS/MS with a 1.8 µm column. Refer to Agilent Publication 5990-7824EN for more information regarding this specific pplication.

Peak capacity and baseline separation are not always the most important factors to mass spectrometrists. However, in analyses like this, there are four epimer pairs that are detected by the same mass unit, which must be baseline separated for accurate, reproducible quantitation; most notably are the last two peaks, epicatechin gallate (ECG) and catechin gallate (CG) which elute closely and share *m/z* 443 as their mass. Figure 7 shows a rapid analysis with more than adequate MS detector speed of catechins in tea where the epimer pairs are shown in matching colors; the black peak pair eluting around 0.9 min is the critical pair of this analysis. With the highly efficient Agilent Poroshell 120 column used for this analysis, all 10 peaks can be baseline separated in 1 min, a feat that would likely not be possible with a slightly larger 3.5 µm column.



Instrument	Agilent 1200/6410 LC/MS/MS		
A	0.2% acetic acid in water		
B	acetonitrile		
Flow rate	1.5 mL/min		
T	0	0.36	0.71
%B	10	15	27
Stop time	1 min		
Post run time	1.5 min		

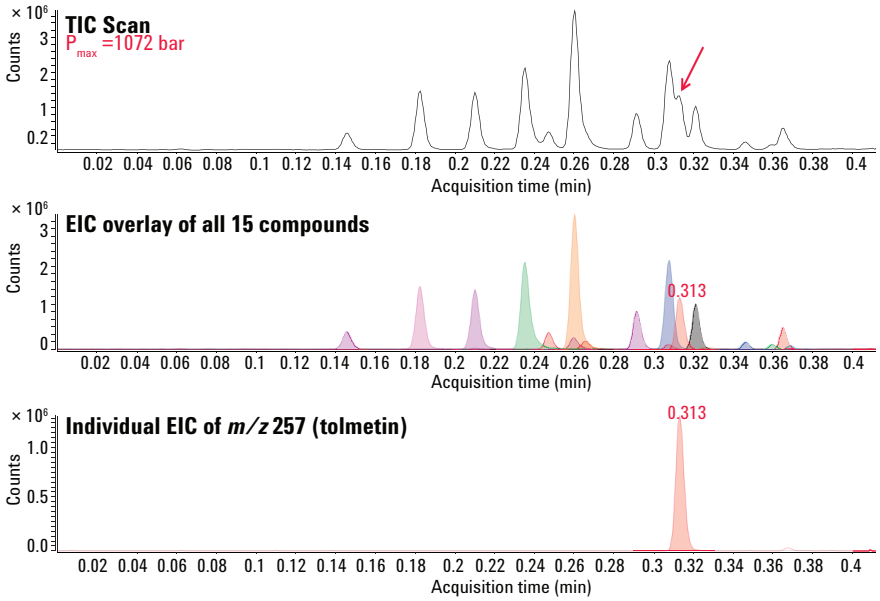
Sample	1.5 µL injection of 3 µg/mL standard in water
TCC	40 °C
Column	Agilent Poroshell 120 SB-C18 2.1 × 50 mm, 2.7 µm
DAD	Sig = 210, 4 nm; Ref = Off
Source	ESI+, 350 °C, 10 L/min, 50 psi, ± 3500 V
Fragmentor	135 V

MS, SIM- (169, 305, 289, 457, 441), SIM+ (195)

Sample:
Gallic acid: *m/z* 171
(Epi)gallocatechin: *m/z* 307
(Epi)catechin: *m/z* 291
Caffeine: *m/z* 195
(Epi)gallocatechin gallate: *m/z* 459
(Epi)catechin gallate: *m/z* 443

Figure 7. Example of fast MS scan analysis possible with a highly efficient superficially porous 2.7 µm column. Refer to Agilent Publication 5990-7824EN for more information regarding this specific application.

Figure 8 shows an ultra fast separation of 15 analgesic compounds in 0.4 minutes on an RRHD Eclipse Plus C18 column. As with Figure 7, the MS is fast enough to collect enough data points across all peaks, even in cases with coeluting peaks. The total ion chromatogram (TIC) on top shows several partially coeluting peaks, particularly around tolmetin, shown with the red arrow. Because each compound is identified by a unique mass, extracted ion chromatograms (EICs) allow the analyst to view each component separately, as seen with just the EIC of *m/z* 257 for tolmetin in the bottom chromatogram. Viewing EICs separately allows for simple integration and quantification of any compound. Again, it should be noted that using this G6410A MS with an ESI source at 2.75 mL/min is not recommended. The flow rate was increased to demonstrate sufficient scan rate by the MS under extreme conditions. When increased throughput is needed, the new Agilent Jet Stream Thermal Gradient Focusing Technology with ESI source can be used to extend the flow rate range beyond 2 mL/min without negatively impacting the analysis. For more information refer to Agilent Publication 5990-3494EN.



Instrument	Agilent 1290/6410 LC/MS/MS			
A	0.2% formic acid in water			
B	acetonitrile			
Flow rate	2.75 mL/min			
T	0	0.27	Source	ESI+, 350 °C, 12 L/min, 50 psi, 3500 V
%B	15	95	Fragmentor	135 V
Stop time	0.4 min		Scan	100–400, 5 ms scan time, 0.2 amu step, 28.36 cycles/s, 35.3 ms/cycle
Post run time	0.27 min			
Sample	10 µL injection of 1 µg/mL standard in water			
TCC	Ambient, no temperature control (approx 24 °C)			
Column	Agilent ZORBAX RRHD Eclipse Plus C18 3 × 50 mm, 1.8 µm			

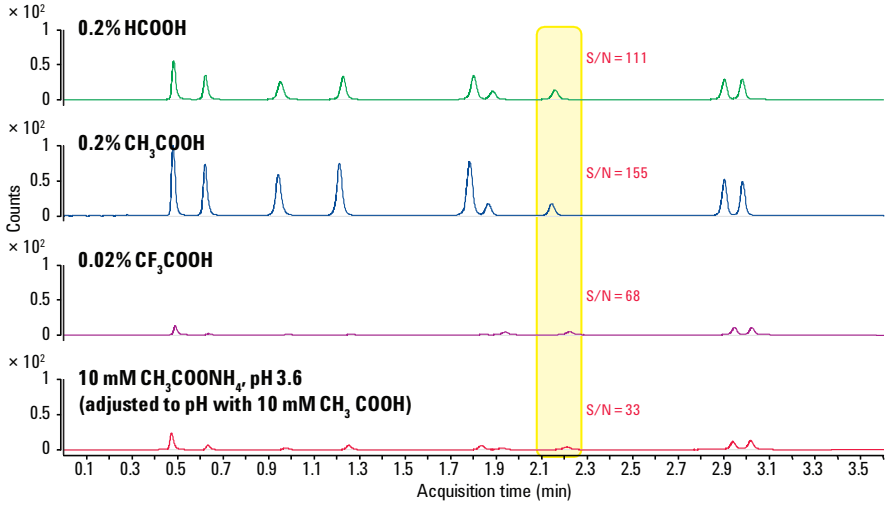
Compounds (in elution order) with Identifying Mass:

acetaminophen: *m/z* 109	acetylsalicylic acid: *m/z* 120	sulindac: *m/z* 356	diflunisal: *m/z* 332
caffeine: *m/z* 194	phenacetin: *m/z* 179	piroxicam: *m/z* 332	diclofenac: *m/z* 295
2-acetamidophenol: *m/z* 109	salicylic acid: *m/z* 120	tolmetin: *m/z* 257	celecoxib: *m/z* 381
acetanilide: *m/z* 135		ketoprofen: *m/z* 254	ibuprofen: *m/z* 160

Figure 8. Example of ultra fast MS scan analysis possible with a highly efficient 1.8 µm column. Refer to Agilent Publication 5990-8428EN for more information regarding this specific application.

Optimizing sensitivity by selecting the best MS mobile phase

Figure 9 shows several MS-friendly mobile phases that were screened for use with a tea analysis on an Agilent Poroshell 120 SB-C18 column. Selectivity and peak shape remained constant regardless of the mobile phase used, consequently, the optimal mobile phase was selected based on signal strength of the analytes. Significant ion suppression is present with the ammonium acetate buffer, as well as with the trifluoroacetic acid mobile phase. The two best contenders were formic and acetic acid, with acetic acid producing a slightly more intense signal. Comparing the most sensitive acetic acid mobile phase to the least sensitive ammonium acetate mobile phase shows that the sensitivity of the acetic acid was five times the sensitivity of the ammonium acetate.



Instrument	Agilent 1200/6410 LC/MS/MS		
A	acidified water		
B	acetonitrile		
Flow rate	0.729 mL/min		
T	0	1.43	2.86
%B	10	15	27
Stop time	3.6 min		
Post run time	1.7 min		
Sample	3 µL injection of 3 µg/mL standard in water		
TCC	40 °C		
Column	Agilent Poroshell 120 SB-C18 2.1 × 100 mm, 2.7 µm		
DAD	Sig = 210, 4 nm: Ref = Off		
Source	ESI+, 350 °C, 10 L/min, 50 psi, ± 3500 V		
Fragmentor	135 V		

MS, SIM- (169, 305, 289, 457, 441), SIM+ (195)

Sample:

Gallic acid: *m/z* 171

(Epi)gallocatechin: *m/z* 307

(Epi)catechin: *m/z* 291

Caffeine: *m/z* 195

(Epi)gallocatechin gallate: *m/z* 459

(Epi)catechin gallate: *m/z* 443

Figure 9. Comparison of the effect of mobile phase on signal-to-noise (S/N) with a superficially porous 2.7 µm column and MS scan detection. Refer to Agilent Publication 5990-7824EN for more information regarding this specific application.

Conclusions

Small particle size columns, including sub-2 µm and superficially porous 2–3 µm, enhance LC/MS (Scan and SIM) and LC/MS/MS results. Improvements in sensitivity, resolution and peak capacity are observed, as compared to 3.5 and 5 µm columns with MS detection. High efficiency columns are particularly useful when samples contain isomers that require baseline resolution for accurate quantitation, or when samples are in complex matrices and separation from the background is desirable to avoid the possibility of ion suppression with coeluting peaks. Additionally, mobile phase considerations should be taken with MS detection to prevent ion suppression and optimize for analyte peak shape, selectivity and sensitivity.

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Printed in the USA
January 3, 2012
5990-8623EN

Agilent Technologies