Discovery Sciences

High Speed Chromatographic Analysis Using Sub-2 Micron HPLC Packings

KVCV studiedag – 12 Oktober 06

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GRACE

Outline

Introduction

Particle size evolution

Column volume and efficiency

1.5 µm media

Column hardware

Conclusions

The Need for Speed and Resolution

Today's Laboratories Demand:

- Greater Productivity – Do more "faster" - Speed
- Greater Sample Complexity - Resolution
- Increasing instrumentation technology

The need to produce results faster means a need for products that can produce equal or better results in less time.

Resolution

Resolution (Rs) is directly proportional to selectivity and the square root of column efficiency.

$$Rs = \frac{1}{4}\sqrt{N*((\alpha-1/\alpha)*(k'1+k'))}$$

Increasing resolution allows:

- Greater sample complexity
- Faster flow rates
- Shorter columns

Balancing Speed and Resolution

The Trade Off

- Faster flow rates – More speed, less resolution
- Slower flow rates – Less speed, greater resolution

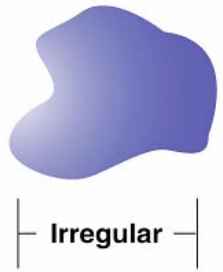
Solution = Efficiency and Selectivity.

Higher efficiency combined with the right selectivity allows faster flow rates and shorter columns.

By decreasing particle size, column efficiency is increased and greater resolution is achieved.

↓ µm > N

Particle Size Evolution

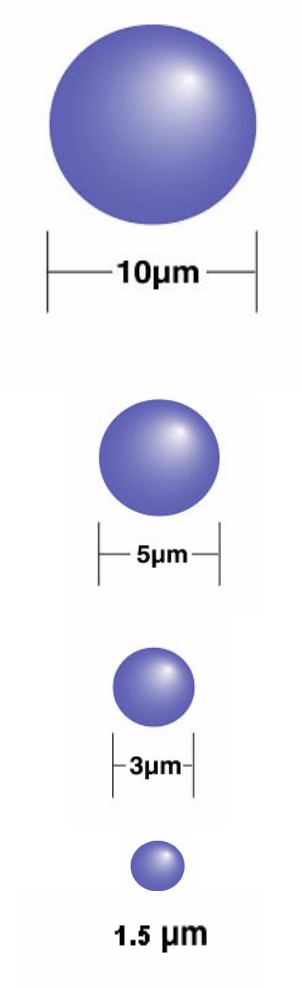


1970's
10 µm Irregular micro-porous
Efficiency = 45,000 plates/meter

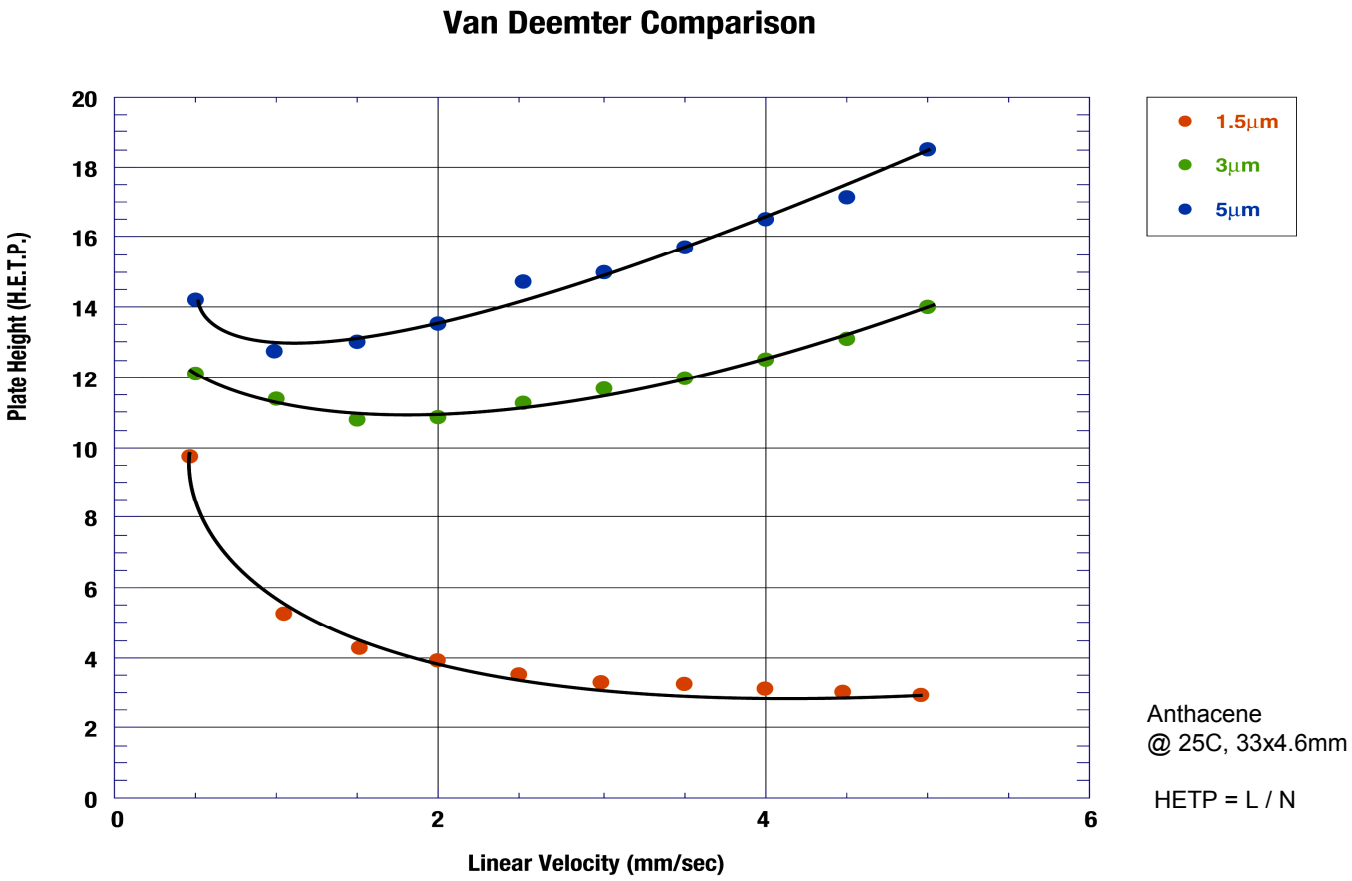
1980's
3-5µm Spherical micro-porous
Efficiency = 85,000-120,000 plates/meter

Early 1990's
1.5µm Spherical Non-porous
Efficiency = 150,000 plates/meter

1996 – Alltech Introduces
Spherical micro-porous
1.5µm Spherical micro-porous
Efficiency = >200,000 plates/meter

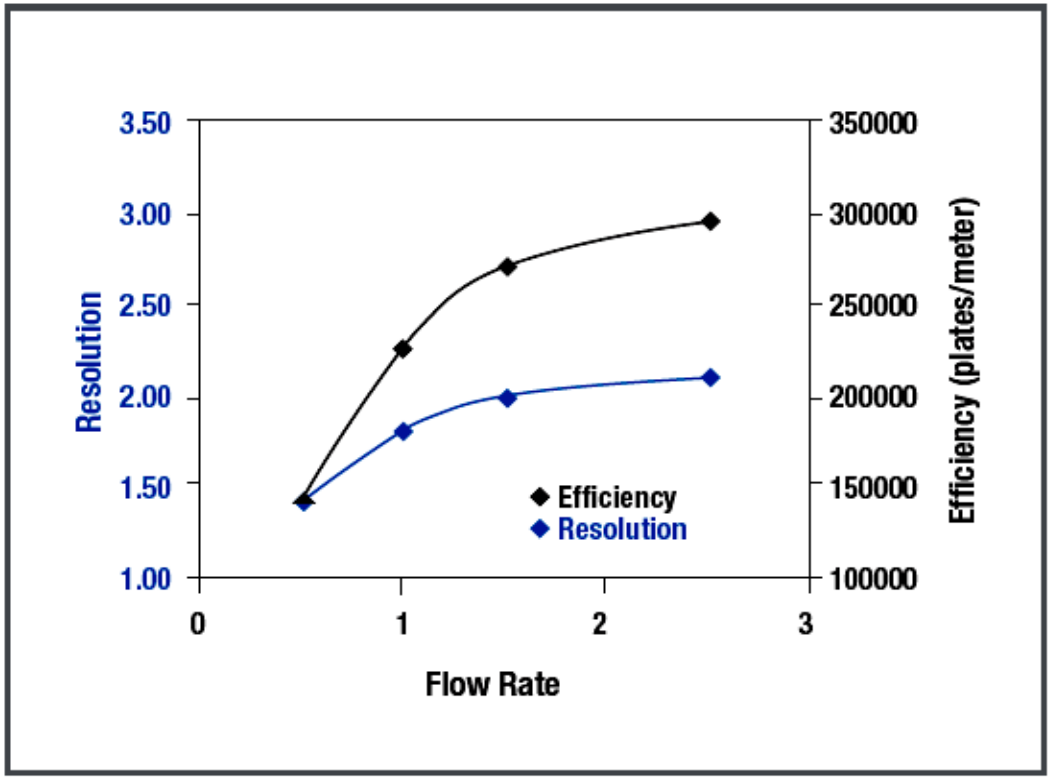


Particle Size and Linear Velocity



Reducing particle size extends optimal linear velocities allowing faster run times without reducing efficiency.

Efficiency and Resolution vs. Flow Rate 1.5µm



Platinum ™ C18 Column 1.5µm, 33 x 7mm
Butyl Paraben Propyl Paraben

With optimal linear velocities extending over a wider range, efficiency and resolution is maintained allowing higher flow rates.

Balancing Speed, Resolution, and Sensitivity

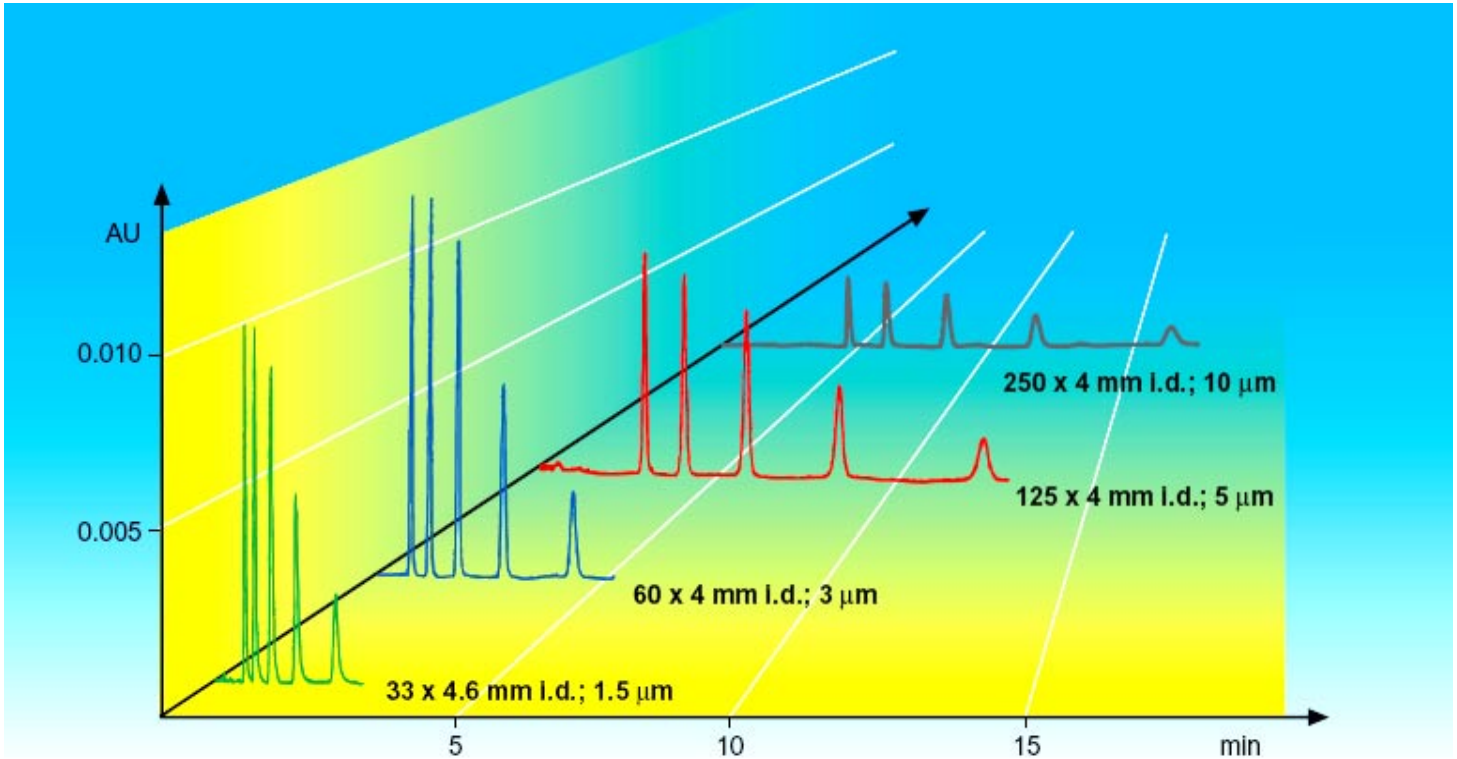
Col Length	Part. Size	Analysis Time	N/m Peak 5*	N/col	Solv. use	Res	Sensitivity	Pressure
mm	µm	min	1/m	1/Col	mL	1&2	Peak 1 AU	psi
250	10	18.1	36000	9000	10.9	4.8	0.004	200
125	5	9.4	75000	9375	5.6	5.2	0.008	500
60	3	4.8	120000	7200	2.9	4.6	0.013	1100
33	1.5	2.5	225000	7425	2	3.9	0.011	2500

Column I.D. 4mm
Homologus Series – Alkyl benzoates at equivalent flow rates
*pentyl benzoate

By reducing column length and particle size proportionally, overall analysis time is reduced and sensitivity is increased.

Comparison of speed, sensitivity, and resolution

Homologus Series – Alkyl Benzoates at equivalent flow rates



Stationary phase: 100 A ODS-2 FE; **Linear velocity:** 1.8 mm/s; **Eluent:** ACN:H2O = * 65:35, resp. ** 60:40; **Flow cell:** 1.2 µl / 3 mm with quick connector (100 x 0.1 mm capillary); **Injection:** 5 µl benzoate test mixture / 1:100 dil. (methyl-, ethyl-, propyl-, butyl-, pentyl benzoate; 10–20 mg/ml)

Effect of Extra Column Volume on Efficiency

Reducing column length increases the effect of additional system volume outside the column.

This will erode benefits gained from increased efficiency of small particles in short columns.

Two approaches can be taken to minimize this effect.

1. Increase column volume without increasing length, allowing higher system flow rates.

2. Decrease system volume.

Effect of Extra Column Volume on Efficiency

Approach #1 – Increase column i.d.

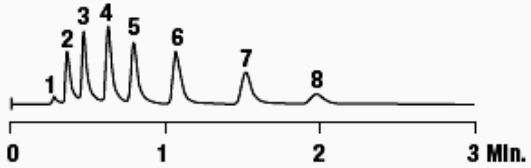
Chromatographic comparison on standard LC system.

Column: Standard Platinum™ C18, 1.5µm, 30 x 7mm
Flow rate: 2.31mL/min

CHROM 8725

Column: Standard Platinum™ C18, 1.5µm, 30 x 4.6mm
Flow rate: 1.0mL/min

CHROM 8726



Mobile Phase: 0.025M NaH_2PO_4, pH3:Acetonitrile, Platinum™ C18 (50:50),

Detector: UV at 254nm

Hydrophilic Preservatives

1. Kathon® Impurity
2. 2-methyl-4-isothiazolin-3-one
3. 5-chloro-2-methyl-4-isothiazolin-3-one
4. Methylparaben
5. Ethylparaben
6. Propylparaben
7. Butylparaben
8. BHA or 3-(t-butyl)-4-hydroxyanisole

Even though these two columns were run on the same system, the larger column ID format runs at higher flow rates reducing the over all effect of system volume and dramatically improving peak shape and efficiency allowing for even faster run times.

4.6mm vs. 7mm Rocket™ id– standard system

Standard Platinum™ C18 1.5µm 30x**4.6mm**	k'	N/meter	N/column	Assymetry
2-Methyl-4-isothiazolin-3-one	0.30	28,567	857	2.5
5-Chloro-2-methyl-4-isothiazolin-3-one	0.67	38,700	1161	3
Methylparaben	1.25	56,133	1684	2.2
Ethylparaben	1.83	65,367	1961	2
Propylparaben	2.82	78,400	2352	2
Butylparaben	4.44	90,567	2717	1.7
BHA or 3-(t-butyl)-4-hydroxyanisole	6.07	93,633	2809	1.24
Standard Platinum™ C18 1.5µm 30x**7mm**	k'	N/meter	N/column	Assymetry
2-Methyl-4-isothiazolin-3-one	0.35	131,367	3941	1.9
5-Chloro-2-methyl-4-isothiazolin-3-one	0.72	166,767	5003	1.7
Methylparaben	1.16	208,733	6262	1.5
Ethylparaben	1.65	231,433	6943	1.4
Propylparaben	2.46	238,067	7142	1.3
Butylparaben	3.74	250,500	7515	1.2
BHA or 3-(t-butyl)-4-hydroxyanisole	4.88	233,267	6998	1.1

Larger column i.d. reduces effects of extra column volume and improves performance on standard LC systems by allowing faster flow rates, “sweeping” extra column volume faster.

Effect of Extra Column Volume on Efficiency:

Approach #2 – Decrease system volume

Requirements of microbore and analytical columns, driven by need for greater sensitivity and limited sample volumes, have dramatically reduced modern system volumes.

This reduction in system volume now allows for greater benefits from shorter columns and minimizes extra column effects. This allows for short analytical and microbore column formats without compromising efficiency.

Effect of Extra Column Volume on Efficiency

Approach #2 - Decrease system volume

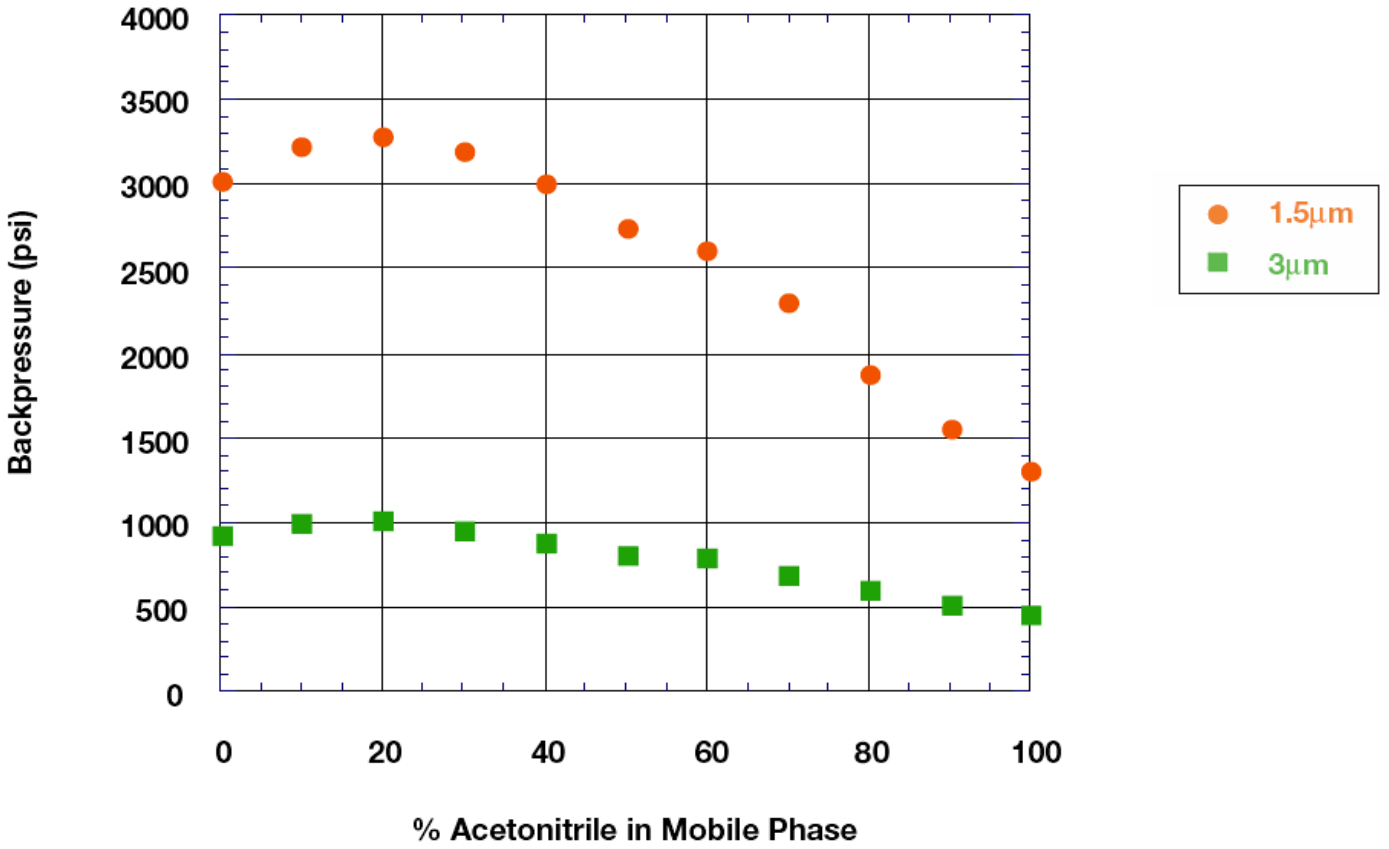
Column Efficiency vs. System Volume Comparison

Platinum™ EPS Porous Silica-C18 1.5µm, 33 x 4.6mm	Methyl Paraben (N/m)	Ethyl Paraben (N/m)	Propyl Paraben (N/m)	Butyl Paraben (N/m)
Optimized System 1:	146272	156000	165788	173030
Standard System 2 with no additional attachments:	74909	81424	94182	109000
Standard System 2 with guard	44121	50393	61818	73667
Standard System 2 with 0.5µm filter	59879	70788	86303	105697

Optimized System = Microbore ~ 100uL total volume
Standard System = Standard valve and Flow Cell ~ 400uL volume
Standard w/ Guard = Total volume ~ 700uL

Effect of optimizing system volumes and reducing extra column volume.

Back Pressure Correlation



Platinum™ C18 Column, 30 x 4.6mm

Observed back pressure of 3µm vs 1.5µm particle sizes at various mobile phase concentrations. 1.5µm produce higher backpressures but at 30mm column lengths are still well under maximum system pressures.

Grace 1.5µm Media

•Platinum™	Designed for high throughput and selectivity of small molecule polar and non polar compounds.
•Alltima™ HP HILIC	Hydrophilic Interaction for high retention of polar compounds.
•ProSphere™ ZAP! C18	500Å C18 for biomolecule separations

Grace 1.5µm Media

Platinum™

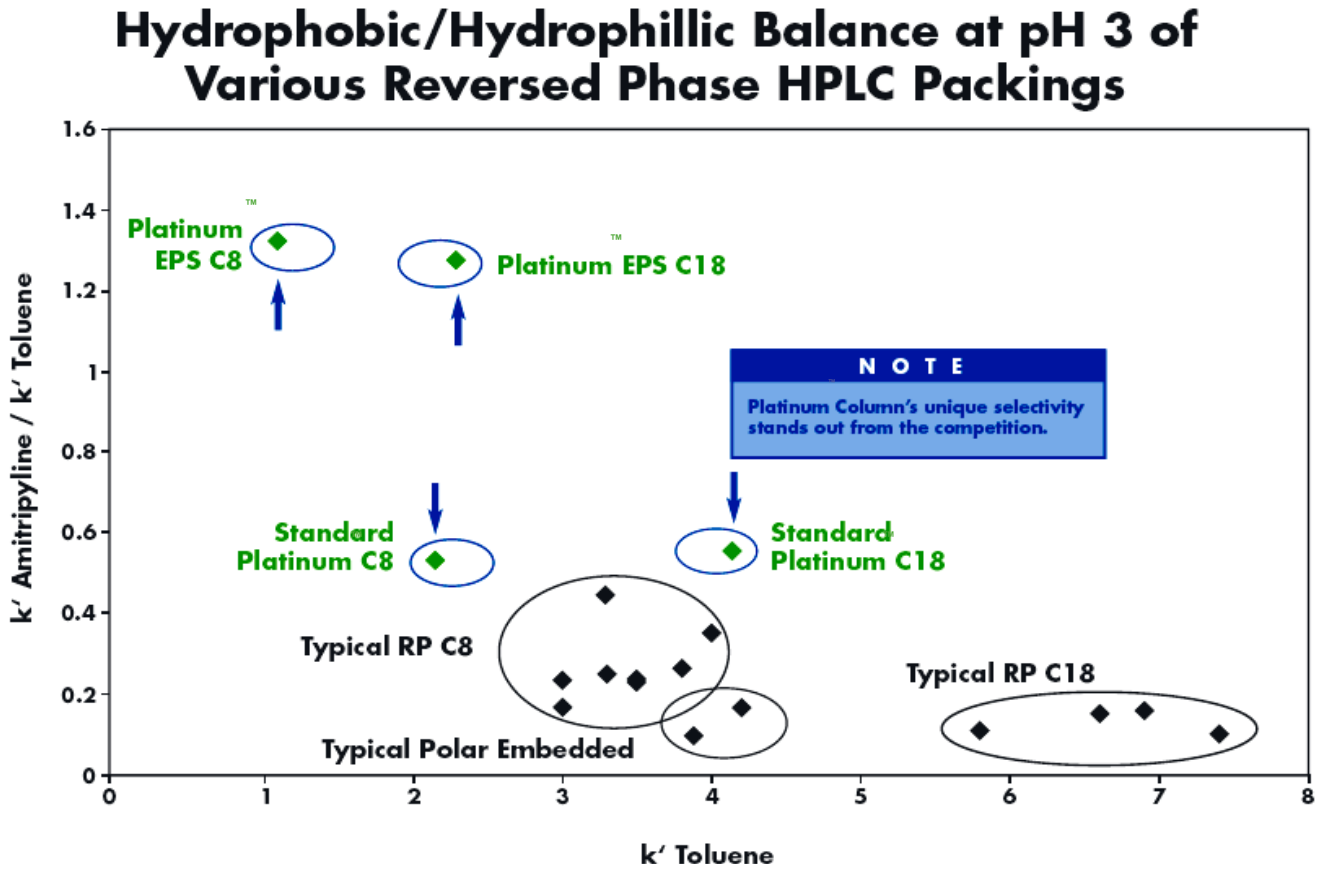
High Throughput Media Optimized for High Speed Applications.

Unique Selectivity of Polar and Non-polar Compounds

- Porous Spherical Silica Base
- 100A pore size, 200m^2/g surface area
- Available in C18 and C18EPS phases
- 1.5, 3, 5, & 10µm particle sizes

Grace 1.5µm Media

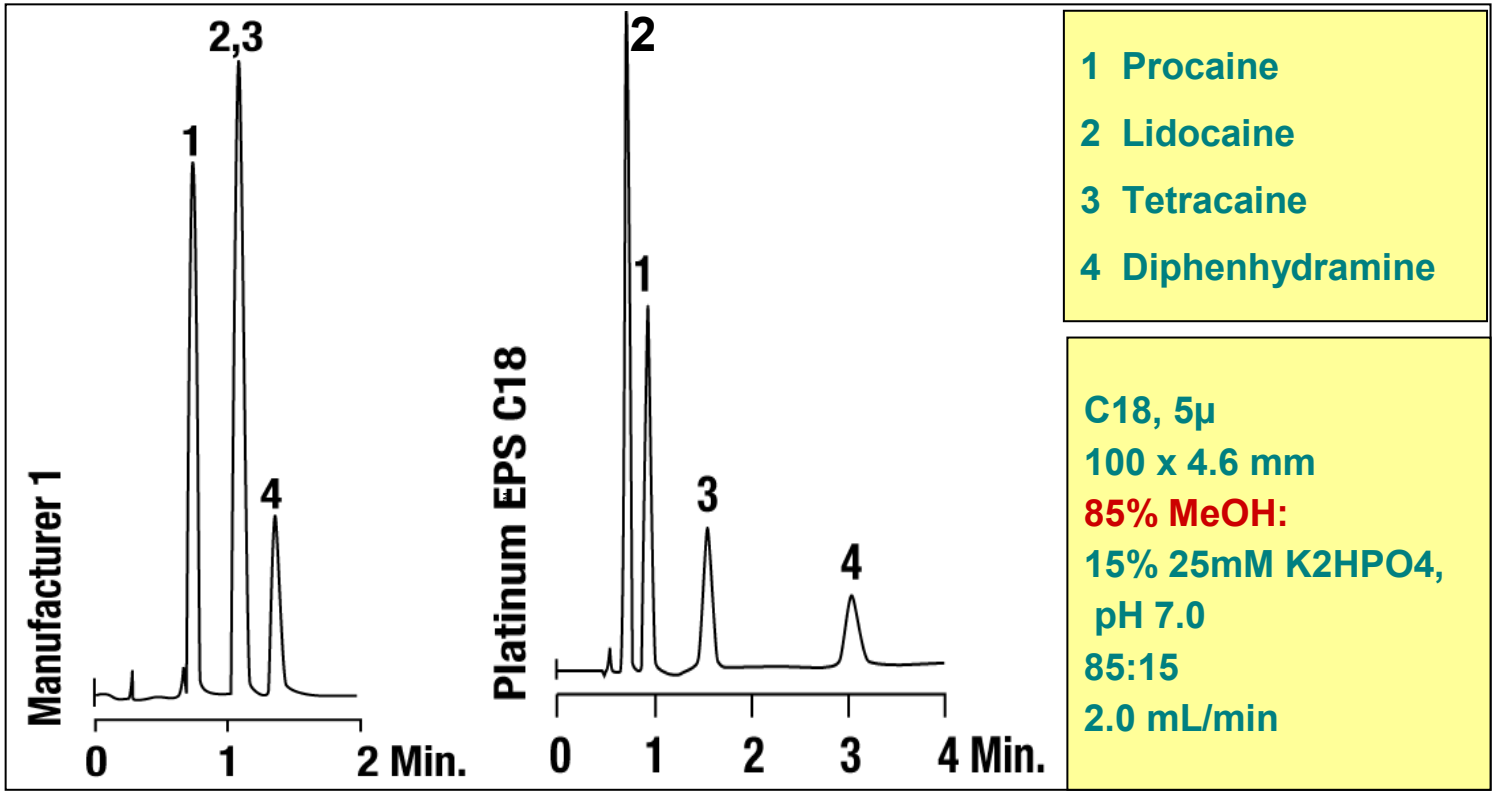
Platinum™ – Extended Polar Selectivity



Controlled Silica Exposure – Utilize silica's hydrophilic surface for unique selectivity of polar compounds and high throughput of non-polar compounds.

Grace 1.5µm Media

Platinum™ – Extended Polar Selectivity



1 Procaine
2 Lidocaine
3 Tetracaine
4 Diphenhydramine

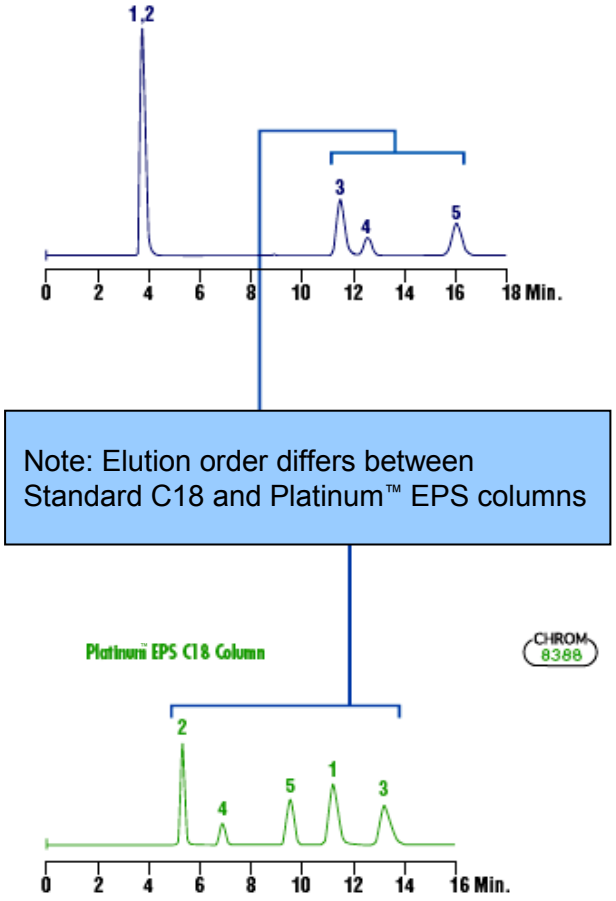
C18, 5µ
100 x 4.6 mm
85% MeOH:
15% 25mM K2HPO4,
pH 7.0
85:15
2.0 mL/min

NH2>COOH>CHO>CN=OH>NO2>OCH3>Cl>F>

Platinum™ EPS C18's ability to retain polar compounds is strong enough that under 85% organic conditions selectivity differences are seen with basic compounds.

Grace 1.5µm Media

Platinum™ – HTS phases reduce the “gap”



Column: 5µm, 150 x 4.6mm

Mobile Phase: 0.025M KH2PO4,

pH 3.0:Methanol (50:50)

Flow Rate: 1.0mL/min

Detector: UV at 220nm

1. Pindolol

2. Pentoxifylline

3. Isoxsuprine

4. Nifedipine Degradant

5. Nifedipine

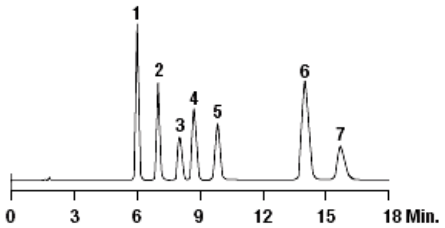
Lower %C and increased polar interactions reduces the “gap” normally observed with RP HPLC separations as neutral non-polar compounds elute faster and polar compounds retain.

Grace 1.5µm Media

Platinum™ – Applications

Triazine Herbicides

CHROM 8669

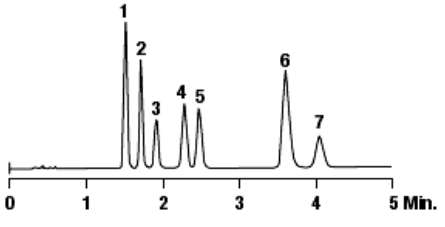


1. Simazine
2. Simetryn
3. Prometon
4. Atrazine
5. Ametryn
6. Prometryn
7. Terbutryn

Column: Platinum EPS 100Å C18, 5µm, 150 x 4.6mm
Mobile Phase: 0.025M Potassium Phosphate, Monobasic pH3: Acetonitrile (65:35)
Flow rate: 1.0mL/min
Detector: UV at 254nm

70% Faster

CHROM 8671



1. Simazine
2. Simetryn
3. Prometon
4. Atrazine
5. Ametryn
6. Prometryn
7. Terbutryn

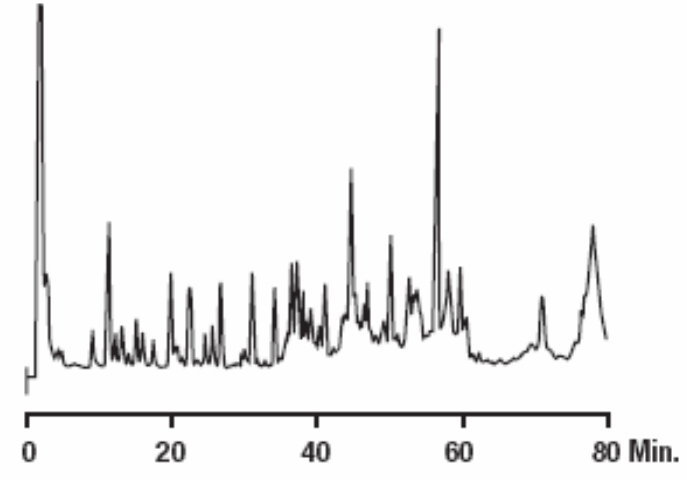
Column: Platinum EPS 100Å C18, 1.5µm, 30 x 7mm
Mobile Phase: 0.025M Potassium Phosphate, Monobasic pH3: Acetonitrile (65:35)
Flow rate: 2.0mL/min
Detector: UV at 254nm

Grace 1.5µm Media

Platinum™ – Applications

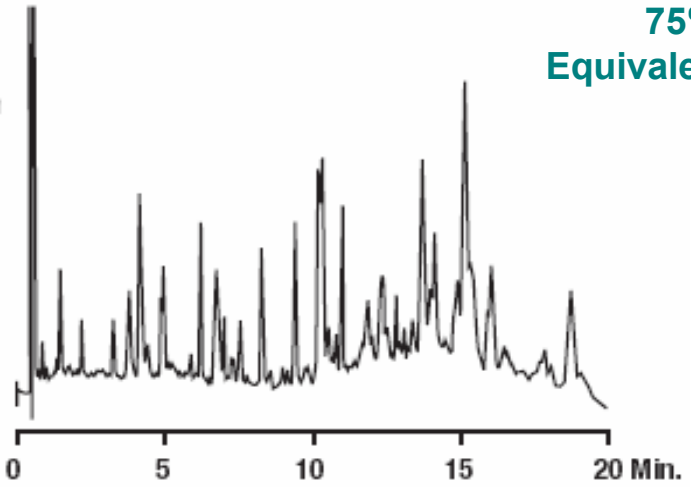
Peptide Mapping

Column: Conventional Porous Silica-C18, 150 x 2.1mm
Mobile Phase: **A:** 0.065% TFA in Water
B: 15% Water/85% Acetonitrile + 0.05% TFA
Gradient: **Time:** 0, 80
%B: 10, 90
Flow rate: 0.25mL/min
Detector: UV at 220nm



75% Faster
Equivalent peak count

Column: Standard Platinum™ 100Å C18, 1.5µm, 30 x 7mm
Mobile Phase: **A:** 0.065% TFA in Water
B: 15% Water/85% Acetonitrile + 0.05% TFA
Gradient: **Time:** 0, 20
%B: 10, 90
Flow rate: 1.85mL/min
Detector: UV at 220nm



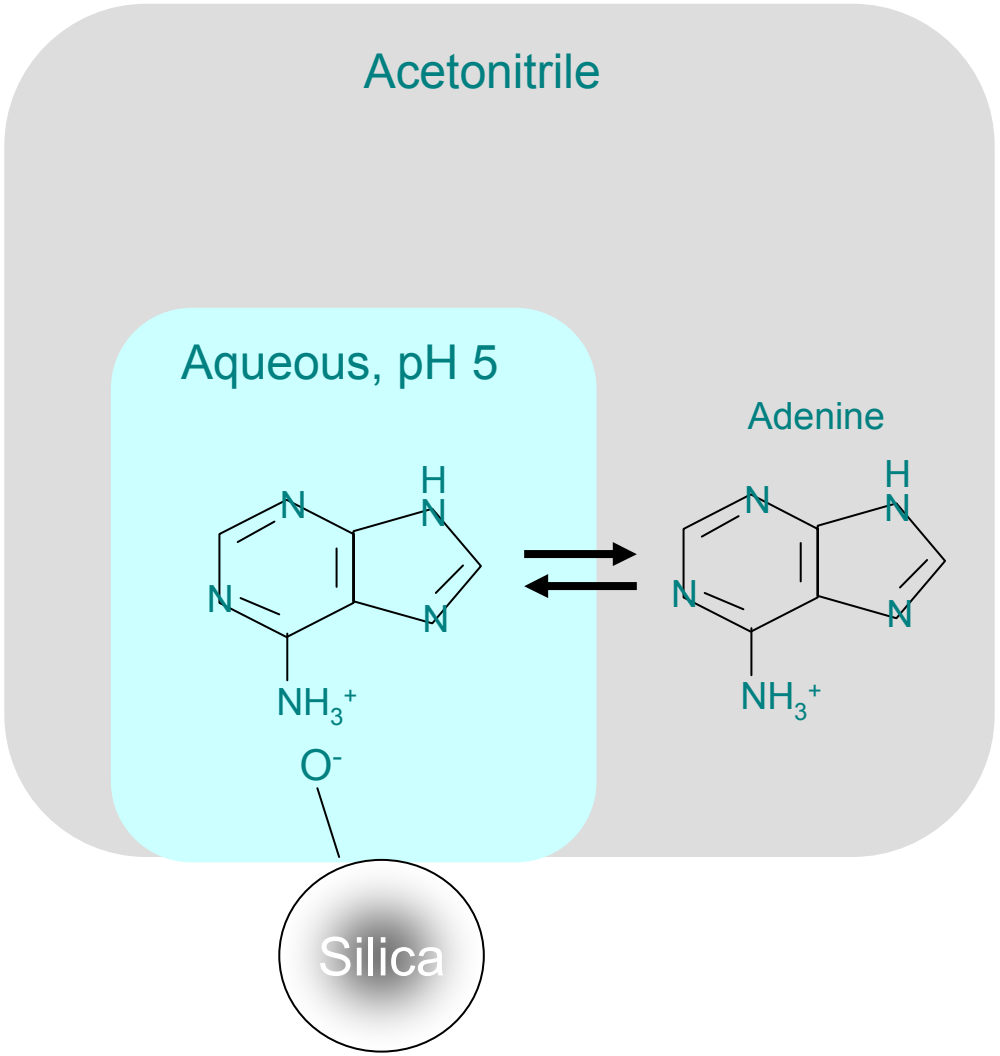
Chromatogram Courtesy of Dr. John C. Le, Amgen Inc., Thousand Oaks, CA

Alltima™ – HP HILIC

Hydrophilic Interaction of Polar Compounds

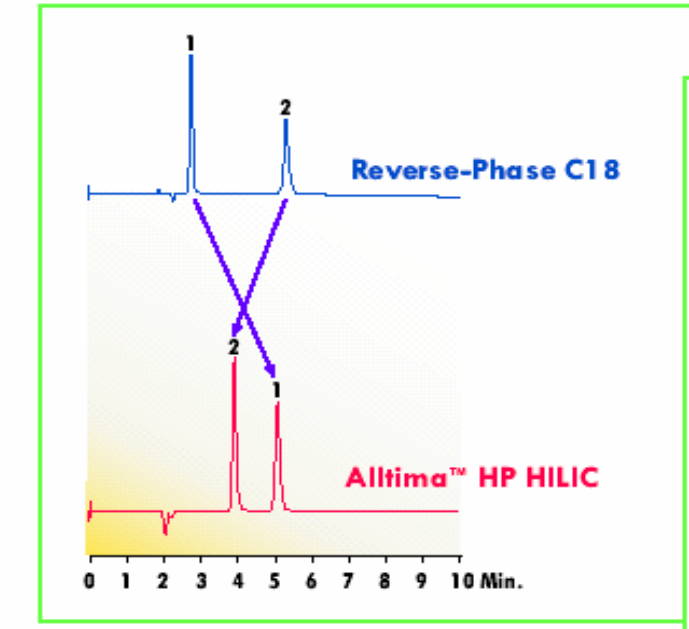
- Porous Spherical Silica Base
- 120A pore size, 200m^2/g surface area
- Un-bonded high purity silica designed for HILIC separation of polar compounds.
- 1.5, 3, & 5 µm particle sizes.

HILIC Mechanisms on Silica



- **Polar analyte partitions into and out of adsorbed water mono-layer.**
- **Charged polar analyte can undergo cation exchange with charged silanol groups.**
- **Combination of these mechanisms results in enhanced polar retention.**

RP vs. HILIC



Basic Drugs

Peak ID: 1. Albuterol, 2. Bamethan

Reverse Phase Column:
Alltech Prevail™ C18,
150 x 4.6mm, 5µm

HILIC Column:
Alltech Alltima™ HP HILIC,
150 x 4.6mm, 5µm

Reverse Phase Mobile Phase:
0.5M Ammonium Formate,
pH 3.0: Acetonitrile (88:12)

HILIC Mobile Phase:
Acetonitrile: 0.5M Ammonium
Formate, pH 3.0 (85:15)

Flow Rate: 1.0mL/min

Injection: 10µL

Detection: UV at 220nm

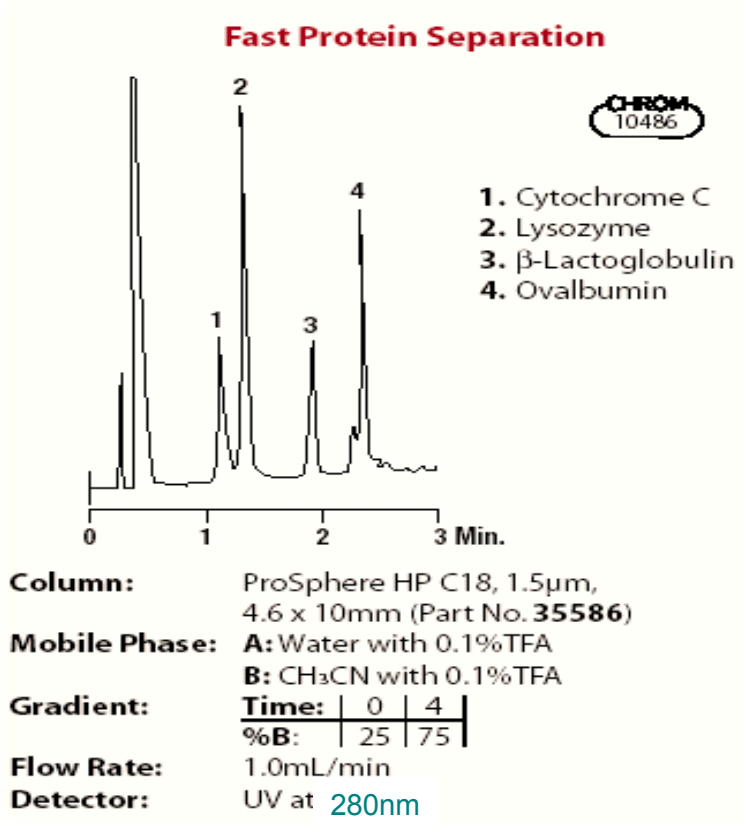
Use of a buffer can be beneficial for

- **retention time reproducibility**
- **peak shape**
- **obtaining different selectivity**

ProSphere™ – ZAP! C-18

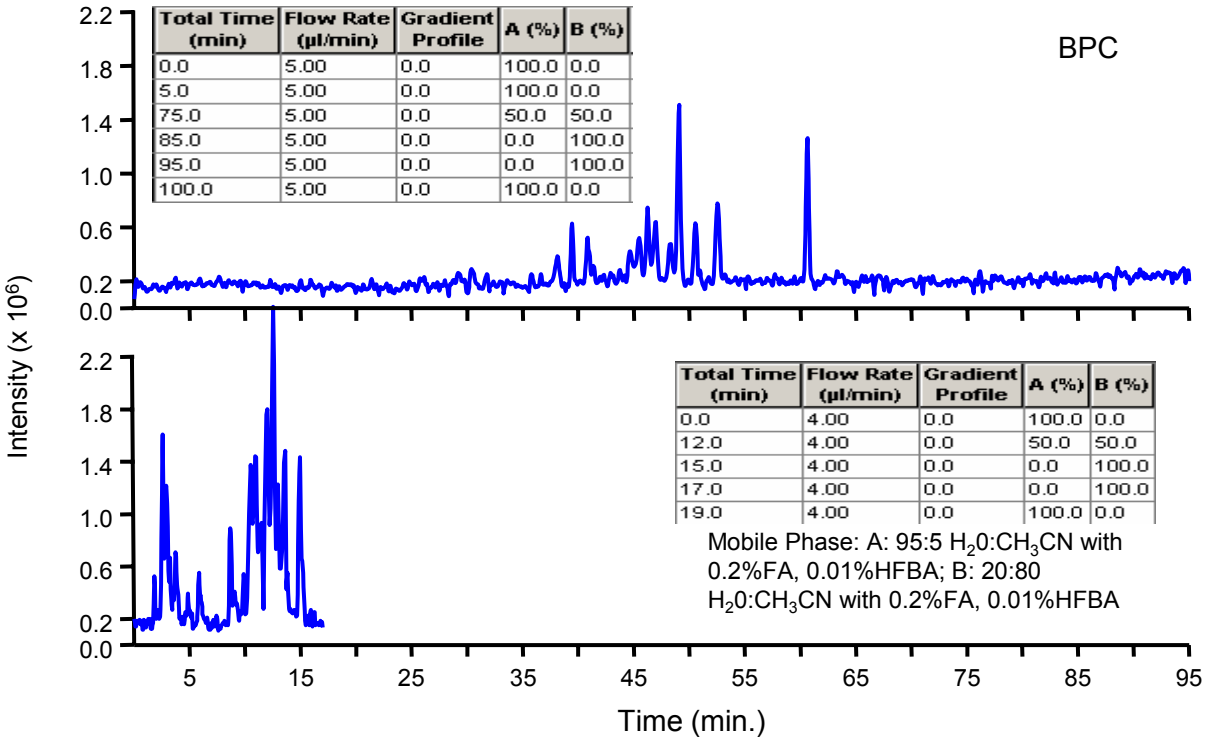
Large Porosity Reversed Phase for Fast Protein Separations.

- Porous Spherical Silica Base
- 500A pore size, 60m²/g surface area
- 1.5µm particle sizes.



5µm, 300Å vs. 1.5µm, 100Å Capillary LCMS

Tryptic Digest of BSA, 7pmol



Total Time (min)	Flow Rate (µl/min)	Gradient Profile	A (%)	B (%)
0.0	5.00	0.0	100.0	0.0
5.0	5.00	0.0	100.0	0.0
75.0	5.00	0.0	50.0	50.0
85.0	5.00	0.0	0.0	100.0
95.0	5.00	0.0	0.0	100.0
100.0	5.00	0.0	100.0	0.0

Total Time (min)	Flow Rate (µl/min)	Gradient Profile	A (%)	B (%)
0.0	4.00	0.0	100.0	0.0
12.0	4.00	0.0	50.0	50.0
15.0	4.00	0.0	0.0	100.0
17.0	4.00	0.0	0.0	100.0
19.0	4.00	0.0	100.0	0.0

Mobile Phase: A: 95:5 H_20:CH_3CN with 0.2%FA, 0.01%HFBA; B: 20:80 H_20:CH_3CN with 0.2%FA, 0.01%HFBA

Column: C18, 300Å, 5µm, 300µm i.d. x 150mm VYDAC® 238EV5.315

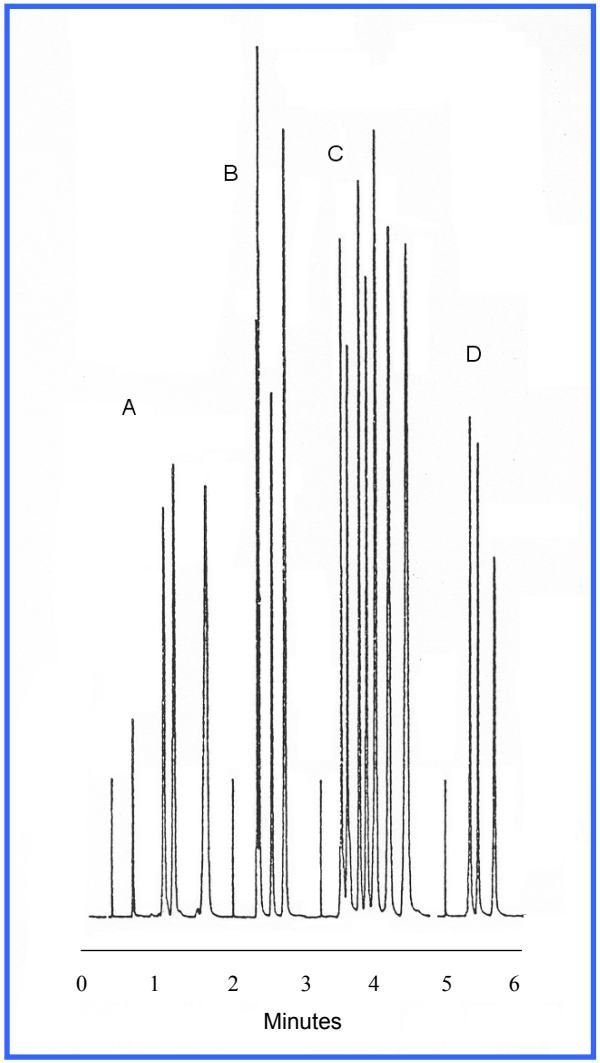
Mascot Results
Score: 928
Queries matched: 22
Sequence coverage: 34%

Column: C18, 100Å, 1.5µm, 300µm i.d. x 33mm

Mascot Results
Score: 1164
Queries matched: 24
Sequence coverage: 42%

• Faster analysis time and better Mascot score with higher sequence coverage using 1.5µm, 100Å column.

Additional Applications



Four multi-component samples in 6 minutes

Phase: **1.5µm C18**
Column: **30 x 4.6 mm**
Eluent: **50% MeCN/50% 50mM KH_2PO_4 pH 3 @ 1mL/min**

A - Uracil, Amitriptyline, Phenylvaleric Acid and Toluene

B - Procaine, Lidocaine, Diphenhydramine and Amitriptyline

C - Nicotine, Quinine, Diphenhydramine, Nortriptyline, 2,4-Dichlorophenoxyacetic, propionic and butyric acids.

D - Pyridine, 8-Hydroxyquinoline and 2,2'-Dipyridyl.

Providing suitable precautions are taken to limit off column band spreading (e.g. <1µL flow cell/ 0.005" connecting tubing or 7mm i.d. columns), 1.5µm phases can give excellent resolution with short analysis times.

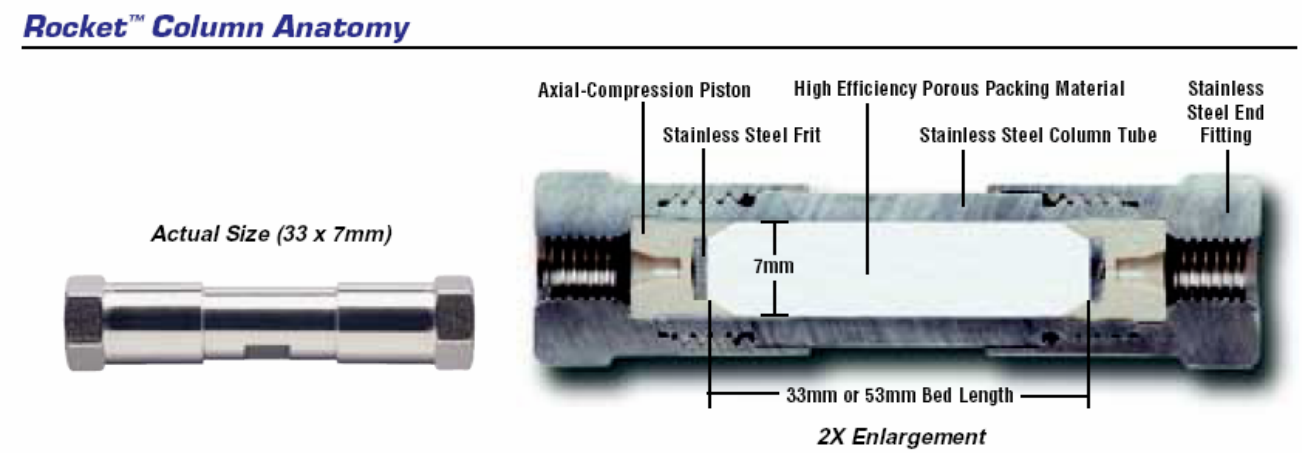
High Speed 1.5µm HPLC Columns

Expedite™ Column – Low Volume HTS Format

10, 20, 30, and
50mm Lengths in
2.1 or 4.6mm i.d.'s

Analytical Column
30 & 50mm Lengths

Rocket™ Column – High throughput on standard LC systems



33 or 53mm Lengths
with 7mm i.d. – designed
For HTS on standard
LC systems

Nano-Microbore 1.5µm HPLC Columns

1.0mm i.d.

0.3 and 0.5mm i.d.

0.075 and 0.15mm i.d.

capillary guard/trap and holder assembly

Hardware Specifications		
Format (i.d.)	**Column Material**	**Fitting Connection**
1.0mm	1/4" 316 Stainless Steel	1/16" 10-32 thread
0.3–0.5mm	1/16" Glass-lined Stainless Steel	1/16" 10-32 thread
0.075–0.15mm	Fused Silica housed in PEEK	1/16" 10-32 thread

Platinum™ High Speed HPLC Columns

Platinum™ 1.5µm Columns

Packing	Phase	Dimensions	Particle Size	Format	Catalog Number
Platinum™	C18	30x4.6mm	1.5um	Analytical	505117
Platinum™	C18	50x4.6mm	1.5um	Analytical	505112
Platinum™	C18	10x2.1mm	1.5um	Expedite™	505100
Platinum™	C18	10x4.6mm	1.5um	Expedite™	505106
Platinum™	C18	20x2.1mm	1.5um	Expedite™	505102
Platinum™	C18	20x4.6mm	1.5um	Expedite™	505108
Platinum™	C18	30x2.1mm	1.5um	LC/MS	505114
Platinum™	C18	50x2.1mm	1.5um	LC/MS	505104
Platinum™	C18	33x7mm	1.5um	Rocket™	50527
Platinum™	C18	53x7mm	1.5um	Rocket™	50529
Platinum™	C18 EPS	30x4.6mm	1.5um	Analytical	505118
Platinum™	C18 EPS	50x4.6mm	1.5um	Analytical	505113
Platinum™	C18 EPS	10x2.1mm	1.5um	Expedite™	505101
Platinum™	C18 EPS	10x4.6mm	1.5um	Expedite™	505107
Platinum™	C18 EPS	20x2.1mm	1.5um	Expedite™	505103
Platinum™	C18 EPS	20x4.6mm	1.5um	Expedite™	505109
Platinum™	C18 EPS	30x2.1mm	1.5um	LC/MS	505116
Platinum™	C18 EPS	50x2.1mm	1.5um	LC/MS	505105
Platinum™	C18 EPS	33x7mm	1.5um	Rocket™	50577
Platinum™	C18 EPS	53x7mm	1.5um	Rocket™	50579

Conclusions

Use of Sub-2 micron packings can dramatically reduce analysis time and increase sample throughput on any HPLC system provided the right considerations are taken.

1. **Correct Media – Selectivity is appropriate for analytes**

2. **Minimize system volume**

3. **Appropriate column format and dimensions**

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Questions ?