การเลือกใช้งาน LC คอลัมน์และการแก้ปัญหาเบื้องต้น

จันทร์เพ็ญ ชัยวงศ์ขจร

บ.สิทธิพร แอสโซซิเอส จำกัด

21 กุมภาพันธ์ 2566

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HOW HPLC COLUMNS WORK

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LC COLUMN TYPE

- NORMAL PHASE COLUMN
- REVERSE PHASE COLUMN
- ION EXCHANG COLUMN
- SIZE EXCLUSION COLUMN
- CHIRAL COLUMN

มีให้เลือกใช้มากที่สุด

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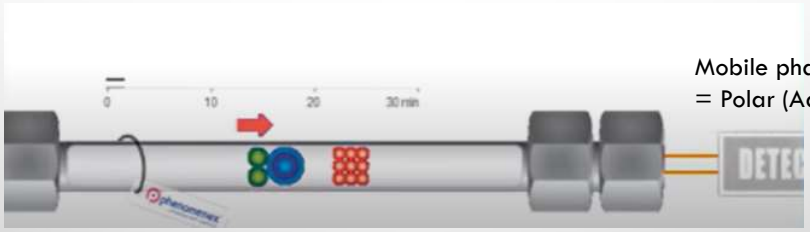
3

REVERSE PHASE CHROMATOGRAPHY

Stationary phase = Non-Polar (C18)



RED คือ สารที่ Polar มากที่สุด
BLUE คือ สารที่ Polar รองลงมา
GREEN คือ สารที่ polar น้อยที่สุด

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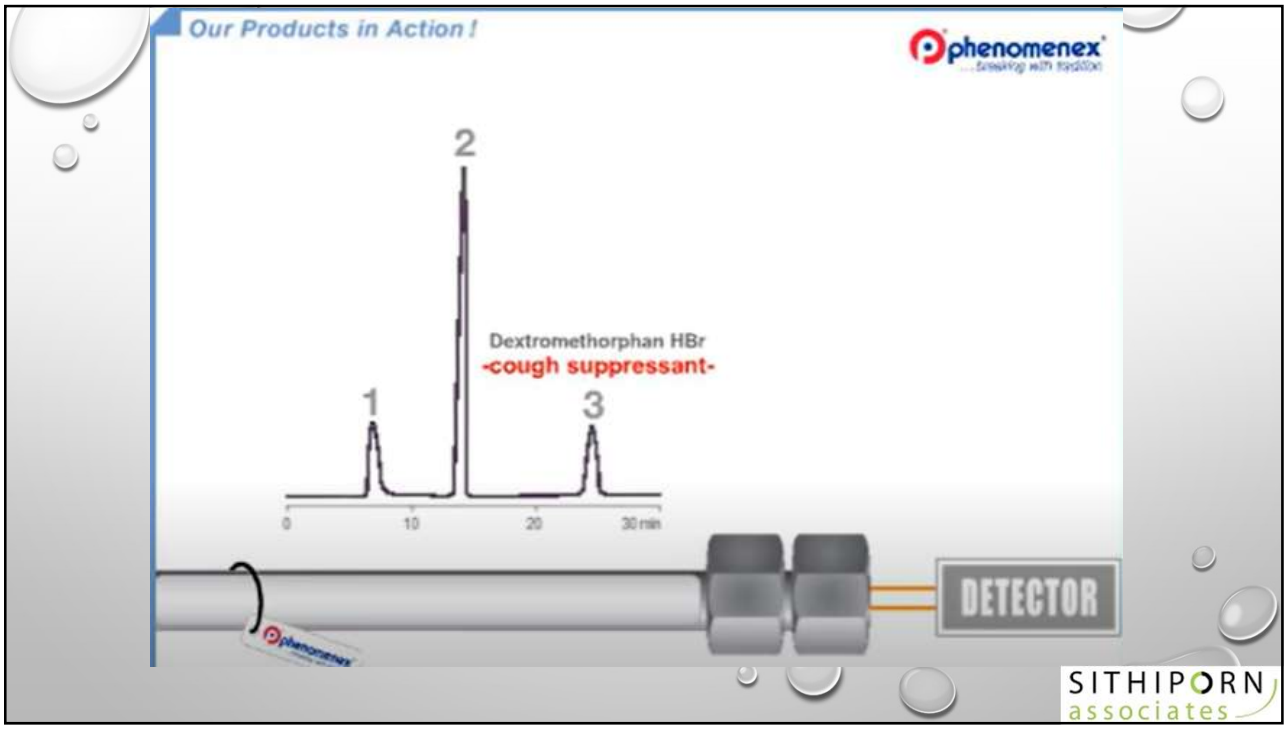

COLUMN CHARACTERISTICS

- STRUCTURE OF SILICA
- BONDING TYPE
- PORE SIZE
- SURFACE AREA
- END CAPPING
- PARTICLE SIZE
- ID & COLUMN LENGHT

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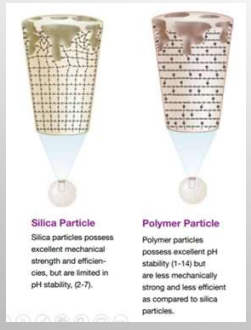
SILICA BASE



Two-In One (TWIN) Technology

การพัฒนาคุณภาพ ของ Siliga Base เพื่อ

- Pressure resistant
- pH Resistant
- Strong condition use
- Retention

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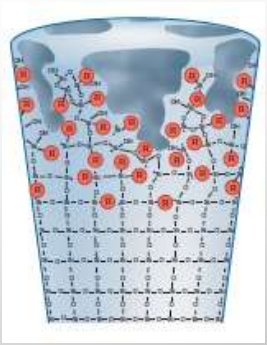
PORE SIZE

The smaller the pore size, the greater the surface area

การแยกของ Reverse Phase จะเกิดที่ Surface area



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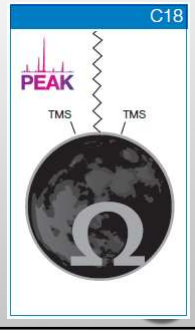
END CAPPING

- REDUCES SILANOL ACTIVITY
- SECONDARY BONDING STEP TO “COVER” UNREACTED SILANOL SITES AFTER FIRST STEP

SILANOL ACTIVITY ทำให้เห็นพีค ไม่ SYSMETRY ได้



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BONDING CHEMISTRY

- C4, C6
- C8 >> USP L7
- C18 >> USP-L1-L2
- PHENYL >> USP L11
- CN >> USP L10
- NH2 >> USP L8
- อื่นๆ

USP Column Classification		Recommended Phenomenex Column	Particle Shape	Page
L1	Octadecyl silane chemically bonded to porous or non-porous silica or ceramic microparticles, 1.5 to 10 µm in diameter, or a monolithic rod.	Kinetex® C18	Core-Shell	235
		Kinetex EVO C18	Core-Shell	235
		Kinetex Polar C18	Core-Shell	235
		Kinetex PS C18	Core-Shell	235
		Kinetex XB-C18	Core-Shell	235
		Luna® C18(2)	Spherical	265
		Luna Omega C18	Spherical	279
		Luna Omega PS C18	Spherical	279
		Luna Omega Polar C18	Spherical	279
		Gemini® NX-C18	Spherical	223
		Gemini C18	Spherical	223
		Synergi™ Hydro-RP	Spherical	332
		Synergi Fusion-RP	Spherical	332
		bioZen™ Peptide PS-C18	Spherical	208
		Onyx™ C18	Monolith	302
		Jupiter® C18	Spherical	233
		Clarity® Oligo-RP	Spherical	394
		Clarity Oligo-MS	Core-Shell	394
		Clarity Oligo-XT	Core-Shell	394
		Aeris™ WIDEPORE XB-C18	Core-Shell	202
		bioZen Peptide XB-C18	Core-Shell	208
L2	Octadecyl silane chemically bonded to silica gel of a controlled surface porosity that has been bonded to a solid spherical core, 30 to 50 µm in diameter.			
L3	Porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.	Kinetex HILIC	Core-Shell	235
		Luna Silica(2)	Spherical	265
		Onyx Silica	Monolith	302
L4	Silica gel of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter.			
L5	Alumina of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter.			
L6	Strong cation-exchange packing: sulfonated fluorocarbon polymer coated on a solid spherical core, 30 to 50 µm in diameter.			
L7	Octyl silane chemically bonded to totally or superficially porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.	Kinetex C8	Core-Shell	235
		Luna C8(2)	Spherical	265
		Onyx C8	Monolith	302
		bioZen Intact XB-C8	Core-Shell	208
L8	An essentially monomolecular layer of aminopropyl-silane chemically bonded to totally porous silica gel support, 1.5 to 10 µm in diameter, or a monolithic silica rod.	Luna NH2	Spherical	265
		Luna Omega SUGAR	Spherical	279
L9	Irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating, 3 to 10 µm in diameter.	Luna SCX	Spherical	265
L10	Nitrile groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.	Luna CN	Spherical	265
L11	Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.	Kinetex Biphenyl	Core-Shell	235
		Kinetex Phenyl-Hexyl	Core-Shell	235

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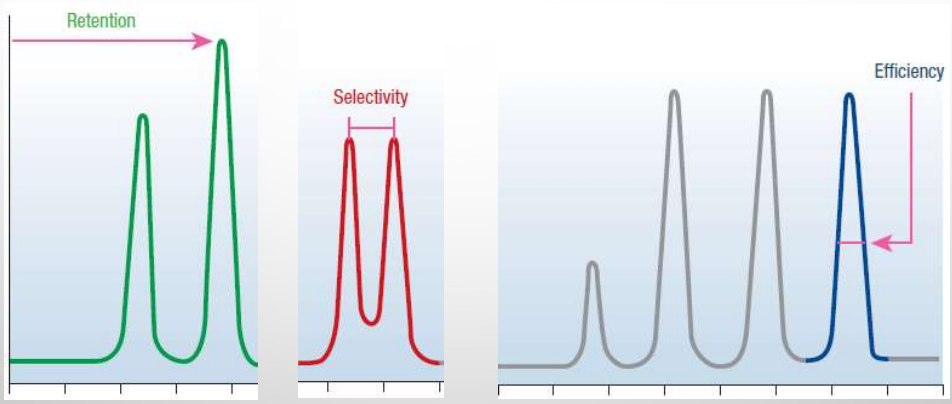
ทบทวนความรู้พื้นฐาน VAN DEEMTER THEORY



Mobile Phase
Column bonding
Temperature
Column????

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การเลือกคอลัมน์ มีปัจจัยอย่างไรบ้าง เมื่อ BOND PHASE เหมือนกัน

- PARTICAL SIZE
- ID COLUMN
- COLUMN LENGTH

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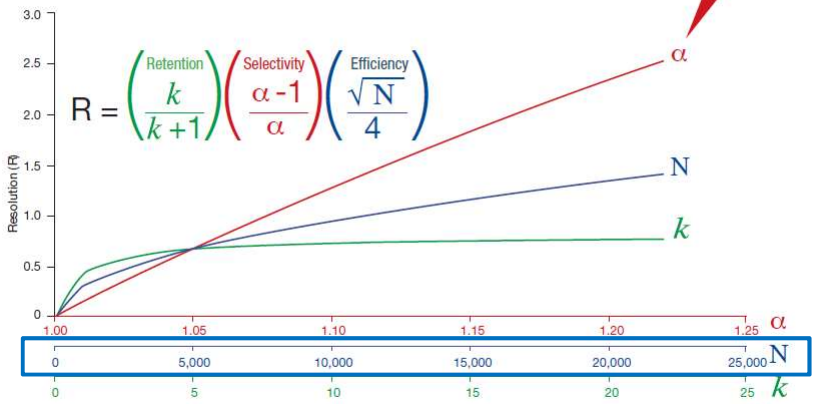
The Impact of Selectivity on Resolution

Selectivity is the most important parameter for increasing resolution.

$$R = \left(\frac{k}{k+1}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\sqrt{N}}{4}\right)$$

Retention · Selectivity · Efficiency



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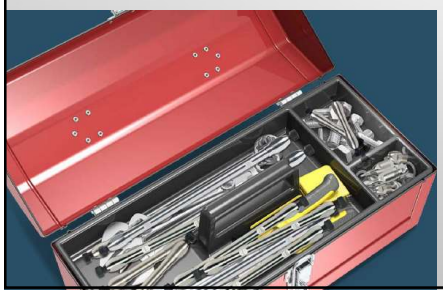
COLUMN SIZE VS. EFFICIENTCY

Column Length (L) = 250 mm = 250,000 um

Dp = 5um

$$N = \frac{L}{Dp} = \frac{250{,}000}{5} = 50{,}000$$



Length (L, mm)	Column Diameter (d_c, mm)	Particle Size (d_p, µm)	L/d_p
250	4.6	10	25,000
150	4.6	5	30,000
150	2.1	5	30,000
100	4.6	3.5	28,600
100	2.1	3.5	28,600
75	4.6	2.5	30,000
75	2.1	2.5	30,000
50	4.6	1.7	29,400
50	2.1	1.7	29,400

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UPDATE COLUMN ใหม่ๆ มีอะไรบ้างที่ต้องพิจารณา

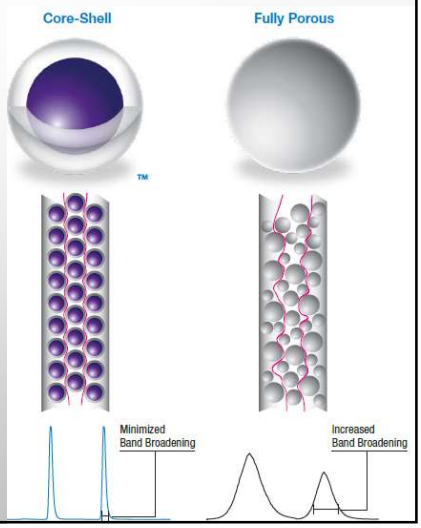
Core–shell particles consist of a **solid core** coated with a layer of porous silica that is deposited either in layers or a single coating, depending on the manufacturer.

ประโยชน์ที่ได้ เมื่อเทียบกับ Fully Porous ทั่วไป

narrower particle size distribution

significantly lower back pressures

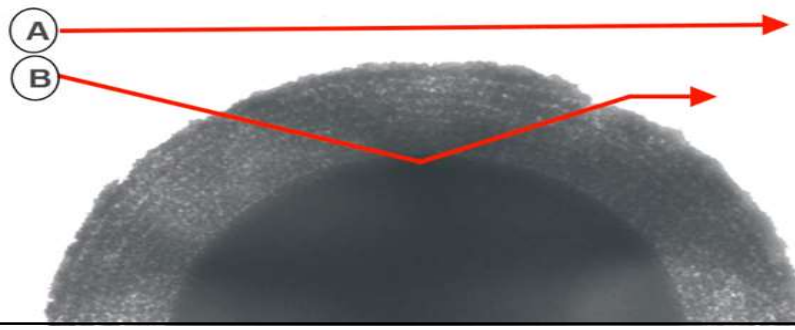
ปัจจุบัน **Core shell column** มีหลากหลายให้เลือกทุกยี่ห้อ มีตั้งแต่ **Analytical** ถึง **Preperative scale**



phenomenex ...breaking with tradition™

Retention on Core-Shell Media

Surface Area



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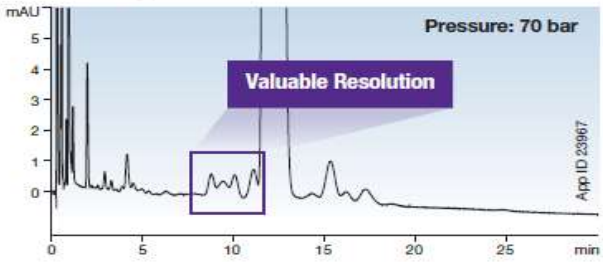
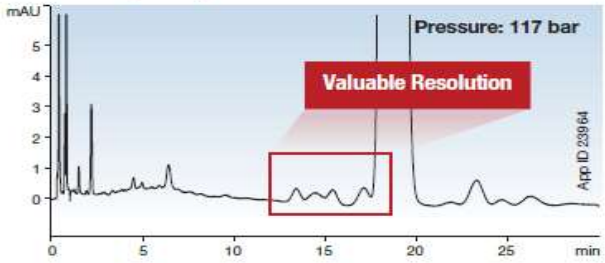
UHPLC Performance – Cyclosporine Impurity Profile

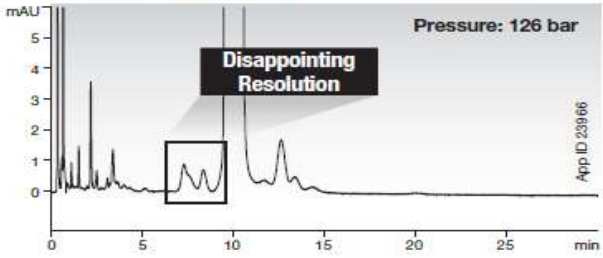
Kinetex 2.6µm Polar C18



Luna Omega 1.6µm Polar C18



Conventional 1.7µm C18



Conditions for all columns same except where noted:

Columns:	Kinetex 2.6µm Polar C18 Luna Omega 1.6µm Polar C18 Conventional Fully Porous 1.7µm C18
Dimensions:	50 x 2.1 mm
Mobile Phase:	Acetonitrile/Tert-butyl methyl ether/Water/ Phosphoric acid (430:50:520:1)
Flow Rate:	0.30 mL/min
Temperature:	80 °C
Detection:	UV @ 210 nm
Sample:	Cyclosporine

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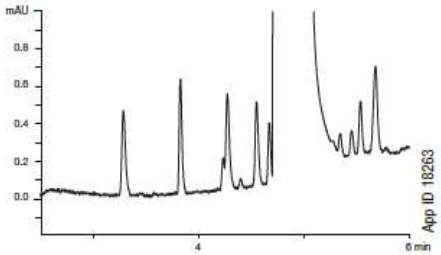
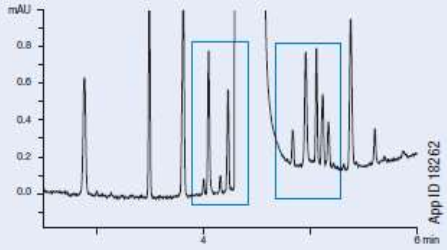
DECREASE PARTICLE SIZE- LOWER LEVEL OF DETECTION AND QUANTITATION

Agilent Technologies® ZORBAX® 3.5µm SB-C18



Kinetex 2.6µm C18



Conditions for both columns:

Dimensions:	150 x 4.6 mm
Mobile Phase:	A: Water B: Acetonitrile
Gradient:	(95:5) A/B for 1.16 min, then to (5:95) A/B
Flow Rate:	1.5 mL/min
Temperature:	45 °C
Detection:	UV @ 254 nm
Instrument:	Agilent 1200

Sample:

1. Pyridine
2. Acetaminophen
3. Pindolol
4. Quinine
5. Acebutolol
6. Chlorpheniramine
7. Triprolidine
8. Prednisolone
9. Nortriptyline
10. 4-Chlorobenzoic acid
11. 5-Methyl-2-hydroxy benzaldehyde
12. 4-Chlorocinnamic acid
13. Diazepam
14. Diflunisal
15. Niflumic acid
16. Hexanophenone

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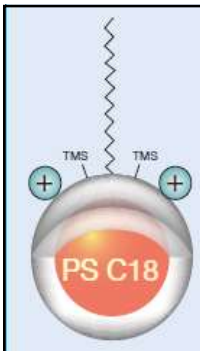
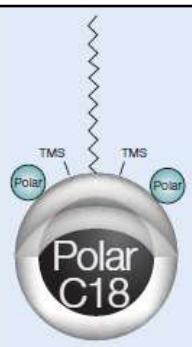
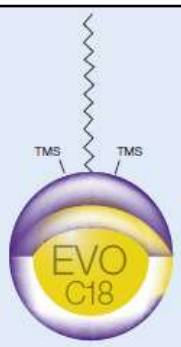
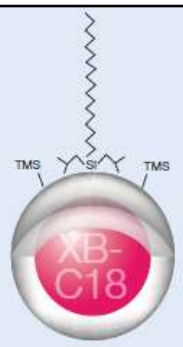
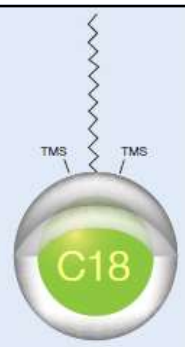
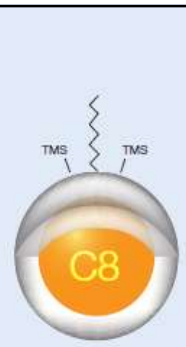
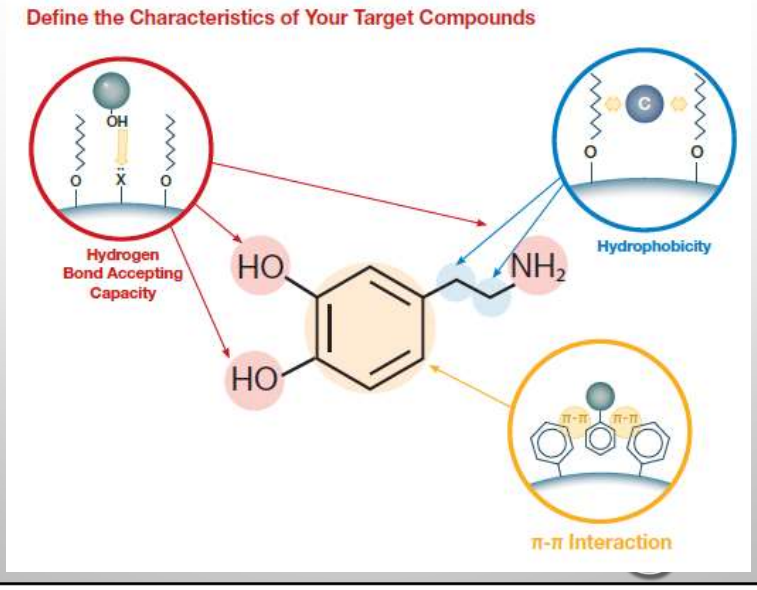
					
100% Aqueous , good peak shape for basic compound	**Polar & Non-Polar retention , for 100% Aqueous mobile phase**	**High pH stable C18**	**Improved peak shape of basic compound and increased retention of acidic compound**	**Core-Shell C18 universal**	**Core-Shell C8 universal**
pH Range 1.5-8.5 USP L1	pH Range 1.5-8.5 USP L1	pH Range 1-12 USP L1	pH Range 1.5-8.5 USP L1	pH Range 1.5-8.5 USP L1	pH Range 1.5-8.5 USP L7

RELATING SELECTIVITY TO UHPLC STATIONARY PHASE



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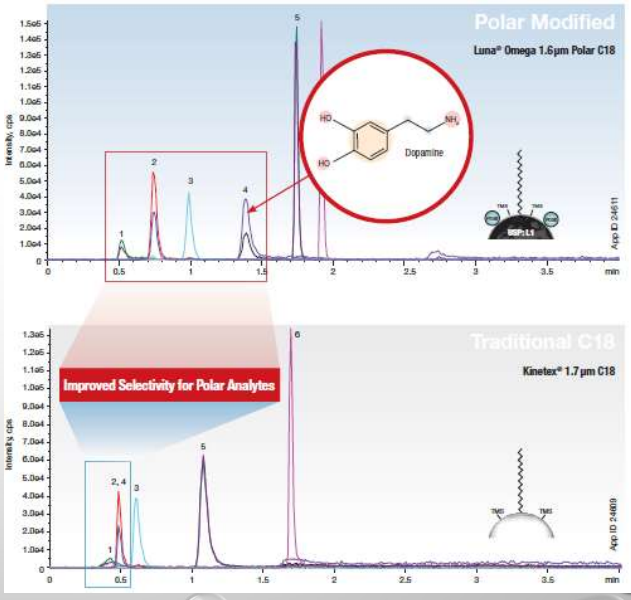
POLAR SELECTIVITY OF CATECHOLAMINES

Conditions for both columns:

Columns: Luna Omega 1.6 µm Polar C18
Kinetex 1.7 µm C18
Dimensions: 50 x 2.1 mm
Mobile Phase: A: 10 mM Ammonium Formate with 0.1% Formic Acid
B: Acetonitrile with 0.1% Formic Acid
Gradient:

Time (min)	% B
0	0
3	90

Flow Rate: 0.4 mL/min
Injection Volume: 1 µL
Temperature: 22°C
Detection: MS/MS (SCIEX API 4000™)
Sample: 1. Norepinephrine
2. Epinephrine
3. Normetanephrine
4. Dopamine
5. Metanephrine
6. Serotonin



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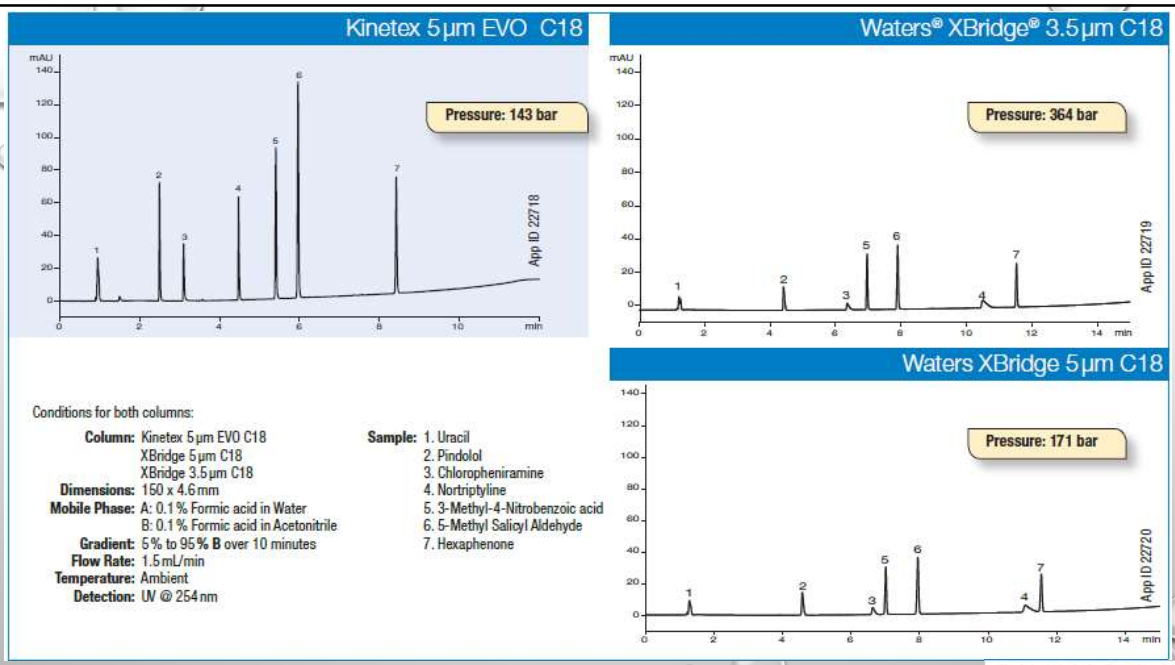

Conditions for both columns:

Column: Kinetex 5 µm EVO C18
XBridge 5 µm C18
XBridge 3.5 µm C18
Dimensions: 150 x 4.6 mm
Mobile Phase: A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Acetonitrile
Gradient: 5% to 95% B over 10 minutes
Flow Rate: 1.5 mL/min
Temperature: Ambient
Detection: UV @ 254 nm

Sample: 1. Uracil
2. Pindolol
3. Chlorpheniramine
4. Nortriptyline
5. 3-Methyl-4-Nitrobenzoic acid
6. 5-Methyl Salicyl Aldehyde
7. Hexaphenone

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Avoiding Problems

"Chromatographic Hygiene"

- GUARD COLUMNS
  - Particulate matter from piston seals and injection valve rotors can clog columns if they are not removed
- 0.2 µm SYRINGE FILTER
  - With sub-2 µm columns, every sample must be filtered through a 0.2 µm porosity filter
- MOBILE PHASE FILTERS
  - Fresh buffers must be made daily and filtration through a 0.2 µm porosity filter is required
- INLINE FILTERS:
  - I strongly recommend using a 0.2 µm porosity in-line filter between the autosampler and guard column

LC/GC Magazine
November 1, 2010
UHPLC Tips and Techniques
By John W. Dolan

Benefits of Guard Columns

- Why Use Guard Columns?
  - Protect valuable analytical columns by removing particulates and strongly retained sample components that may accumulate on column
  - Increase lifetime of analytical column
  - Maintain
    - High column efficiencies
    - Resolution
    - Peak shape
  - Cost-effective

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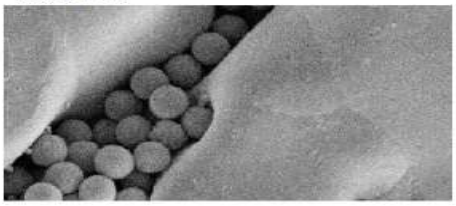
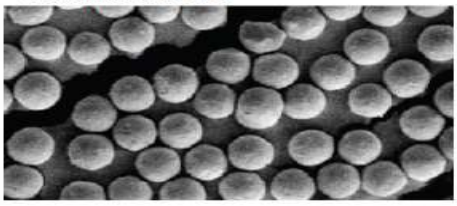
Benefits of Guard Columns

Protected Column

Inlet Frit



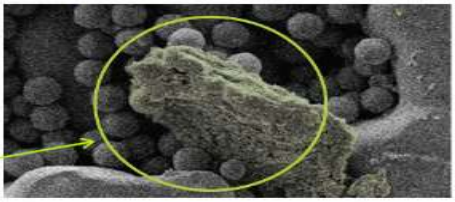
Column Media



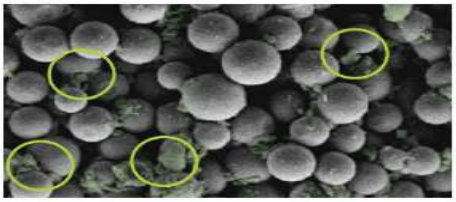
Unprotected Column

Enrichment of particles and impurities

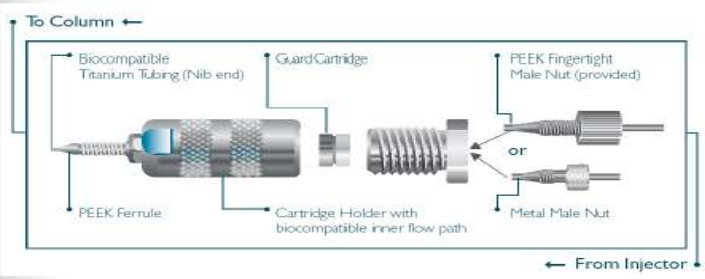
Inlet Frit



Column Media



SecurityGuard™ SecurityGuard™ ULTRA



- SecurityGuard (left) for fully porous ≥3 µm columns
  - Cartridge only 4 mm long
- SecurityGuard ULTRA (right) for core-shell and sub-2 µm columns (2.0 to 4.6 mm ID)
  - Low dead volume (<0.3 µL)
  - Pressure rated up to 20,000 psi (1378 bar)
- Protects against damaging chemical contaminants and microparticulates
- Will NOT alter chromatography & Easy to Use

When Should I Replace Guard Column?

Symptoms	Criteria
• Increasing system backpressure	• >20 % or before system auto-shutdown
• Loss of peak efficiency	• Efficiency (N) decreases >20 %
• Loss of peak resolution (merging, shifting)	• Resolution (R_S) decreases 10 %
Every time the analytical column is replaced, the guard column should also be replaced.	

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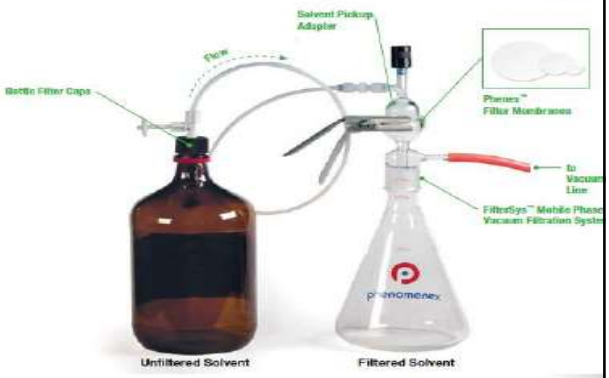
Mobile Phase Filtration

Phenex™ Filter Membrane

- 47 mm diameter
- 0.2 + 0.45 µm
- RC, PTFE, PES, NY

Funnel for successive filtration



Solvent pickup adaptor for continuous filtration

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Solvent Inlet Filter

- Unfiltered or contaminated solvents (microbiological growth) can clog solvent inlet filters
- This has an impacting on pump performance
- HPLC system has to work harder to pull mobile phase into the pump or even sucks air
- Clean the solvent inlet filters whenever preparing new mobile phase and replace it once every 3-6 months

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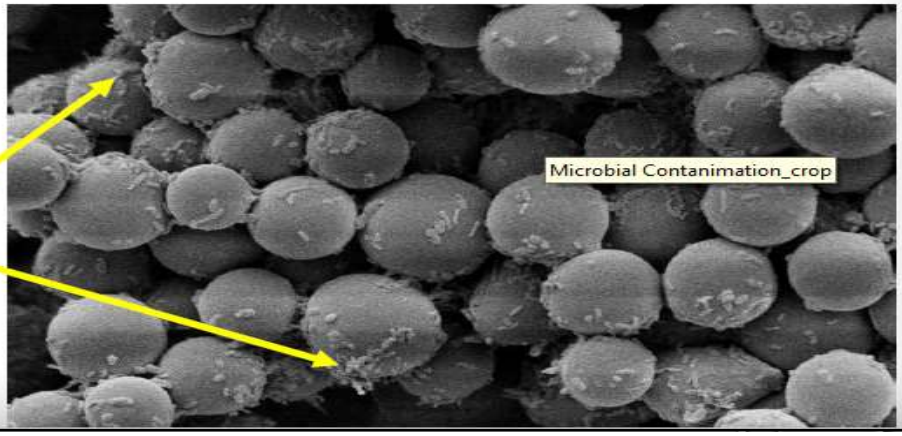
Mobile Phase Filtration

- Strongly recommend preparing aqueous buffers fresh DAILY to minimize microbial growth, which can foul in-line filters and columns

Microbial growth on packing material at inlet of column



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Sample Filtration

Syringe Filter

Select filter diameter based on sample volume

If your sample volume is:		
< 2 mL Sample Volume	2 to 10 mL Sample Volume	10 to 100 mL Sample Volume
4 mm Diameter	15 - 17 mm Diameter	25 - 28 mm Diameter

Preparing Isocratic Mobile Phase

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Isocratic Mobile Phase Preparation

- What is the correct way to mix isocratic mobile phase; and how does it impact chromatography?
- Let's run the following experiment and see if it matters how an isocratic mobile phase is prepared:
  - A. Pre-mix
    - i. Measure water volume, then add organic to total volume?
    - ii. Measure organic volume, then add water to total volume?
    - iii. Measure volume for each component separately, then mix?
  - B. Allow HPLC pump to mix A and B and deliver to column

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Isocratic Mobile Phase Preparation

- Pre-Mix
  - Water first:
    - 400 mL water in graduated cylinder, add to 1-Liter volumetric flask, dilute to mark with MeOH
  - MeOH first:
    - 600 mL MeOH in graduated cylinder, add to 1-Liter volumetric flask, dilute to mark with Water
  - MeOH and Water measured individually:
    - 600 mL MeOH in graduated cylinder, transfer mobile phase reservoir; 400 mL Water in same graduated cylinder and transferred to mobile phase reservoir
- Allow pump to mix A = Water with B = MeOH

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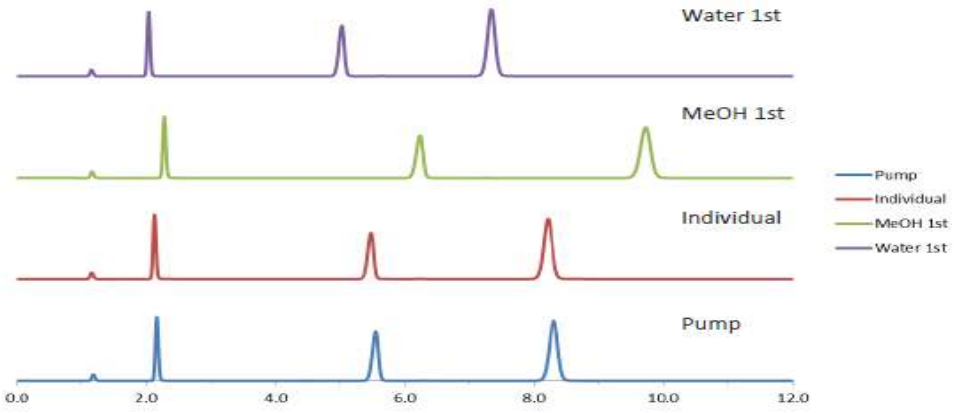
Isocratic Mobile Phase Preparation

Kinetex 5 µm XB-C18
150 x 4.6
60:40 MeOH : H2O
Flow rate: 1.25 mL/min
Inj. Vol: 2 µL
UV @ 254 nm



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Troubleshooting Approach & Diagnostic Tools

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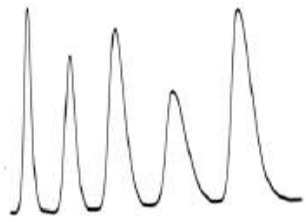
Tailing – All Peaks

Diagnostic Considerations

All Peaks = Mechanical

1. **Column Void (gap)**
2. **Connectivity**
3. **Physical/Particulate Blockage**



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Zero Dead Volume Connection

Figure 1.
Ideal Flush Connection

Figure 2.
Incorrect/Poorly Made Connection

Figure 3.
Chromatogram of Poor Connection and Good Connection Overlay

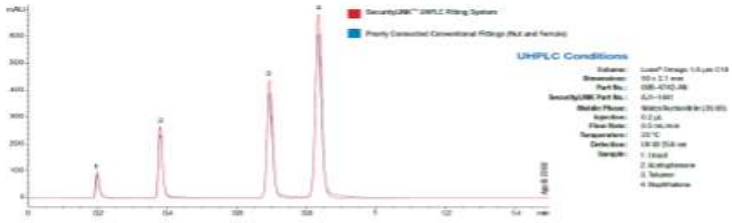
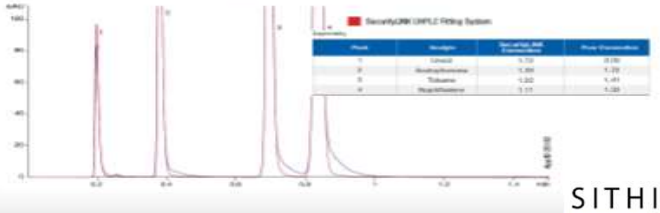


Figure 4.
Zoom In of Poor Connection and Good Connection Overlay



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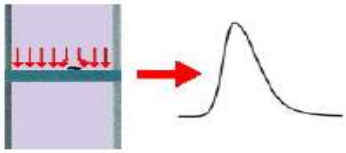
Column Frits

1. Contain media within column while allowing mobile phase to pass
2. Larger particles can block flow path
3. Peak Tailing and High Backpressure



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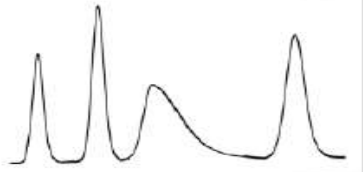
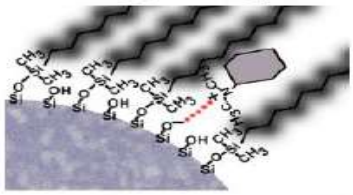
Tailing – Isolated Peak

Diagnostic Considerations

Isolated Peak(s) = Chemical Interaction

1. Free (weak acid) silanols
2. Cation-Exchange with Basic Compounds
3. Secondary Interaction Mechanism





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Column Care

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Column Cleaning

- I am often asked "how can I clean my HPLC/UHPLC column?"
- My typical answer is: "do not get it dirty"
- A bit flippant, however, so let me explain…….

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Column Cleaning

- Consult with column manufacturer for recommended cleaning procedures and limitations
  - Reverse flushing is best if allowed
- Flush column with mobile phase minus buffer to remove all buffer salts
- Flush with 10 column volumes (or more) of mutually miscible solvent such as methanol or acetonitrile
- Flush with 20 column volumes (or more) of strong solvent (THF or IPA) to remove strongly adsorbed sample components
- Reverse the process and equilibrate with mobile phase & retest the column

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Routine Column Cleaning

Reversed phase silica columns

1. **Flush with water:organic (methanol or acetonitrile) 95:5 or the same ratio as used in the analysis**
2. **Increase the organic percentage in a gradient up to 100% (or at least 20% greater than final mobile phase ratio)**
3. **Flush with 40:60 water:organic or any similar ratio for short-term storage**

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Column Storage

Reversed phase silica columns

1. Remove buffer salts before storage
2. Keep pores wetted with at least 30% organic
3. Elute adsorbed components before storage, but do not precipitate out buffer salts in the column
4. Never leave a column static at an elevated temperature

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Tips for Extending Column Lifetimes & Minimizing Problems

- Make sure system is clean before installing a new column
  - This includes flushing old mobile phase and buffer from system before installing new column
- Make fresh mobile phase daily
  - Microbacterial growth can occur in aqueous mobile phase, and this can damage columns and systems
- Use guard columns
- Filter samples before injection, no matter how 'clean' they appear

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Summary

A. Practice Good Chromatographic Hygiene
B. Check the Simple Things First
C. Structured Diagnostic Approach
   1. Mechanical pathway
   2. Chemical interaction pathway
   3. Diagnostic tools – Full Chromatographic Profile, Backpressure, Void Time (t_0)
D. Column Care

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