Optimal Particle Size for High Speed HPLC Analysis

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Introduction

Developing ultra-fast and effective methods for the assay (qualitative and quantitative) of high -throughput samples has become the primary challenge for scientists in combinatorial chemistry syntheses, drug metabolism-pharmacokinetic (DMPK) studies, in clinical, and forensic laboratories. It is well known that reducing the particle diameter of the packing material in a liquid chromatography (LC) column can shorten the analyte's diffusion path, and lower the resistance to mass transfer between the stationary and the mobile phases resulting in improved separation efficiencies at increased mobile phase linear velocities (flow rates). With the introduction of ultra-High Performance Liquid Chromatography (uHPLC) instruments, there has been much interest in using short columns filled with sub-2µm particles to achieve higher column performance and shorter analysis time. At the same time, as particles within the column decrease, the inter-particle void spaces become smaller, and the resistance to flow at the same linear velocity is considerably higher. As a result conventional HPLC instrumentation is limited in the pressure required for operating columns packed with small-particle sorbents.

Based on the van Deemter equation, as the particle size decreases to less than 3 µm, efficiency increases significantly, and is maintained even at increased linear velocities. To retain rapid elution and sufficient resolving power, short columns packed with particles ≤ 3.0 µm can be utilized for the rapid HPLC separations in high-throughput analysis. This allows for fast HPLC separations with maximum performance while maintaining an acceptable level of high pressure at high flow rates without specialized HPLC system. In this presentation we explore the effectiveness of 2.5 µm particle size stationary phases in rapid liquid chromatography separations

Experimental Conditions (1)

Instrumentation

HPLC System:	Jasco X-LC™ System
Pump:	3058PU X-LC™ Semi-micro Pump
Injector:	3050AS X-LC™ Autosampler
Detector:	3075UV X-LC™ UV/VIS Detector
Column Oven:	3067CO X-LC™ Column Oven
Degasser:	3080DG X-LC™ Degasser
Software:	EZ Start Version 3. 1. 7 (Scientific Software)

Experimental Conditions (2)

Compounds		HPLC Conditions
Steroids (10 µg/mL)		
1.Triamcinolone	7. 11α–Hydroxyprogesterone	Mobile phase: A: 0.1 % Formic acid in water B: 0.1 % Formic acid in acetonitrile Gradient: 30 - 70 % B in 5 min, re-equilibrate for 1.5 min Flow Rate: 0.35 mL/min Injection: 1 µL Detection: 254 nm Temperature: 30 ºC
2. Prednisolone	8. Cortisone acetate	
3. Cortisone	9. 11-Ketoprogesterone	
4. Betamethasone	10. 17α–Hydroxyprogesterone	
5. Cortisosterone	11. Betamethasone valerate	
6. Triamcinolone acetonide	12. Progesterone	
Acidic, Neutral, and Basic Compounds (100 µg/mL)		
1.Pyridine (50 µg/mL)	8. Phenol (0.5 mg/mL)	Mobile phase: Same as Steroids Gradient: 5 – 95 % B in 4 min, re-equilibrate for 1 min Flow Rate: 0.6 mL/min Injection: 1 µL Detection: 254 nm Temperature: 50 ºC
2. Acetaminophen	9. Nortriptyline	
3. Quinine	10. Impurity	
4. Impurity	11. 3-Methyl-4-nitrobenzoic acid	
5. Sulfathiazole	12. Methyl-salicylaldehyde (110 µg/mL)	
6. Triprolidine	13. Hexanophenone	
7. Benzyl alcohol (1.2 mg/mL)		

Experimental Conditions (3)

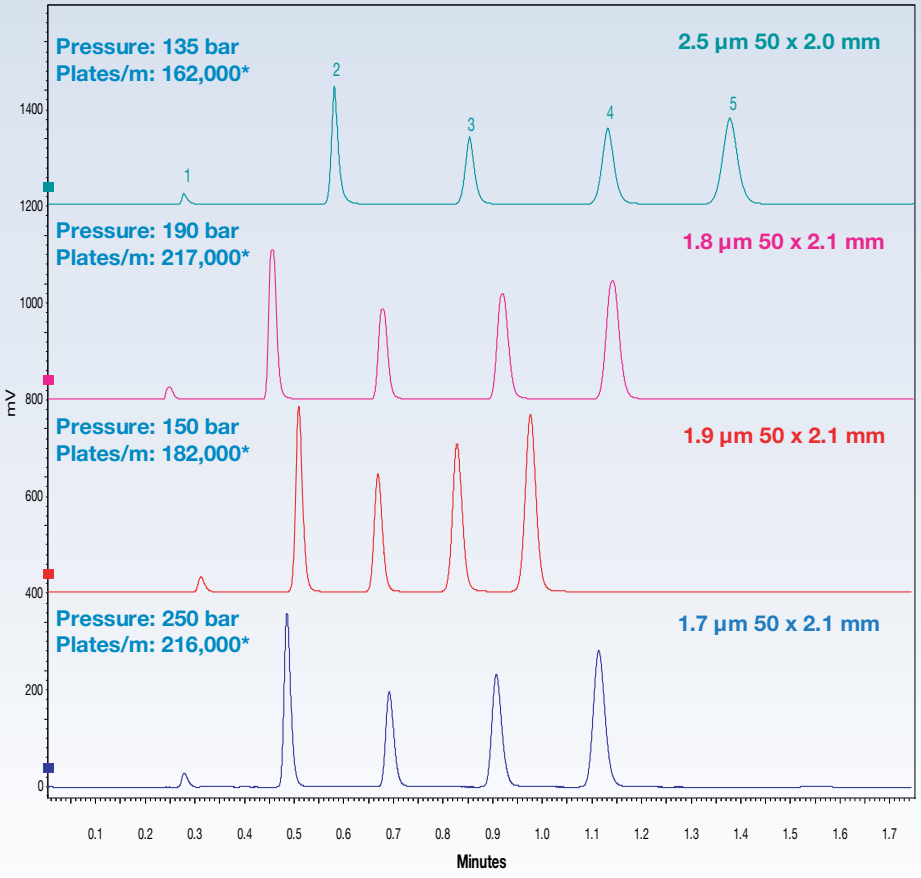
Compounds		HPLC Conditions
Sulfa Drugs (50 µg/mL)		
1.Sulfanilic acid	6. Sulfathiazole	Mobile phase: Same as Steroids Gradient: 5 - 50 % B in 3 min, re-equilibrate for 1 min Flow Rate: 0.6 mL/min Injection: 1 µL Detection: 254 nm Temperature: 35 ºC
2. Sulfaguanidine	7. Sulfamerazine	
3. Sulfanilamide	8. Sulfamethazine	
4. Sulfacetamide	9. Sulfamethoxazole	
5. Sulfadiazine	10. Sulfaquinoxaline	
Benzodiazepines (40 µg/mL)		
1. 7-Aminoflunitrazepam (10 µg/mL)	6. Oxazepam	Mobile phase: Same as Steroids Gradient: 30 - 55 % B in 2.5 min, re-equilibrate for 1 min Flow Rate: 0.6 mL/min Injection: 1 µL Detection: 254 nm Temperature: 35 ºC
2. Chlordiazepoxide (20 µg/mL)	7. Clonazepam	
3. Flurazepam	8. Nordazepam	
4. Bromazepam	9. Diazepam	
5. Flunitrazepam		
Acidic Drugs (100 µg/mL)		
1. Naproxen (10 µg/mL)	4. Ethacrynic acid	Mobile phase: A: 10 mM Ammonium acetate B: Acetonitrile Gradient: 25 to 40 % B in 1.5 min; equilibrate for 1 min Flow Rate: 0.6 mL/min Injection: 1 µL Detection: 254 nm Temperature: 35 ºC
2. Ketoprofen	5. Ibuprofen (50 µg/mL)	
3. Fenprofen	6. Indomethacine (25 µg/mL)	

Characteristics of Evaluated HPLC Columns

Column	Luna® C18(2)-HST	ZORBAX Eclipse XDB – C18	Hypersil™ Gold C18	ACQUITY UPLC® BEH C18
Particle Size (µm)	2.5	1.8	1.9	1.7
Pore Diameter (Å)	100	80	175	130
Surface Area (m^2/g)	400	180	220	185
ID (mm)	50 x 2.0	50 x 2.1	50 x 2.1	50 x 2.1
Expected Efficiency*	181,818	252,525	239,234	267,380
Bonded Phase	Carbon Load: 17.5 % Endcapping: Yes 3.0 µmoles/m^2 **	Carbon Load: 10 % Endcapping: Double	Carbon Load: 10 % Endcapping: Yes	Carbon Load: 18 % Endcapping: Yes
Pressure Limit	400 bar	600 bar	N/A	1000 bar

*The theoretical efficiency is based on **N=20L/2.2d_p** (Plates/m) Neue, U.D. *HPLC Columns*: *Theory Technology, and Practice;* John Wiley and Sons, Inc., New York, **1997**.
** Calculated bonded phase coverage

Figure 1: Preliminary Efficiency of Evaluated Columns



HPLC Conditions

Mobile Phase: Acetonitrile: Water (65:35)
Flow Rate: 0.35 mL/min
Injection: 1 µL
Detection: 254 nm
Temperature: Ambient
Sample: $^{1}/_{10}$ Prodigy mixture

1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

*Based on Peak #5, Naphthalene

Figure 2: Comparison of Expected and Actual Efficiency

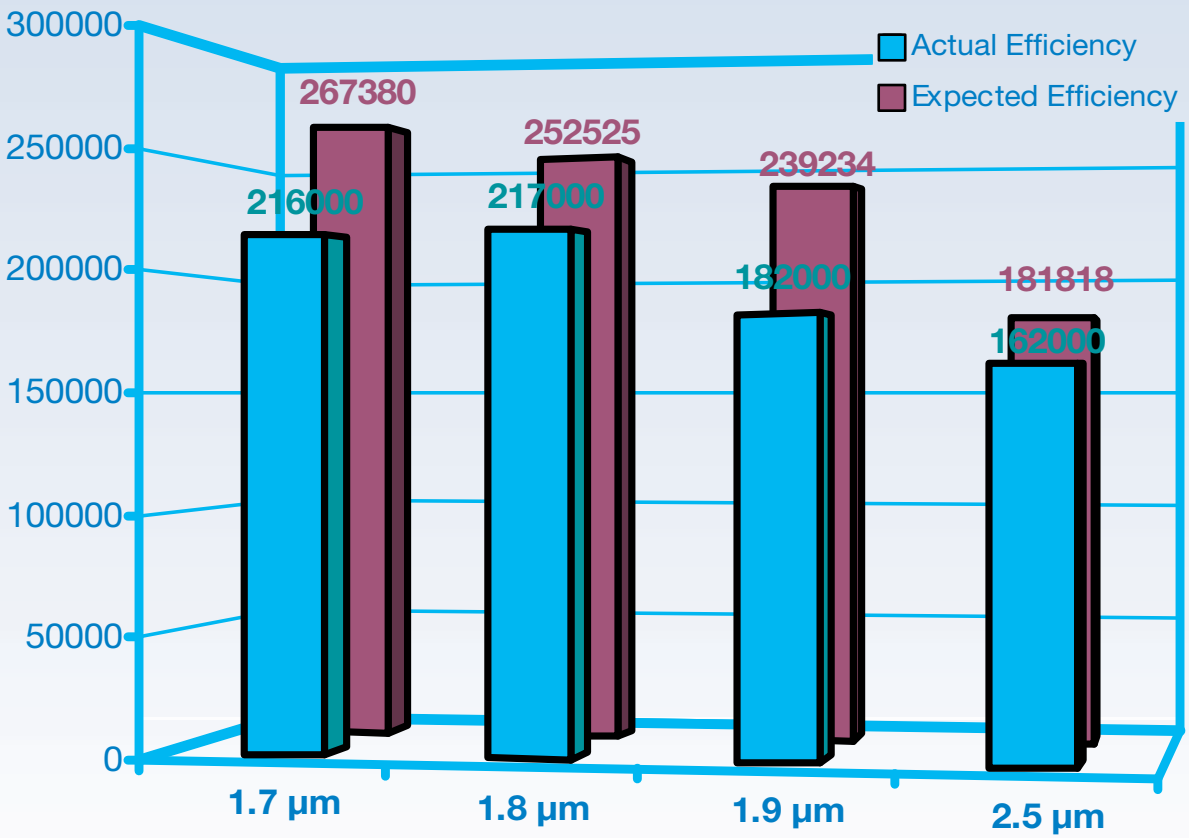
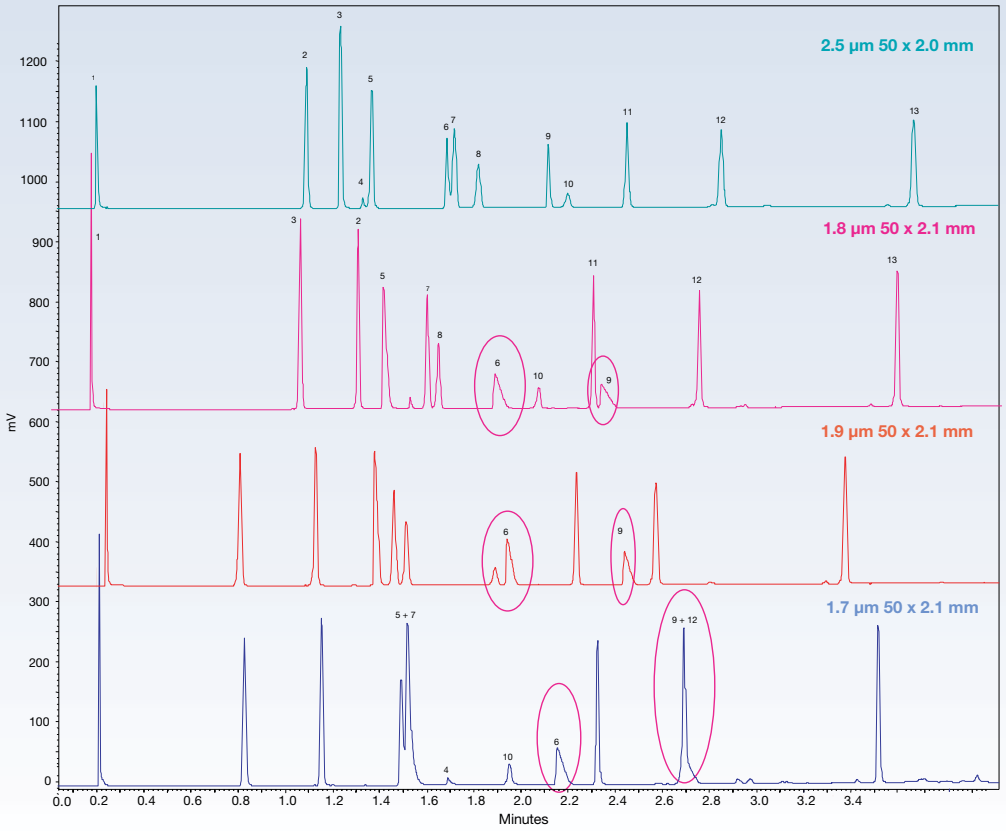


Figure 3: Separation of Acidic, Neutral, and Basic Compounds



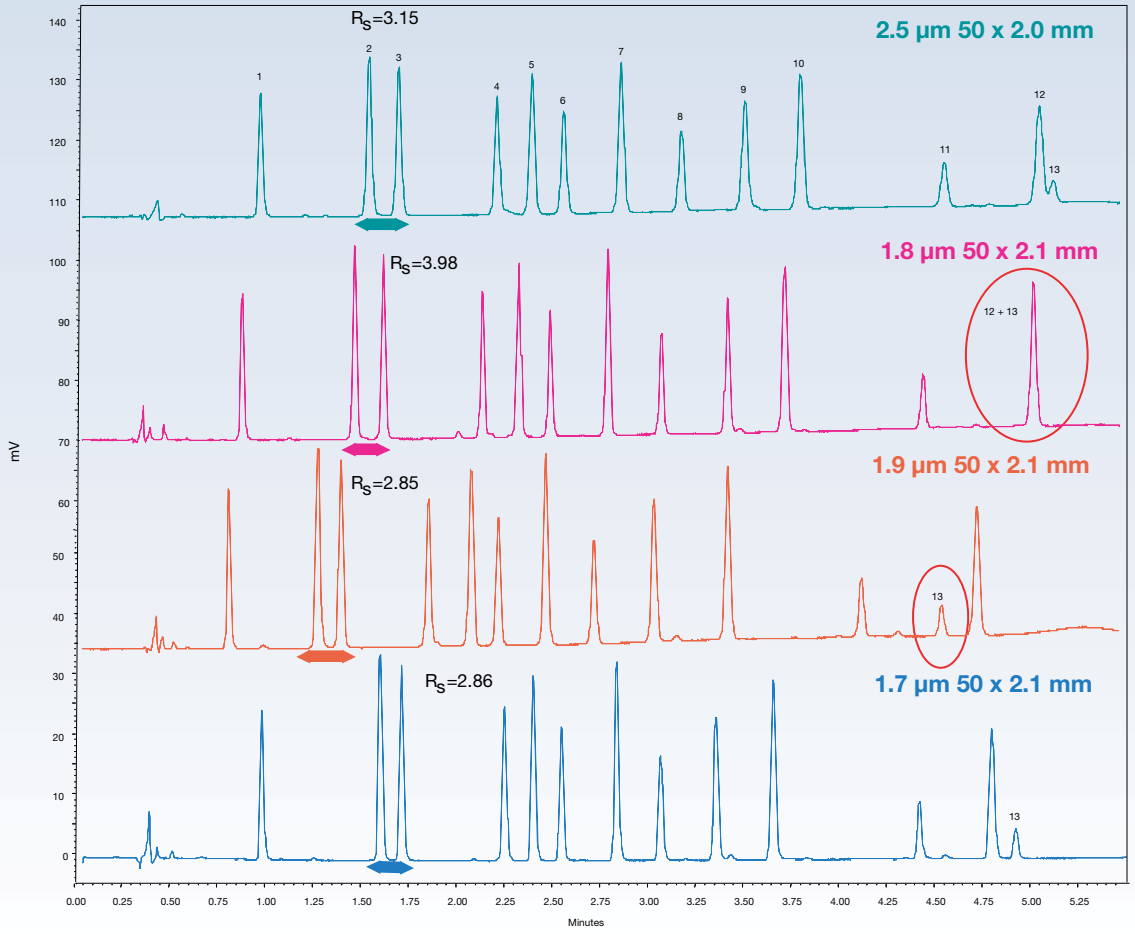
Pressure: 275 bar
Elution window: 3.47 min
N_c: 71

Pressure: 382 bar
Elution window: 3.39 min
N_c: 56

Pressure: 290 bar
Elution window: 3.14 min
N_c: 52

Pressure: 540 bar
Elution window: 3.31 min
N_c: 59

Figure 4: HPLC Separation of Steroids



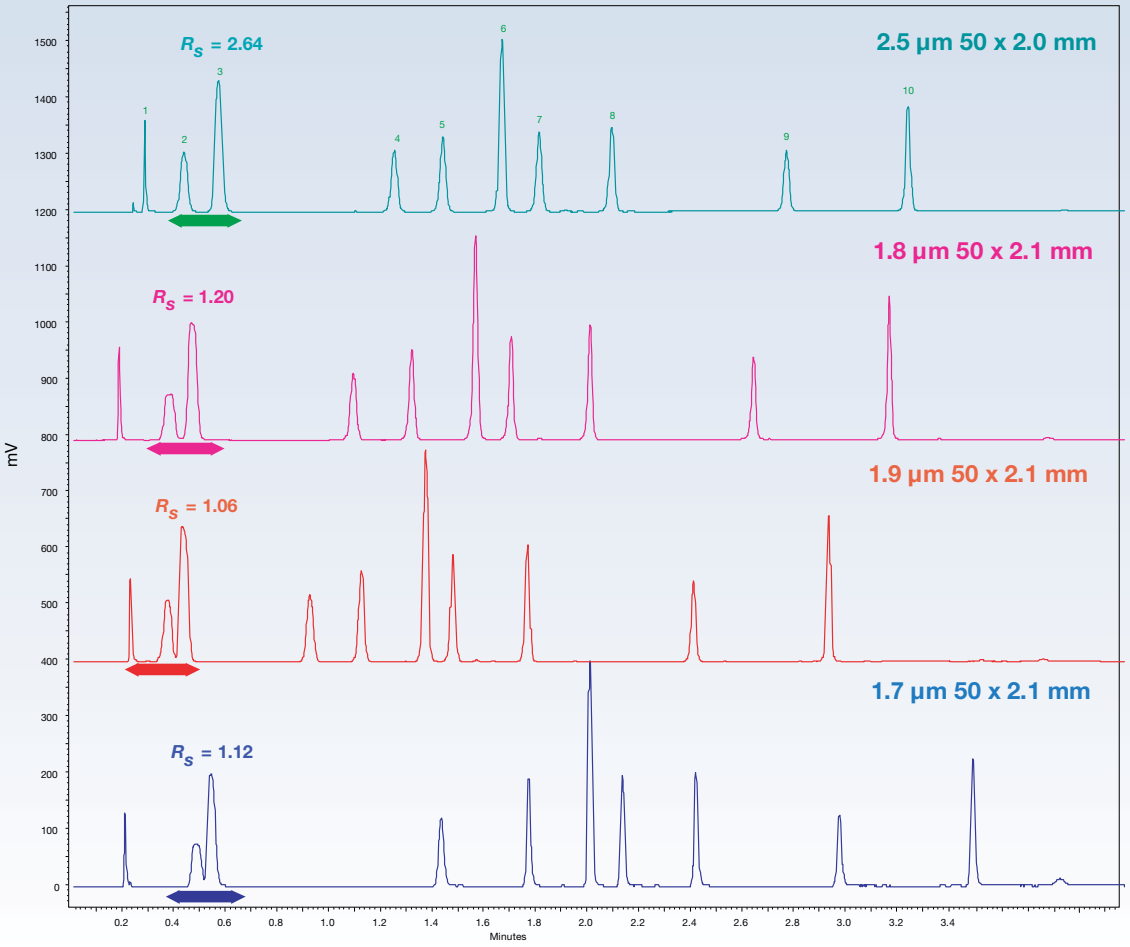
Pressure: 187 bar
Elution window:
4.74 min
N_c: 44

Pressure: 290 bar
Elution window:
4.64 min
N_c: 41

Pressure: 209 bar
Elution window:
4.34 min
N_c: 38

Pressure: 350 bar
Elution window:
4.55 min
N_c: 41

Figure 5: Separation of Sulfa Drugs



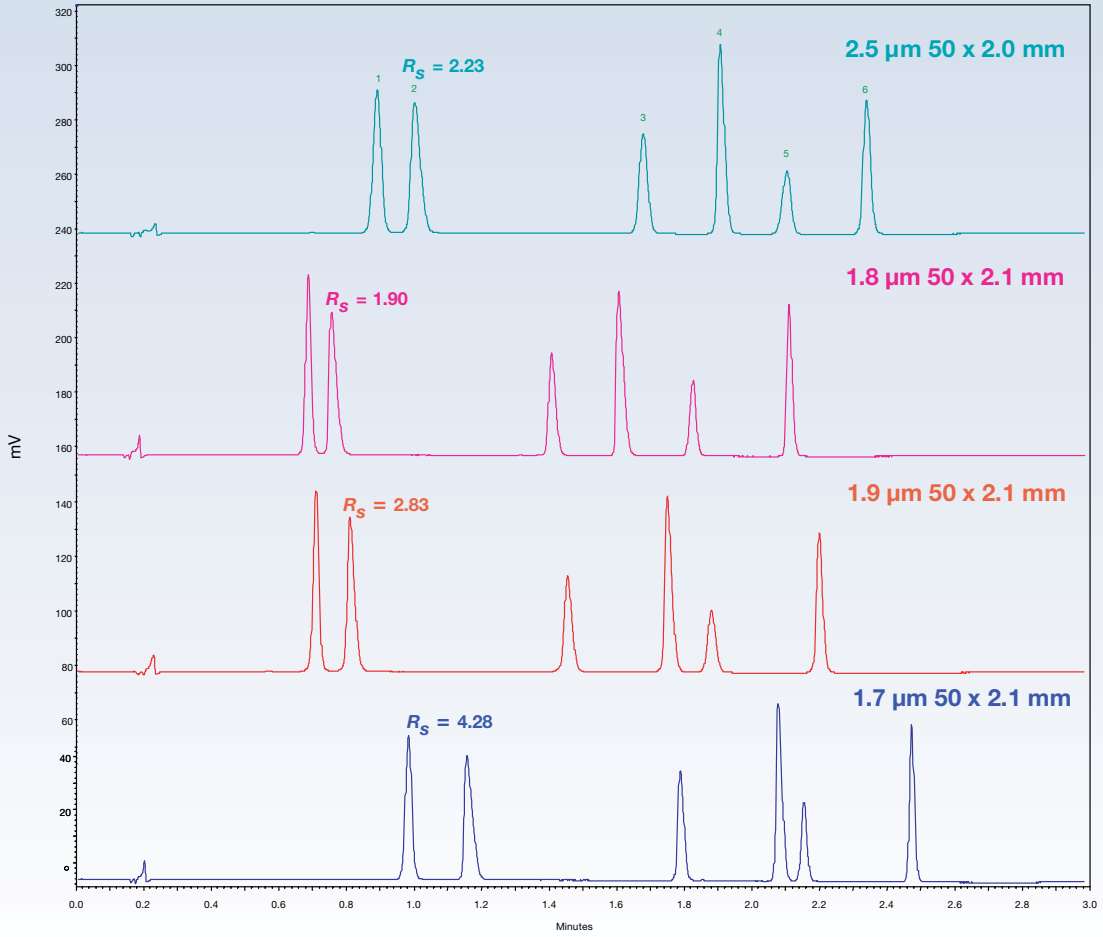
Pressure: 320 bar
Elution window:
2.54 min
N_c: 36

Pressure: 459 bar
Elution window:
2.56 min
N_c: 38

Pressure: 352 bar
Elution window:
2.32 min
N_c: 24

Pressure: 598 bar
Elution window:
2.82 min
N_c: 35

Figure 6: Separation of Acidic Drugs



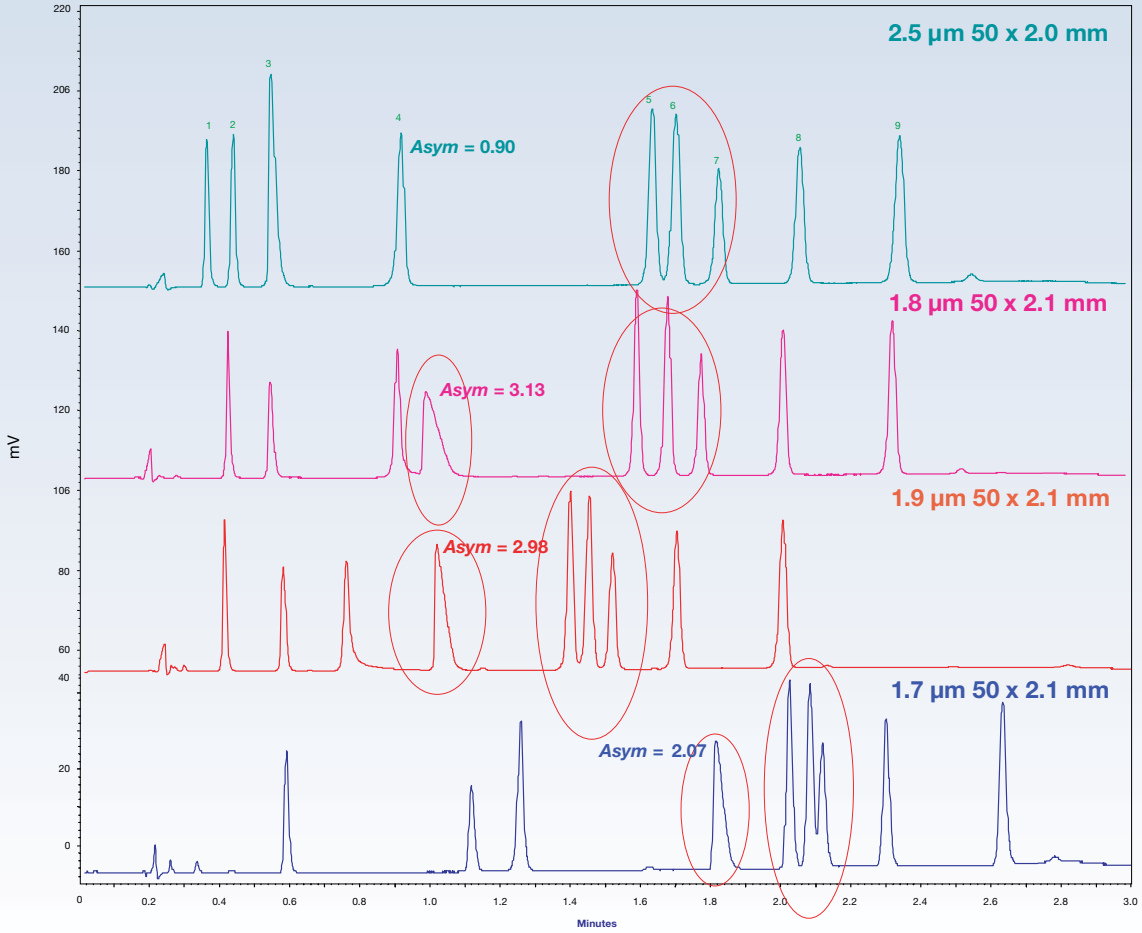
Pressure: 337 bar
Elution window:
2.10 min
N_c: 23

Pressure: 470 bar
Elution window:
1.92 min
N_c: 24

Pressure: 372 bar
Elution window:
2.01 min
N_c: 24

Pressure: 582 bar
Elution window:
2.29 min
N_c: 31

Figure 7: Separation for Benzodiazepines



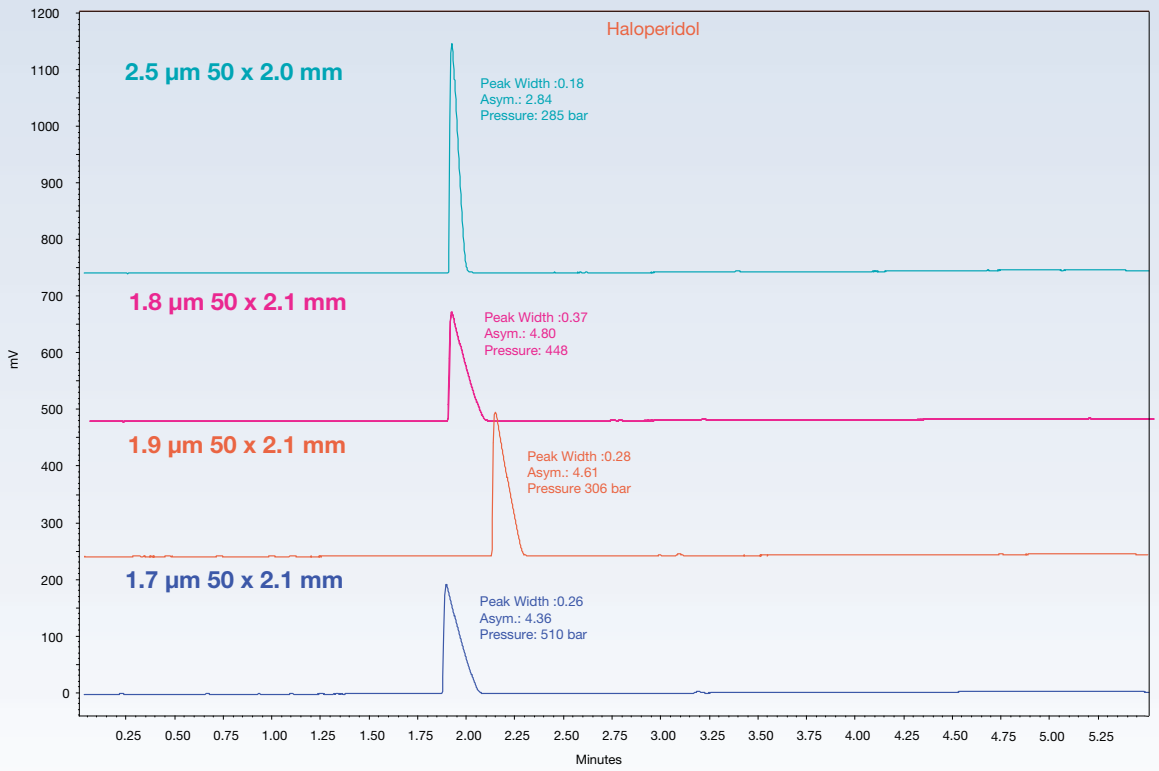
Pressure: 320 bar
Elution window:
2.10 min
N_c: 32

Pressure: 471 bar
Elution window:
2.13 min
N_c: 33

Pressure: 352 bar
Elution window:
1.78 min
N_c: 17

Pressure: 599 bar
Elution window:
2.43 min
N_c: 29

Figure 8: Analysis of Impurity of Haloperidol (1)



HPLC Conditions

Mobile Phase: A: 0.1 % Formc acid in water
B: 0.1 % Formc acid in acetonitrile

Gradient: 15 - 95 % B in 5 min, re-equilibrate for 2 min

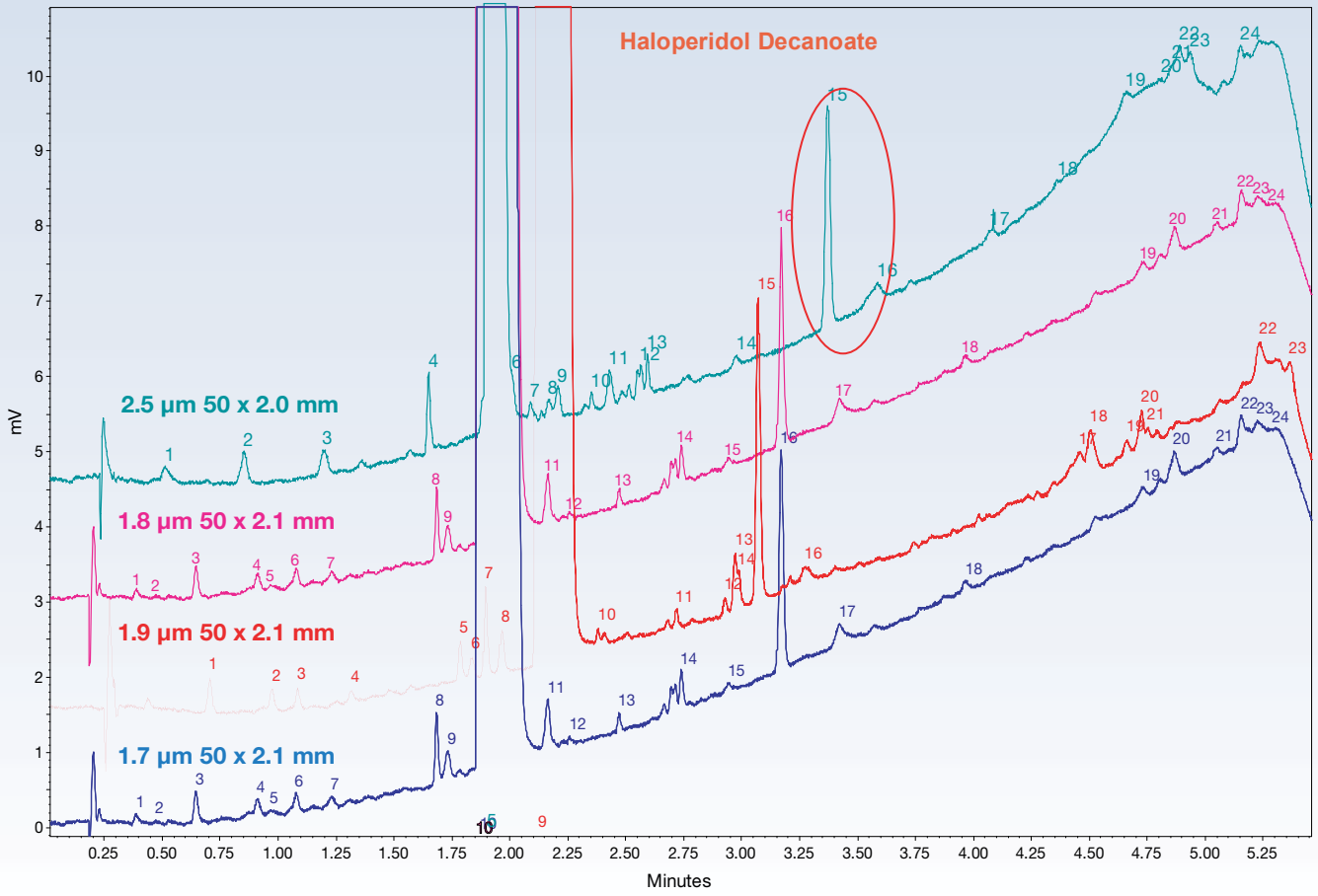
Flow Rate: 0.5 mL/min

Injection: 1 µL
1 mg/mL Haloperidol (in Methanol)

Detection: 254 nm

Temperature: 30 ºC

Figure 9: Analysis of Impurity of Haloperidol (2)



Results and Discussions

Four C18 columns with 2.5 µm and < 2 µm particles from different vendors were randomly picked for this evaluation. The preliminary efficiencies show that the column packed with 2.5 µm particle size sorbent has 75 to 89 % of the efficiency of columns packed with 1.7, 1.8, and 1.9 µm particles, at 54 to 90 % of pressure, respectively **(Figure 1, 2)**, while it is expected that the efficiency of 2.5 µm particles would be only 65 –75 % of that of the other columns.

The high speed separation of complex mixtures of common pharmaceutical compounds (acidic, basic and neutral in nature) such as benzodiazepines, sulfonamide drugs, steroids, and non-polar acids was explored on small particle C18 sorbents. Performance criteria were backpressure, resolution (R_s), width of chromatographic elution window, and peak capacity (N_c) in generic or shallow gradient elution using LC/MS friendly additives in the mobile phase, at various flow rates. The elution window was measured between the unretained solvent peak and the last eluted peak. The peak capacity was calculated by dividing the elution window with the average peak width of all eluted compounds.

In general the performance of 2.5 µm sorbents was found to be similar to that of sub-2 µm materials in regards of resolution, and peak capacity **(Figure 5, 6, 7)**, or even better in regards of peak capacity **(Figure 3, 4)**. This indicates that the efficiency generated by sub–2 µm particles is not the only important factor to determine column performance; other factors like sorbent selectivity and physical characteristics are also important contributors to column performance. Since Luna 2.5 µm C18(2)-HST shows less silanol activity toward basic compounds (nortriptyline) resulting in more narrow peak width (less tailing), and also provides different selectivity for various basic compounds, its peak capacity is equal or larger. Furthermore, Luna 2.5 µm C18(2)-HST has the highest surface area (400 m^2/g) of all sorbents considered here providing large elution windows (longer retention) also contributing to higher peak capacity than the sub–2 µm packing materials.

The speed and efficiency achieved as a function of the increase in backpressure show that 2.5 µm demonstrates the lowest resistance to mass transfer at high linear velocities. Backpressures generated by columns packed with 2.5 µm particles are less than 400 bar, which is compatible with both conventional HPLC and uHPLC instruments.

The assay for impurity profiling for both raw materials and final product is a major task in QC/QA. Fast turnover is often critical to reducing the time-to-market. As an example, the impurity profile of haloperidol was compared in fast gradient elution mode on 2.5 µm and sub–2 µm particles. In order to quantitate 0.05 – 0.1% of impurity in the primary compound, a high concentration of haloperidol was injected onto the column. **Figure 8** shows that columns packed with sub–2 µm particles are overloaded (giving broad peaks) because of their low surface area (less than 250 m^2/g) in contrast to 2.5 µm Luna particles (having a surface area of 400 m^2/g). Such broad peaks may cover closely eluting impurities because of loss of resolution in the elution region of the parent peak.

Conclusions

The high speed separation of complex mixtures of acidic, basic and neutral pharmaceutical compounds was explored on 2.5 µm and sub–2 µm particle size C18 sorbents in LC/MS friendly mobile phases, at different flow rates.

Higher efficiencies of sub–2 µm sorbents do not necessarily lead to separations with the best resolution or peak capacity. Sorbent selectivity and physical characters are also important contributors to column performance.

The performance of Luna 2.5 µm C18(2)-HST columns was found to be similar to sub-2 µm materials in regards to resolution and peak capacity at significantly lower backpressures, or even better in regards to peak shapes of basic compounds.

To achieve rapid elution and retain sufficient resolving power, short columns packed with Luna 2.5 µm C18(2)-HST can be utilized for the rapid HPLC separations in high-throughput analysis. This allows for fast HPLC separations with maximum performance while maintaining an acceptable level of backpressure even at high flow rates without the need for a uHPLC system.