

**SUPELCO**  
Solutions within.

## Important Considerations in Optimizing Capillary HPLC Performance

Richard A. Henry, Technical Advisor to Supelco  
and  
Wendy S. Roe, Supelco Division of Sigma-Aldrich



[Dick.Henry@sial.com](mailto:Dick.Henry@sial.com)

[Wendy.Roe@sial.com](mailto:Wendy.Roe@sial.com)

[sigma-aldrich.com/analytical](http://sigma-aldrich.com/analytical) 1

© 2012 Sigma-Aldrich Co. All rights reserved.

**SIGMA-ALDRICH**

### Capillary HPLC (Cap LC)

- Capillary HPLC is a specialized form of LC employed with reduced diameter columns to improve sensitivity by reducing on-column sample dilution.
- Cap LC does not require large sample amounts and may be qualitative and quantitative, often employing mass spec as a detector.
  - LC-MS: excellent qual; adequate quant; ; high sensitivity; volatile mobile phases and additive requirement.
  - LC-UV: excellent quant; limited qual; moderate sensitivity; UV-transparent mobile phases and additive requirement.
- LC Column Terminology (HPLC and UHPLC):

Name	Dimension	$V_0$ ( $V_M$ )
– Prep (collection of pure solute)	10 mm ID and larger	5 mL up
– Analytical (routine assay)	4.6, 3.0 and 2.1 mm ID	1 mL - 0.2 mL
– Microbore	1 mm ID	50 $\mu$ L
– Capillary	0.5 mm and 0.3 mm ID	10 $\mu$ L - 5 $\mu$ L
– Nano	0.1 mm and smaller	<1 $\mu$ L

2

© 2012 Sigma-Aldrich Co. All rights reserved.

**SIGMA-ALDRICH**

## Critical Aspects of a Cap LC Experiment\*

- Pump design and flow control \* No data provided by vendor

Specification	Vendor 1		Vendor 2	Vendor 3	Vendor 4A	Vendor 4B
	Primary	Loading	Primary	Primary	Primary	Primary
Flow Rate ( $\mu\text{L}/\text{min}$ )	0.05 - 50	50 - 2500	0.2 - 100	0.1 - 5.0	0.01 - 4.0	0.01 - 20.0
Flow Precision (% RSD)	*	*	*	*	*	0.7
Composition Precision (%SD)	< 0.2	N/A	< 0.25	*	< 0.2	< 0.2
Max Pressure (bar)	800	500	689	200	400	400
Delay Volume (nL)	< 25	N/A	*	*	300	3,000

- Sample injection volume and technique (focusing)
- Detector internal volume and response
- Instrument internal volume and sample flow path design
- Column internal volume and uniform bed design

\* Many users improvise to optimize; creating a Cap LC instrument is common at this stage of development.

3

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Capillary HPLC Instrument

Thermo Scientific UltiMate 3000 RSLC Nano System (not an endorsement)

- Pump equipped with capillary flow meter
- Heated column compartment
- UV detector equipped with a 45 nL flow cell
- External loop autosampler equipped with 1  $\mu\text{L}$  sample loop and electronic Peltier elements for sample cooling
- 50  $\mu\text{m}$  I.D. tubing connecting all components

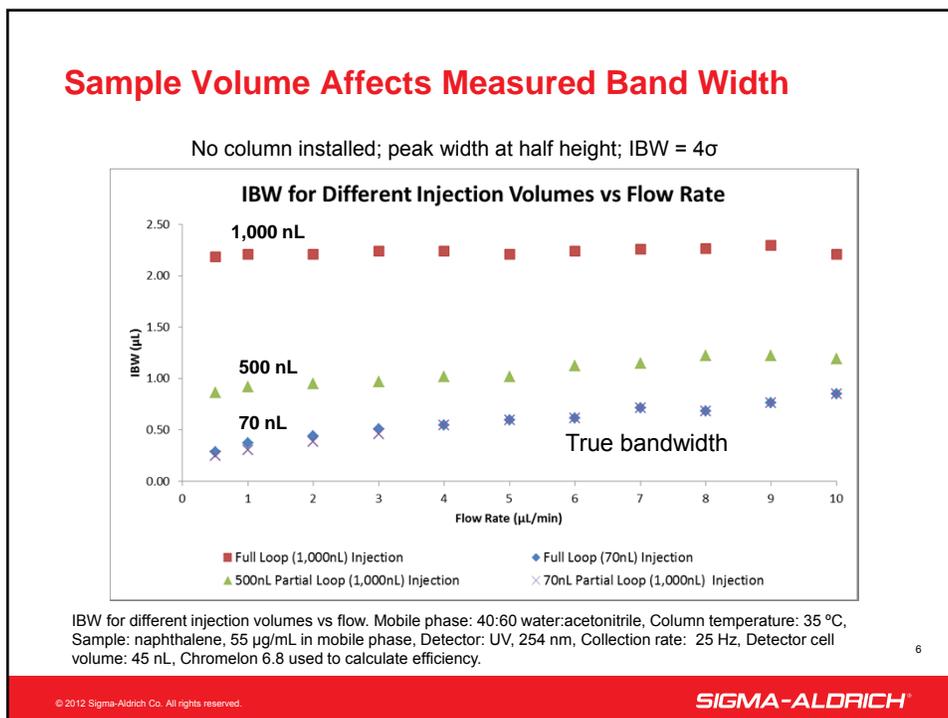
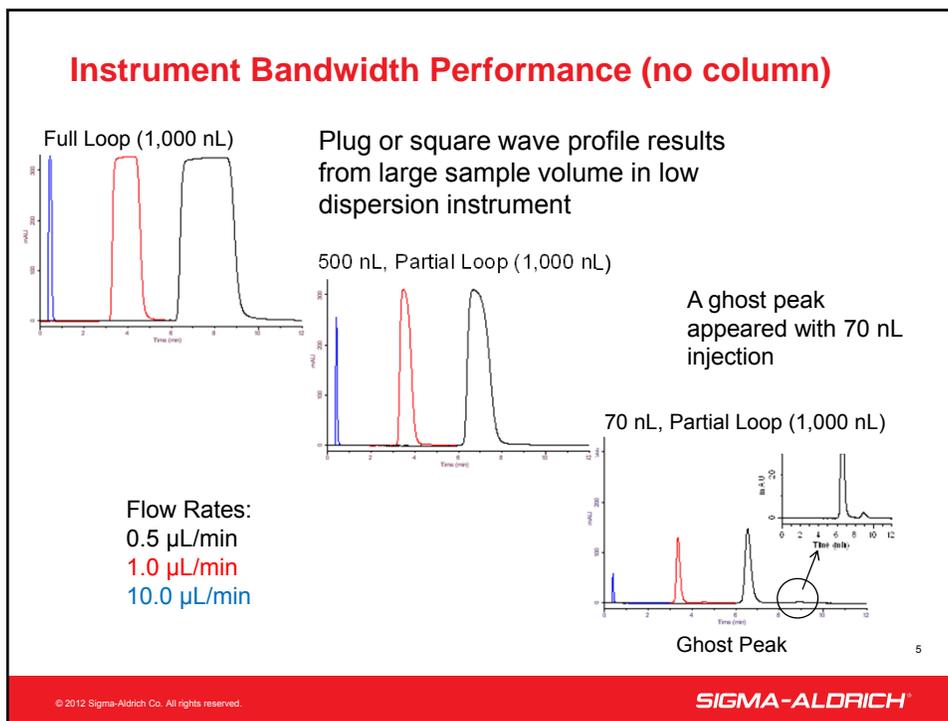
- Wendy Roe, Hillel Brandes and Richard Henry, Impact of Instrument Dispersion on Performance of HPLC Capillary Columns, Poster P-62-W, ISCC May 2013, Palm Springs, CA.



4

© 2012 Sigma-Aldrich Co. All rights reserved.

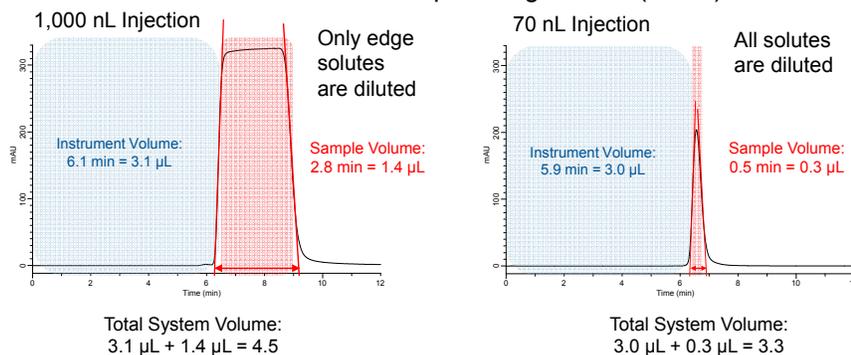
SIGMA-ALDRICH



## Full Loop Injection Volume Impacts System Volume

0.5  $\mu\text{L}/\text{min}$  w/o column

Peak width measured at baseline (use of efficiency formula for PW correlates well when peak is gaussian (70 nL)



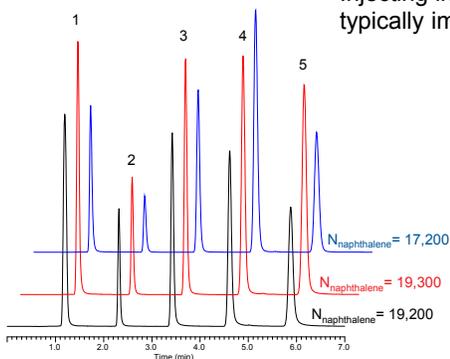
When column is present, large samples can be injected by focusing if sample is dissolved in weaker solvent than mobile phase; sample is extracted, and precolumn volume can be virtually eliminated.

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Minimize Dispersion by Changing Sample Solvent

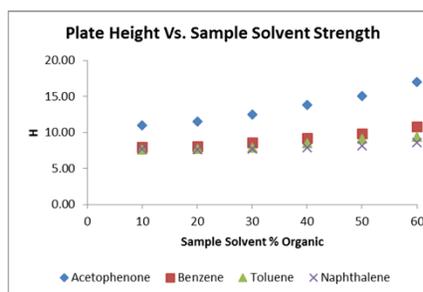
Injecting in weaker solvent than mobile phase typically improves efficiency and resolution.



Elution Order:  
1. Uracil  
2. Acetophenone  
3. Benzene  
4. Toluene  
5. Naphthalene

Sample Solvent (3 Shown):  
90:10 Water:Acetonitrile  
70:30 Water:Acetonitrile  
40:60 Water:Acetonitrile

Mobile Phase:  
40:60 Water:Acetonitrile



Conditions: Column: Acclaim PepMap RSLC C18, 150 mm x 300  $\mu\text{m}$ , 2  $\mu\text{m}$ , Mobile phase: 40:60 water:acetonitrile, Flow rate: 10  $\mu\text{L}/\text{min}$ , Column temperature: 35  $^{\circ}\text{C}$ , Injection: Full loop 70 nL, Detector: UV, 254 nm, Collection rate: 25 Hz, Detector cell volume: 45 nL.

Six examples graphed (three sample runs shown) <sup>8</sup>

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Peptide Analysis by Cap LC- Common Application

### Peptide Test Mix Operating Conditions

Column: Ascentis Express Peptide ES-C18, 15 cm x 300  $\mu$ m I.D., 2.7  $\mu$ m

Mobile phase: (A) 0.1% formic acid, (B) acetonitrile with 0.1% formic acid

Gradient: 1 to 40% B in 30 min, 40 to 90% B in 2, held at 90% B for 2 min, 90 to 1% B in 2 min, held at 1% B for 4 min

Flow rate: 7.9  $\mu$ L/min

Column temperature: 40 ° C

Detector: UV, 210 nm

Injection: 1  $\mu$ L

Sample: MS RT Calibration Mix (MSRT1) (from Sigma-Aldrich)

Sample preparation: Sample extracted from 8 vials using 10  $\mu$ L of 20:80 acetonitrile:water with 0.1% formic acid

9

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Ascentis Express Fused-Core Columns

Follow the hyperlinks below for more information:

<http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=17841436>

<http://sigmaaldrich.tv/ifb/supelco/1-hplc/#/14/>

<http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=17841440>

10

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

### Prepared Sample: MS RT 1 (Sigma-Aldrich)

Peptide Sequence	Retention Order	Monoisotopic MW	pmol*
RGDSPASSP[K]	1	1008.508	132
GLV[K]	2	423.2937	309
LGGNETQV[R]	3	982.5071	81
AEFAEVS[K]	4	887.448	101
SGFSSVSVS[R]	5	1021.5068	67
ADEGISF[R]	6	903.4325	76
DISLSDY[K]	7	947.4691	120
LVNEVTEFA[K]	8	1156.6219	38
DQGGELLSL[R]	9	1096.5752	37
GLFIIDD[K]	10	927.5157	100
LGEYGFQNA[L]	11	1117.5517	82
YWGVASFLQ[K]	12	1205.6324	61
TDELFQI EGLKEELAYL[R]	13	2176.1291	350
AVQQPDGLAVLGIFL[K]	14	1675.9752	46

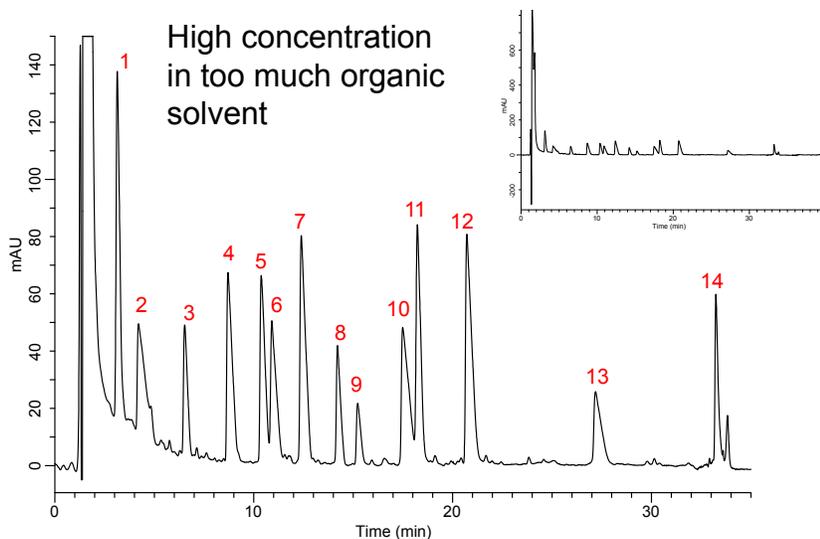
Amino acid in [brackets] denotes site of label incorporation as follows: [K],  $^{13}\text{C}_6$   $^{15}\text{N}_2$ ; [R],  $^{13}\text{C}_6$   $^{15}\text{N}_4$ ; or [L],  $^{13}\text{C}_6$  \*Amount in MSRT1

11

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

### Peptide Calibration Mix (Solvent 20% ACN)

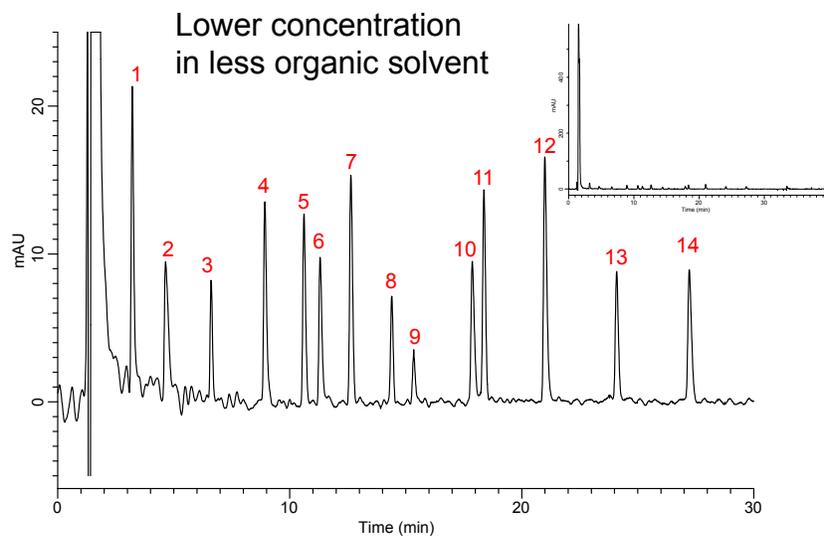


12

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Peptide Calibration Mix –Diluted to 10% ACN



© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## QC Test Mix Conditions

### Column efficiency test:

Column: Ascentis Express Peptide ES-C18, 15 cm x 300  $\mu$ m I.D., 2.7  $\mu$ m

Mobile phase: (A) 0.1% formic acid, (B) acetonitrile with 0.1% formic acid  
55:45 (A:B)

Flow rate: 4  $\mu$ L/min

Column temperature: 35 ° C

Detector: UV, 254 nm

Injection: 0.05  $\mu$ L

Sample: uracil (35  $\mu$ g/mL), acetophenone (35  $\mu$ g/mL), benzene (3,750  $\mu$ g/mL), toluene (3,8750  $\mu$ g/mL), ethylbenzene (4,250  $\mu$ g/mL)

14

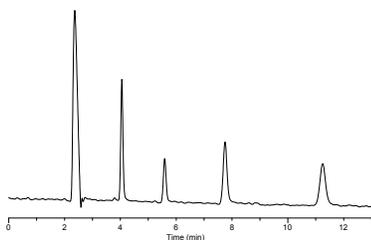
© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Establish Minimum Acceptable Performance Levels

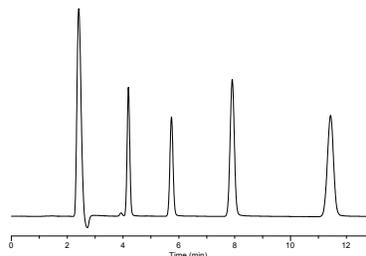
### Isocratic Column QC Test

#### New Prototype Column



Elution Order	Compound	As	N
1	Uracil	1.34	969
2	Acetophenone	1.08	12487
3	Benzene	1.07	15186
4	Toluene	1.06	14954
5	Ethylbenzene	0.99	14934

#### After Gradient Use



Elution Order	Compound	As	N
1	Uracil	1.28	1152
2	Acetophenone	1.05	8909
3	Benzene	1.02	11975
4	Toluene	1	12657
5	Ethylbenzene	0.98	12538

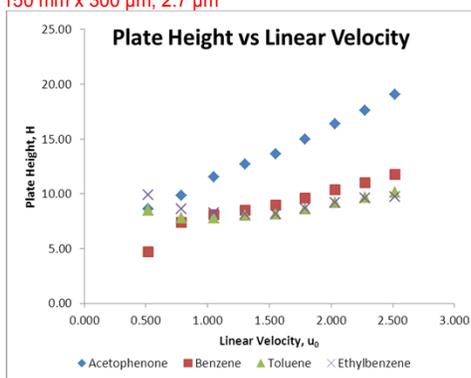
15

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

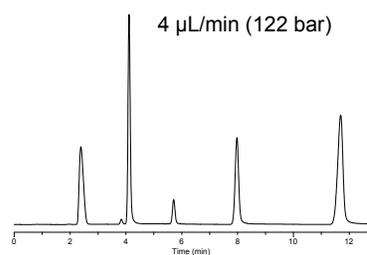
## Combine Efficiency Test and van Deemter Plot to Establish Column Performance

### Ascentis Express Peptide ES-C18 Column 150 mm x 300 $\mu$ m, 2.7 $\mu$ m



Mobile phase: (A) water, (B) acetonitrile 55:45 (A:B); Column temperature: 35 °C; Detector: UV, 254 nm; Injection: 0.05  $\mu$ L; Sample: uracil (35  $\mu$ g/mL), acetophenone (35  $\mu$ g/mL), benzene (3,750  $\mu$ g/mL), toluene (3,8750  $\mu$ g/mL), ethylbenzene (4,250  $\mu$ g/mL)

### Optimum Conditions



Elution Order	Compound	As	N
1	Uracil	1.35	1,102
2	Acetophenone	1.12	12,959
3	Benzene	1.06	18,548
4	Toluene	1.01	19,238
5	Ethylbenzene	0.87	18,320

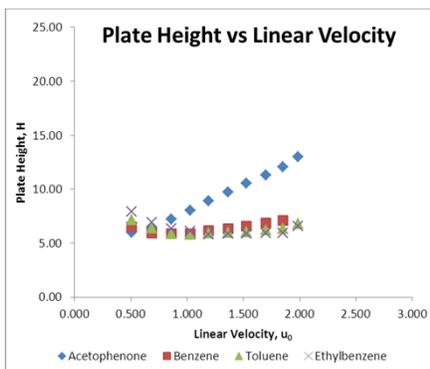
16

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Ascentis Express C18

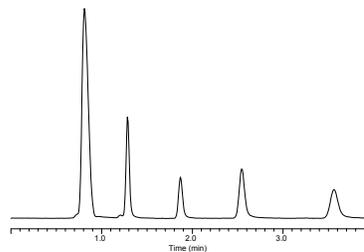
50 mm x 500  $\mu$ m, 2.7  $\mu$ m



Mobile phase: (A) water, (B) acetonitrile 45:55 (A:B); Column temperature: 35 °C; Detector: UV, 254 nm; Injection: 0.05  $\mu$ L; Sample: uracil (35  $\mu$ g/mL), acetophenone (35  $\mu$ g/mL), benzene (3,750  $\mu$ g/mL), toluene (3,8750  $\mu$ g/mL), ethylbenzene (4,250  $\mu$ g/mL)

### Optimum Conditions

12  $\mu$ L/min (17 bar)



Elution Order	Compound	As	N
1	Uracil	1.36	617
2	Acetophenone	1.12	6,235
3	Benzene	1.1	8,580
4	Toluene	1.16	8,665
5	Ethylbenzene	1.2	8,211

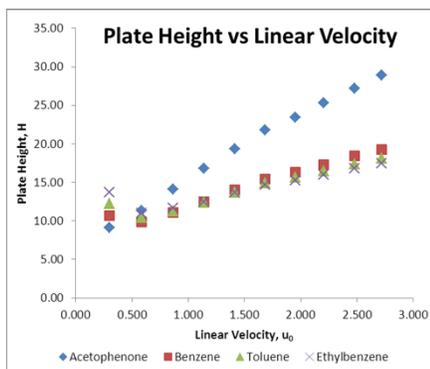
17

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Ascentis Express C18

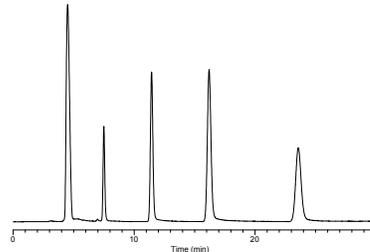
150 mm x 300  $\mu$ m, 2.7  $\mu$ m



Mobile phase: (A) water, (B) acetonitrile 45:55 (A:B); Column temperature: 35 °C; Detector: UV, 254 nm; Injection: 0.05  $\mu$ L; Sample: uracil (35  $\mu$ g/mL), acetophenone (35  $\mu$ g/mL), benzene (3,750  $\mu$ g/mL), toluene (3,8750  $\mu$ g/mL), ethylbenzene (4,250  $\mu$ g/mL)

### Optimum Conditions

2  $\mu$ L/min (59 bar)



Elution Order	Compound	As	N
1	Uracil	1.28	1,151
2	Acetophenone	1.1	13,648
3	Benzene	1.1	15,023
4	Toluene	1.1	14,027
5	Ethylbenzene	1.08	13,037

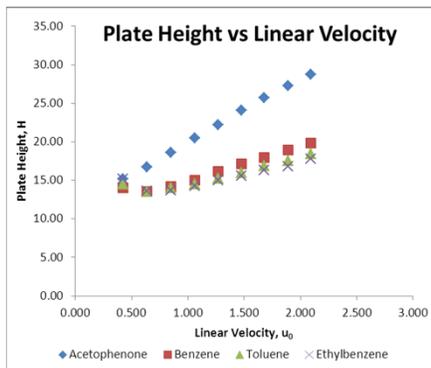
18

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Discovery BIO Wide Pore C18

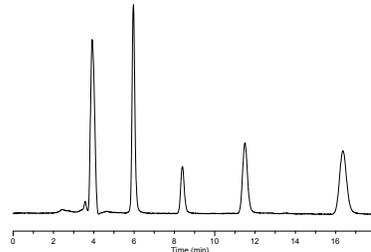
150 mm x 320  $\mu\text{m}$ , 5  $\mu\text{m}$



Mobile phase: (A) water, (B) acetonitrile 55:45 (A:B); Column temperature: 35 °C; Detector: UV, 254 nm; Injection: 0.05  $\mu\text{L}$ ; Sample: uracil (35  $\mu\text{g}/\text{mL}$ ), acetophenone (35  $\mu\text{g}/\text{mL}$ ), benzene (3,750  $\mu\text{g}/\text{mL}$ ), toluene (3,8750  $\mu\text{g}/\text{mL}$ ), ethylbenzene (4,250  $\mu\text{g}/\text{mL}$ )

### Optimum Conditions

3  $\mu\text{L}/\text{min}$  (24 bar)



Elution Order	Compound	As	N
1	Uracil	1.21	1,614
2	Acetophenone	1.14	8,939
3	Benzene	1.11	11,264
4	Toluene	1.07	10,993
5	Ethylbenzene	1.07	10,795

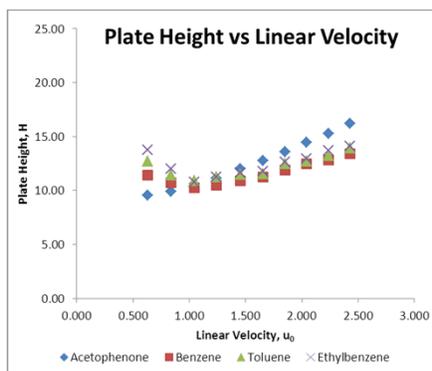
19

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Discovery BIO Wide Pore C18

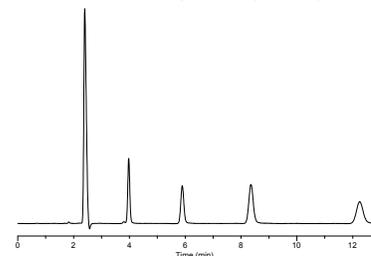
150 mm x 500  $\mu\text{m}$ , 5  $\mu\text{m}$



Mobile phase: (A) water, (B) acetonitrile 55:45 (A:B); Column temperature: 35 °C; Detector: UV, 254 nm; Injection: 0.05  $\mu\text{L}$ ; Sample: uracil (35  $\mu\text{g}/\text{mL}$ ), acetophenone (35  $\mu\text{g}/\text{mL}$ ), benzene (3,750  $\mu\text{g}/\text{mL}$ ), toluene (3,8750  $\mu\text{g}/\text{mL}$ ), ethylbenzene (4,250  $\mu\text{g}/\text{mL}$ )

### Optimum Conditions

10  $\mu\text{L}/\text{min}$  (35 bar)



Elution Order	Compound	As	N
1	Uracil	1.41	3,353
2	Acetophenone	1.04	14,569
3	Benzene	1.04	14,414
4	Toluene	1.03	14,057
5	Ethylbenzene	1.01	13,723

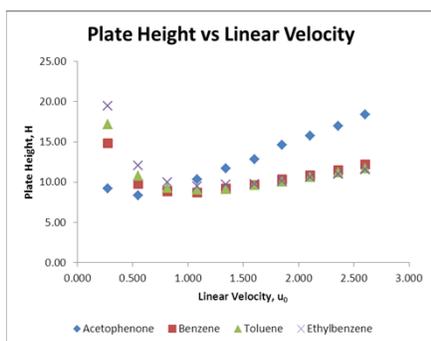
20

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Experimental C18

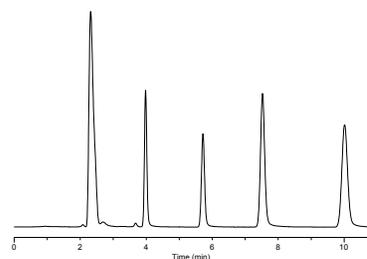
150 mm x 300  $\mu\text{m}$ , 3  $\mu\text{m}$



Mobile phase: (A) water, (B) acetonitrile 40:60 (A:B); Column temperature: 35  $^{\circ}\text{C}$ ; Detector: UV, 254 nm; Injection: 0.05  $\mu\text{L}$ ; Sample: uracil (35  $\mu\text{g}/\text{mL}$ ), acetophenone (35  $\mu\text{g}/\text{mL}$ ), benzene (3,750  $\mu\text{g}/\text{mL}$ ), toluene (3,8750  $\mu\text{g}/\text{mL}$ ), ethylbenzene (4,250  $\mu\text{g}/\text{mL}$ )

## Optimum Conditions

4  $\mu\text{L}/\text{min}$  (52 bar)



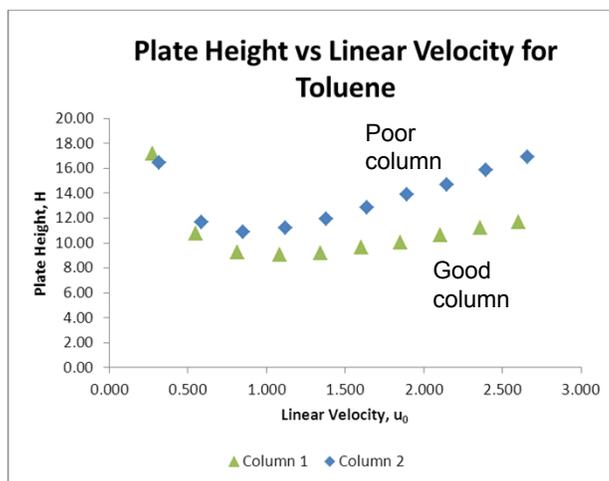
Elution Order	Compound	As	N
1	Uracil	1.54	1,242
2	Acetophenone	1.13	14,410
3	Benzene	1.08	17,297
4	Toluene	1.1	16,551
5	Ethylbenzene	1.08	15,691

21

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Column Comparison using H vs Velocity Plots

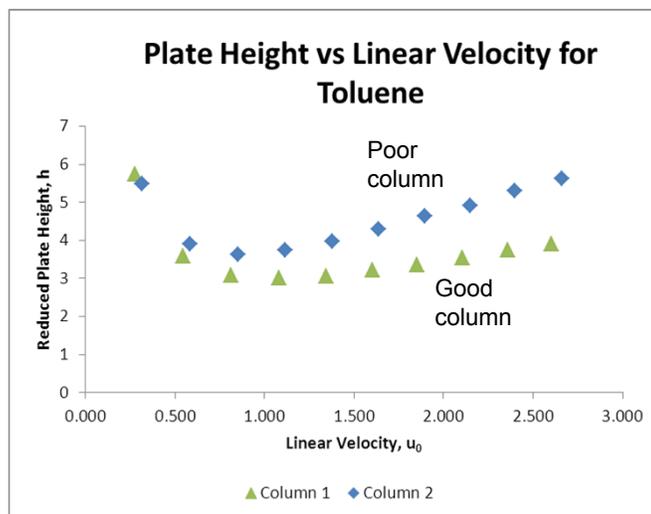


Ascentis C18, 3  $\mu\text{m}$ , 150 mm x 300  $\mu\text{m}$

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Comparison of Columns using h vs Velocity Plots

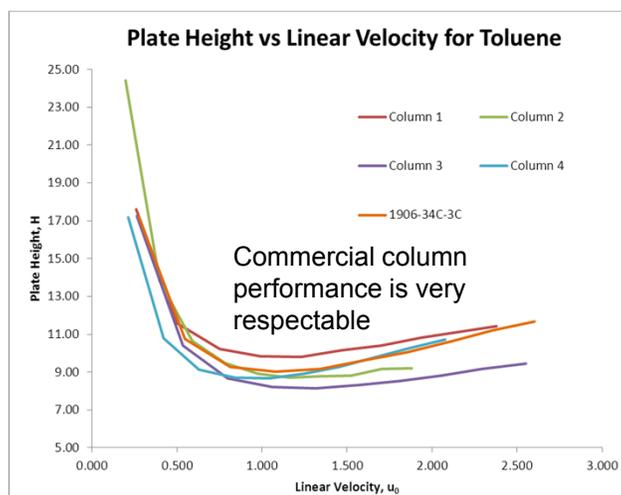


Ascentis C18, 3  $\mu\text{m}$ , 150 mm x 300  $\mu\text{m}$

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

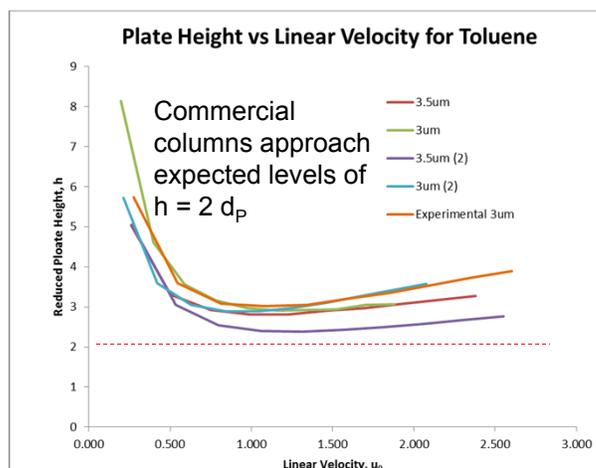
## H - Velocity Plots for Several Commercial Columns



© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## h – Velocity Plots for Several Commercial Columns



© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH

## Peak Dispersion References

1. Wendy Roe, Hillel Brandes and Richard Henry, Impact of Instrument Dispersion on Performance of HPLC Capillary Columns, Poster, ISCC May 2013, Palm Springs, CA.
2. R.E. Majors LCGC NORTH AMERICA, Vol. 21 No. 12, 1124-1133 (December 2003).
3. M. W. Dong, Modern HPLC for Practicing Scientists (Wiley-Interscience, New York 2006).
4. F. Gritti, C.A. Sanchez, T. Farkas and G. Guiochon, J. of Chromatography A, 1217, 3000–3012 (2010).
5. R.A. Henry and D. S. Bell, LCGC NORTH AMERICA, Vol. 23 No. 5, 2-7 (May 2005).
6. R. A. Henry, H. K. Brandes, D. T. Nowlan and J. W. Best, Practical Tips for Operating UHPLC Instruments and Columns, LCGC North America, Vol 31, No. S4a, April 2013, 28-37.

© 2012 Sigma-Aldrich Co. All rights reserved.

SIGMA-ALDRICH