Improve Your USP LC Gradient Separation Methods Following the New <621> Guidelines

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LCGC

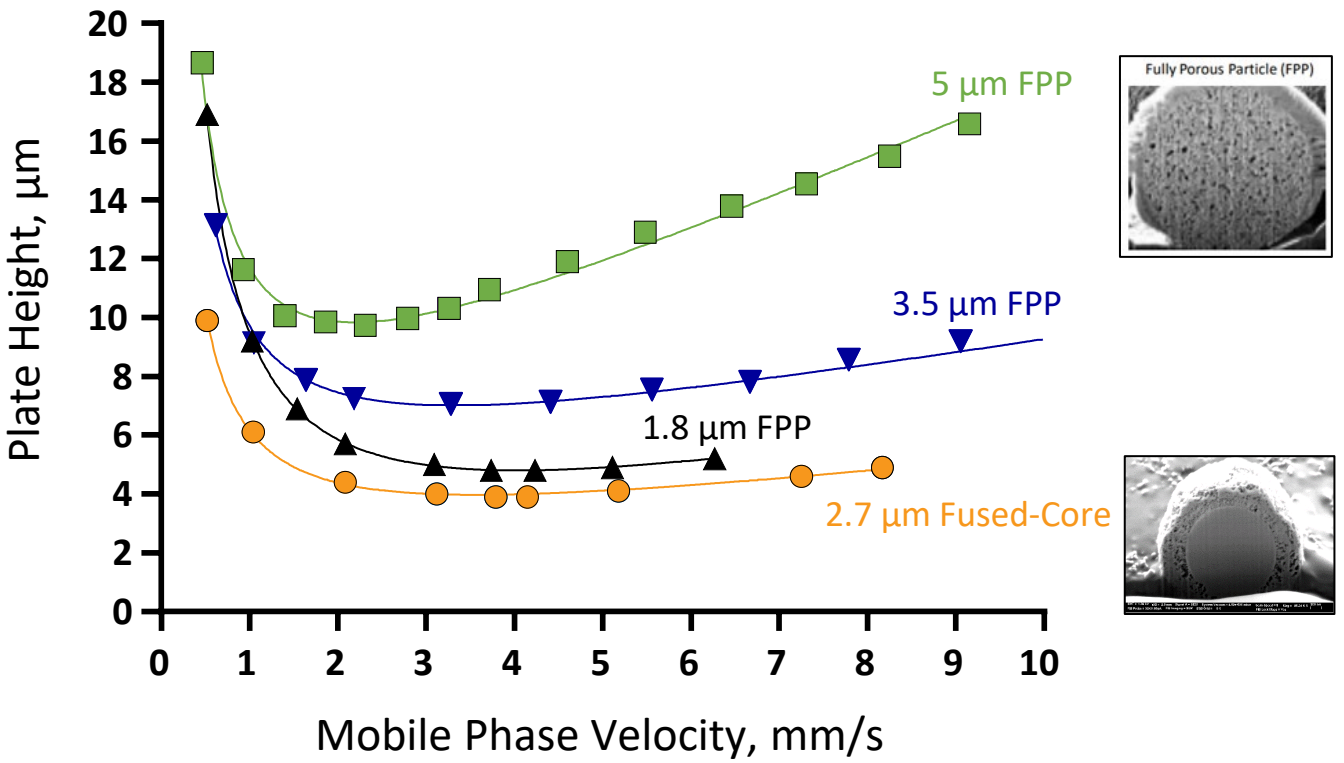
February 28, 2023

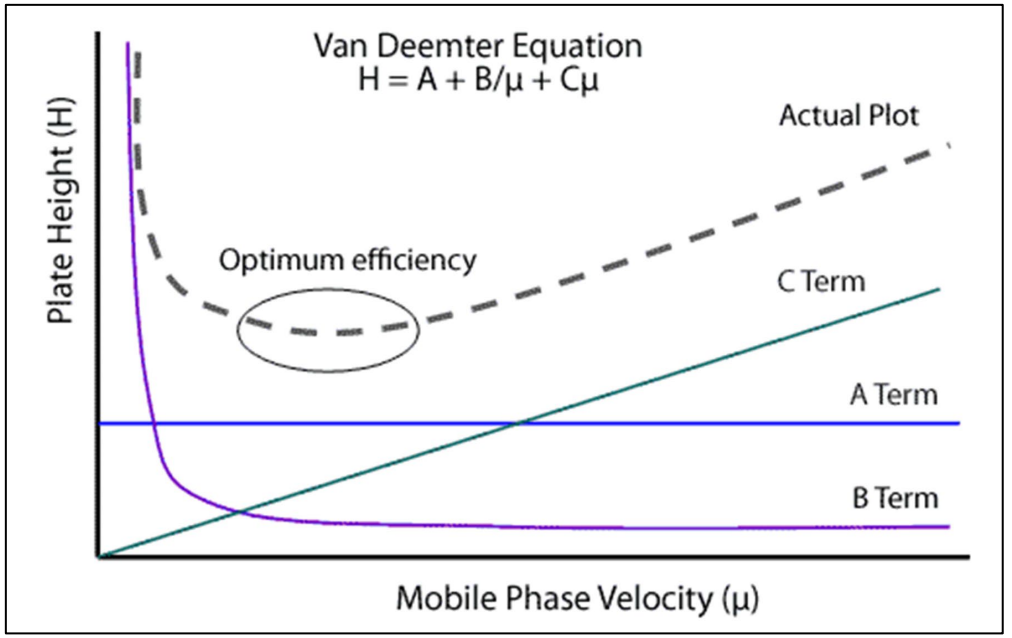
Outline

- Benefits of converting to methods that use superficially porous particle (SPP) columns
- Review guidelines of USP Chapter <621> for changing isocratic and gradient methods
- Case study examples
  - Isocratic
  - Gradient
- Summary

Benefits of Converting to Methods that Use SPP Columns

How SPP Benefits Separations





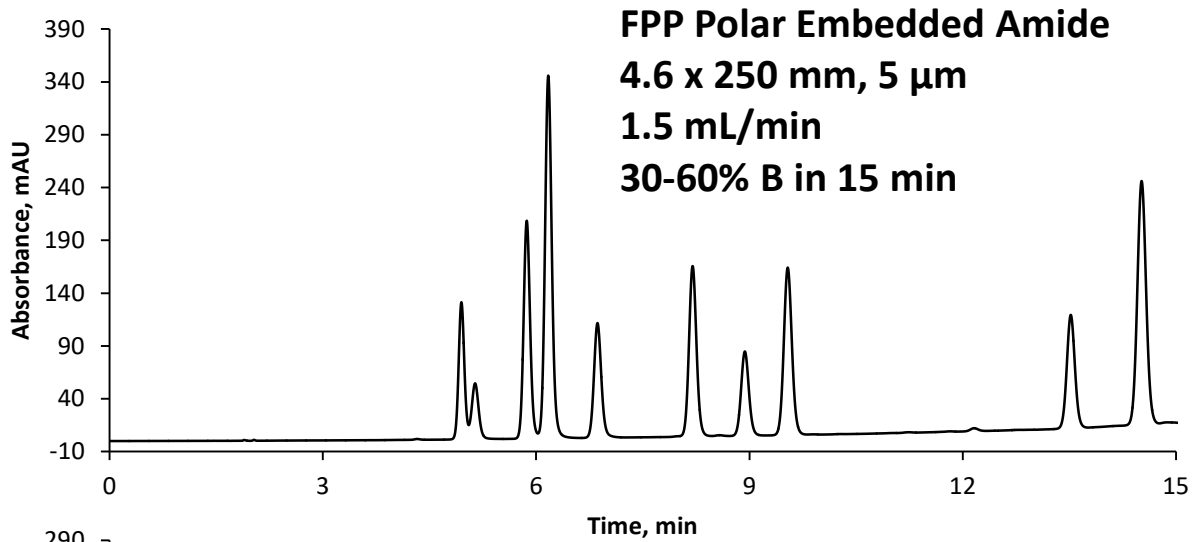
Effect of Particle Size and Type
Columns: 4.6 x 50 mm
5 µm FPP C18
3.5 µm FPP C18
1.8 µm FPP C18
2.7 µm HALO C18
Solute: naphthalene
Mobile phase: 60% ACN/40% water
Temperature:24 °C

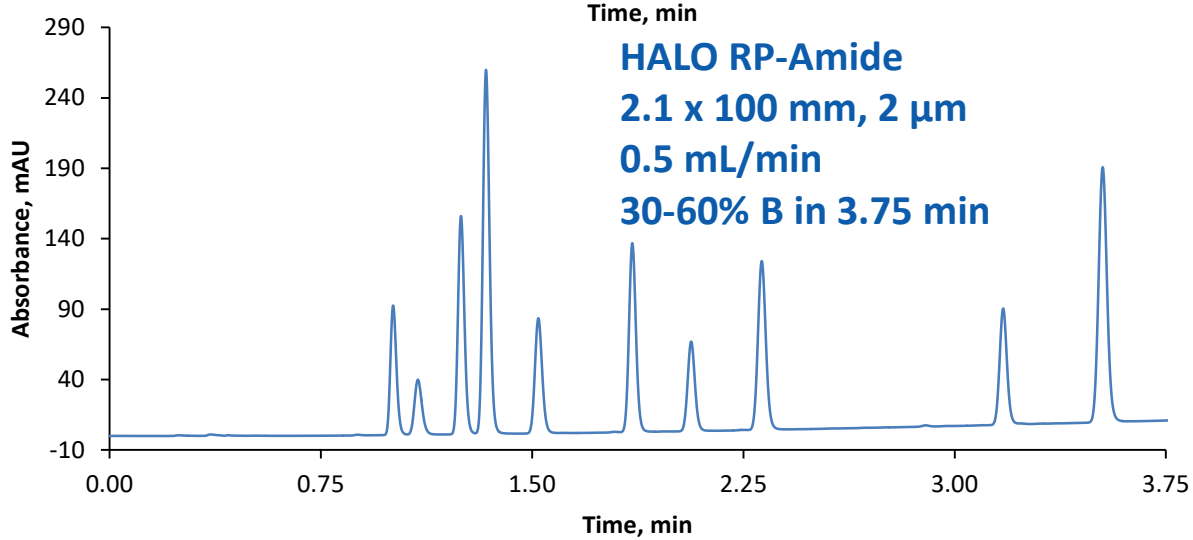
van Deemter Equation

$$H = A + \frac{B}{\mu} + C\mu$$

H = height equivalent to theoretical plate
A = eddy diffusion term (particle size and how well bed was packed) **30 - 40% smaller**
B = longitudinal diffusion term **25 - 30% smaller**
C = resistance to mass transfer term (kinetics of the analyte b/w mobile phase and stationary phase)
µ = mobile phase linear velocity (L/t_0)

Increased Speed & Reduced Mobile Phase Consumption





PEAK IDENTITIES:

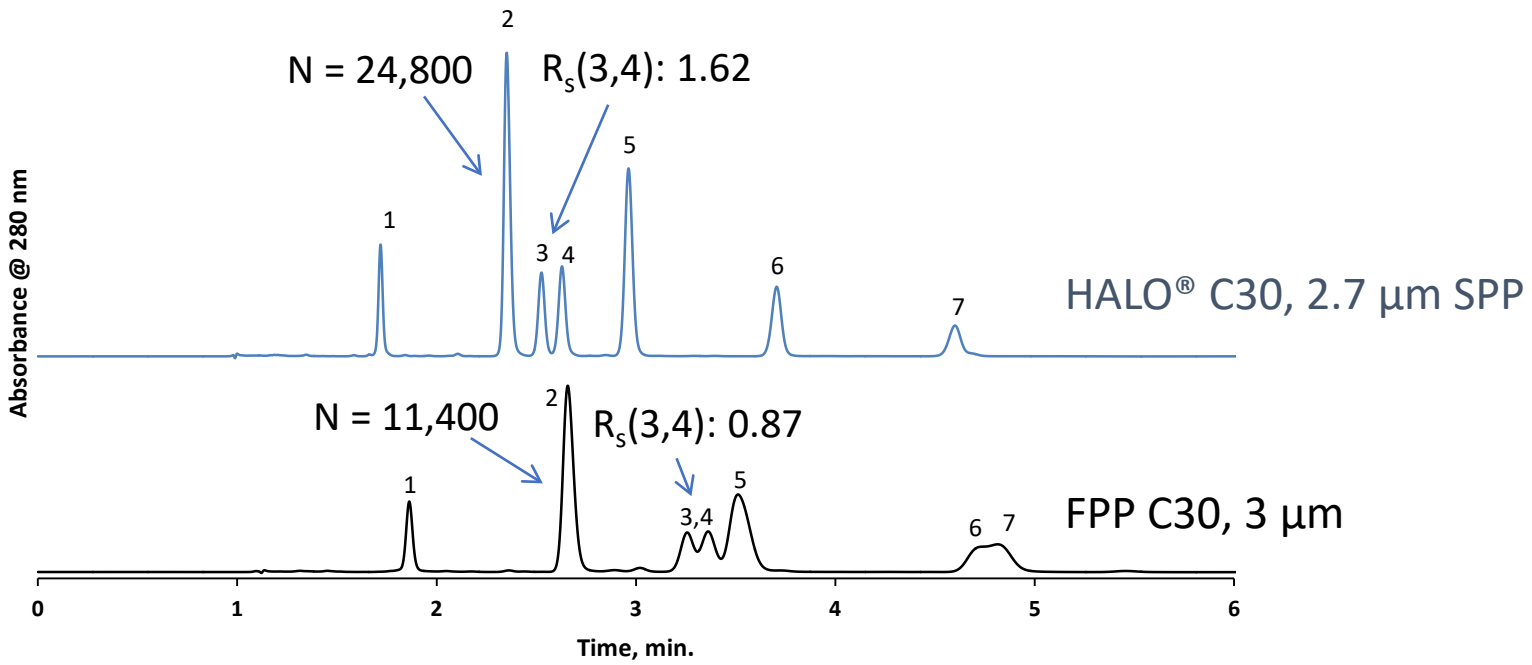
1. homovanillic acid
2. caffeic acid
3. syringic acid
4. vanillic acid
5. chlorogenic acid
6. sinapic acid
7. ferulic acid
8. *p*-coumaric acid
9. *trans*-cinnamic acid
10. resveratrol

SPP method

- 4x faster
- 12x less solvent

Increased Efficiency Demonstrated Using Fat Soluble Vitamins

Sharper peaks and increased resolution with the HALO® C30 column!



PEAK IDENTITIES:

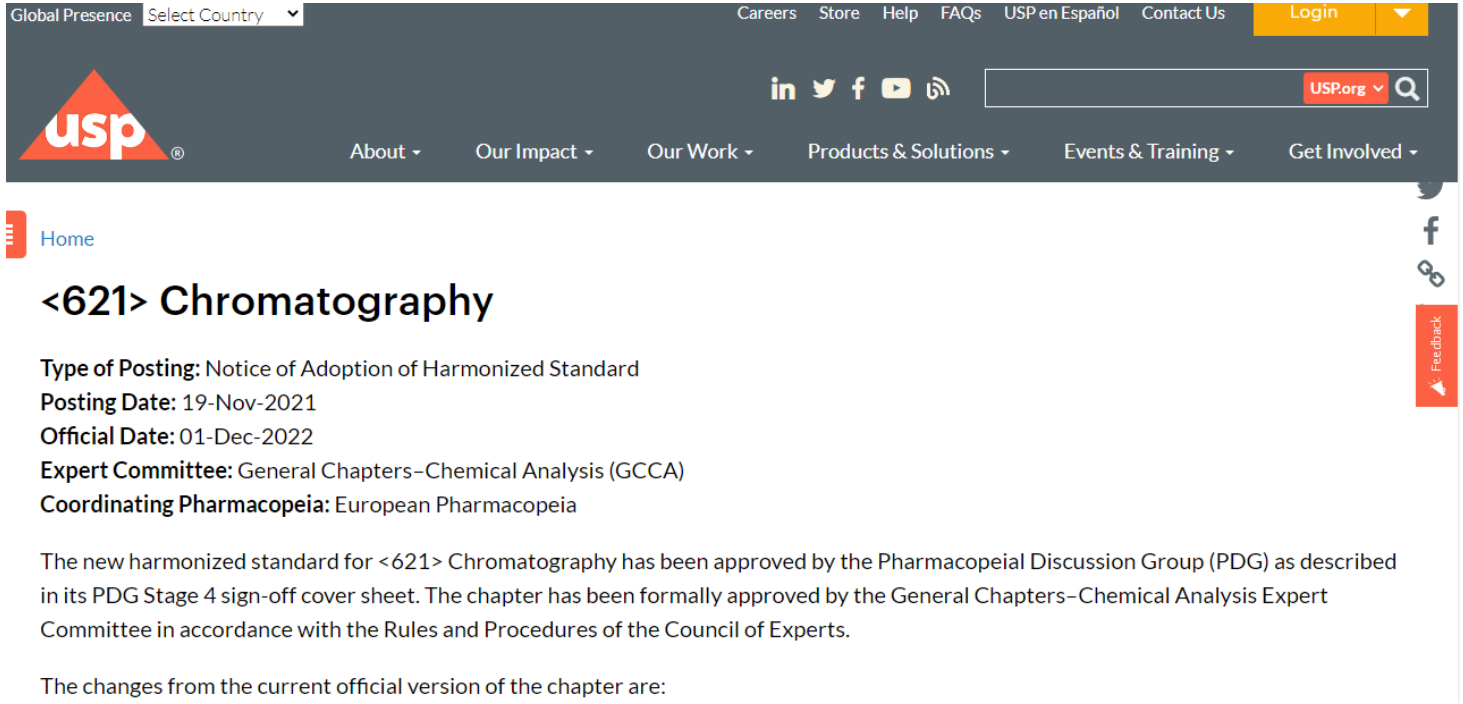
1. Retinyl acetate (A)
2. Delta tocopherol (E)
3. Ergocalciferol (D2)
4. Cholecalciferol (D3)
5. Alpha tocopherol (E)
6. DL-alpha-tocopherol acetate (E)
7. 2,3-*trans*-phylloquinone (K)

Isocratic: 100% Methanol
Wavelength: 280nm
Injection: 2 µL
Temperature: 30 °C
Flow Rate: 1.5 mL/min
Columns: 4.6 x 150 mm

USP Chapter <621> Guidelines for Changing Isocratic and Gradient Methods

When Did Changes Go Into Effect

- For United States Pharmacopeia (USP), changes went into effect December 1, 2022



- For British Pharmacopoeia (BP), European Pharmacopoeia (EP), and Japanese Pharmacopoeia (JP), changes went into effect January 2023

Allowable Changes to USP Methods

Parameter	Isocratic Methods	Gradient Methods
Stationary Phase	Must keep same L category Change from totally porous particle (TPP) to superficially porous particle (SPP) is allowed	Same as isocratic
Column Dimensions	Particle size and/or length of the column may be modified, provided that ratio of the column length (L) to the particle size (d_p) remains constant or in the range between −25% to +50% of the prescribed L/d_p ratio*	Same as isocratic^
Internal Diameter	Change allowed	Change allowed
Flow Rate	$F_2 = F_1 \times [(dc_2^2 \times dp_1) \div (dc_1^2 \times dp_2)]$	Same as isocratic

*For changes from TPP to SPP, other combinations of L and dp can be used, provided that the plate number (N) is within −25% to +50%, relative to the prescribed column. These changes are acceptable, provided that system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent

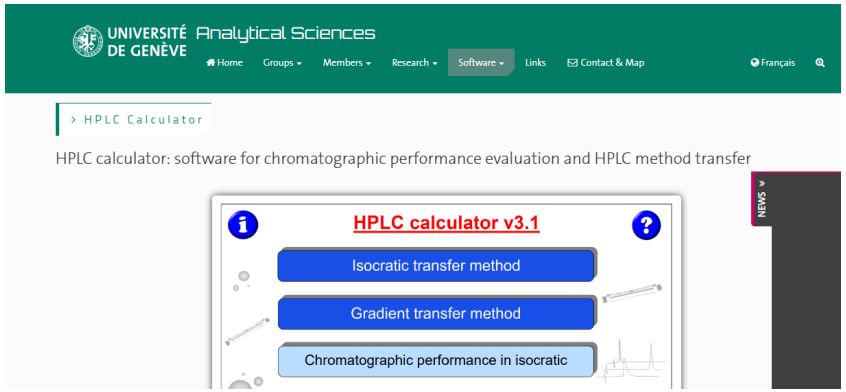
^For changes from TPP to SPP, other combinations of L and dp can be used provided that the ratio $(t_R/W_h)^2$ is within−25% to +50%, relative to the prescribed column for all the peaks used to determine the system suitability parameters. These changes are acceptable provided system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

Allowable Changes to USP Methods - continued

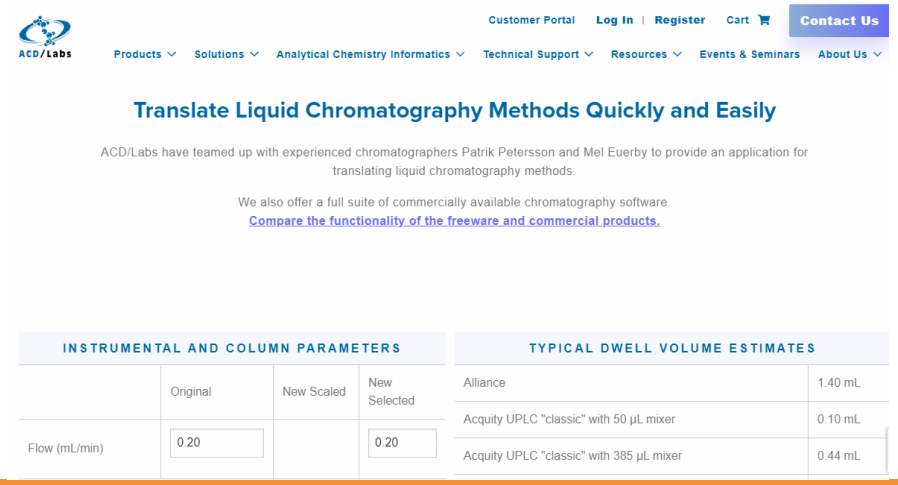
Parameter	Isocratic Methods	Gradient Methods
Detector Wavelength	No change allowed	No change allowed
Mobile Phase Composition	Amount of the minor components of the mobile phase can be adjusted by ±30% relative. However, the change in any component cannot exceed ±10% absolute	Gradient time adjusted by equation $t_{G2} = t_{G1} \times (F_1/F_2)[(L_2 \times d_{c2}^2)/(L_1 \times d_{c1}^2)]$
pH	±0.2 pH units, unless otherwise prescribed	Same as isocratic
Buffer	±10%	Same as isocratic
Injection Volume	$V_{inj2} = V_{inj1} \times (L_2 d_{c2}^2)/(L_1 d_{c1}^2)$	Same as isocratic
Column Temperature	± 10°C	± 5° C

Method Translators

- https://ispso.unige.ch/labs/fanal/hplc_calculator:en



- https://www.acdlabs.com/resources/freeware/translator/index.php



Words of Caution for Moving to Smaller Particle Sizes and Smaller Column I.D.s

- Smaller particle sizes and smaller column I.D.s are more susceptible to extra-column band broadening

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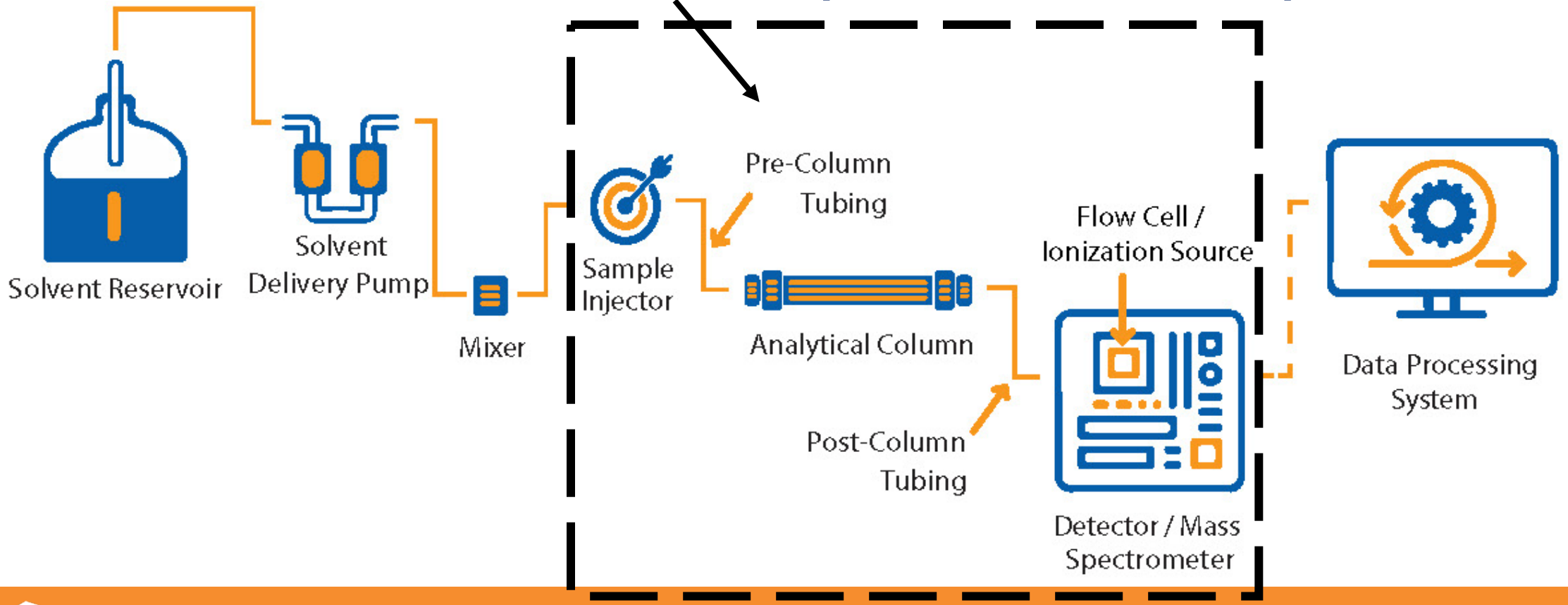
$$\sigma^2_{V,tot} = \sigma^2_{V,pre-col} + \sigma^2_{V,col} + \sigma^2_{V,post-col}$$



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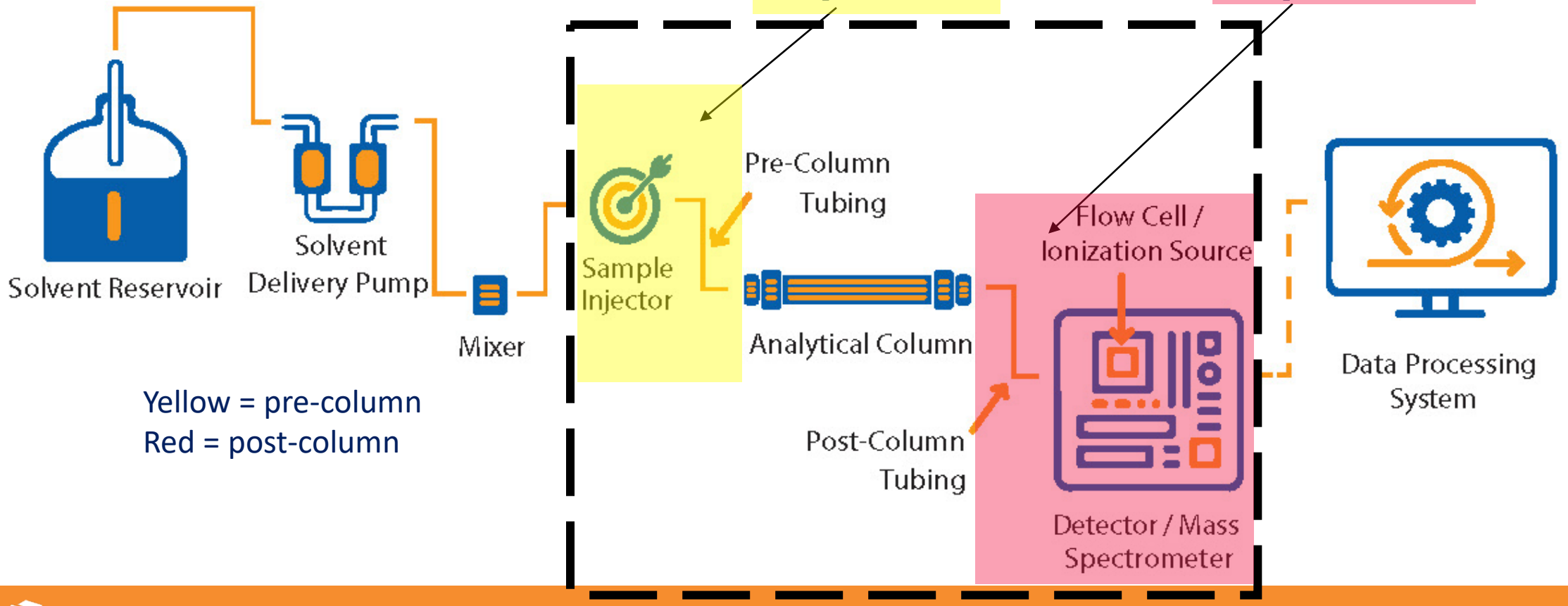


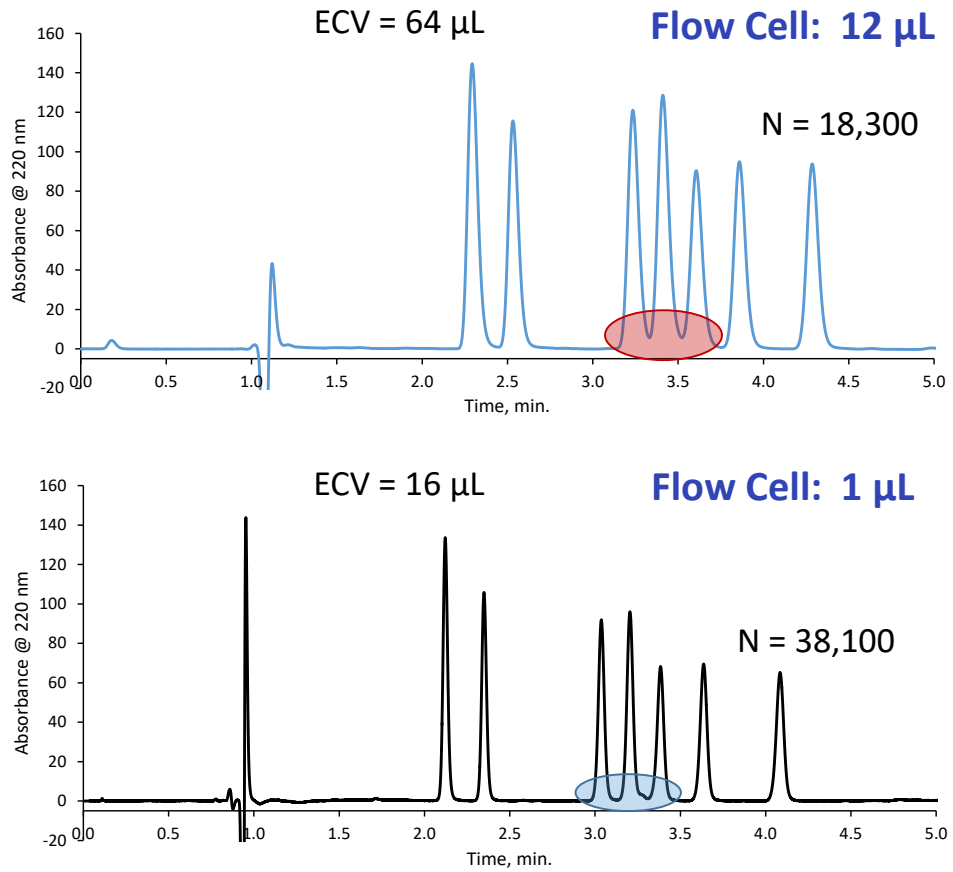
Yellow = pre-column
Red = post-column

Ways to Reduce Extra-Column Band Broadening

- Use the shortest and smallest I.D. connecting tubing between instrument components

- Use a smaller volume detector flow cell (≤ 2.5 µL)
- Increase the data acquisition rate (≥ 20 Hz)
- Decrease the injection volume (more critical for isocratic than gradient separations)

Extra-Column Band Broadening Impact on Resolution and Efficiency

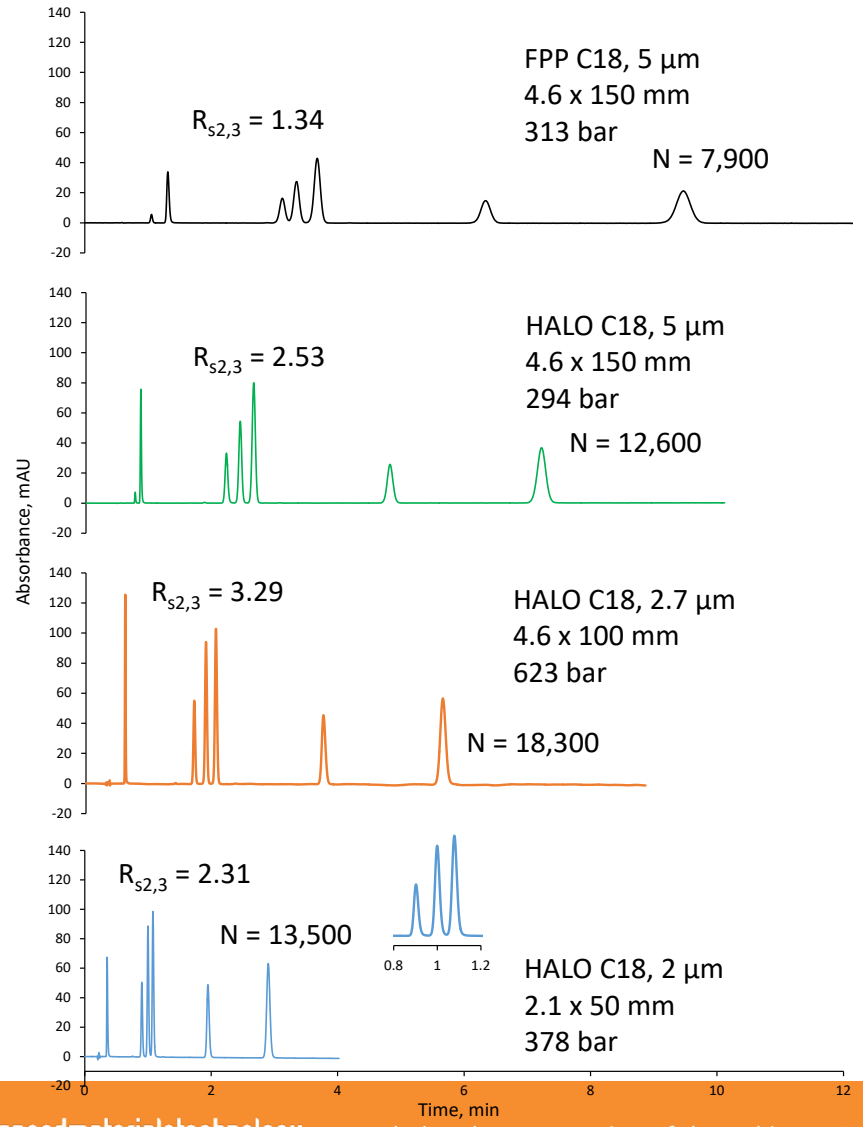


50% average increase in plates is observed by reducing the excess volume in the system!

Isocratic Separations with HALO 90 Å C18, 2 µm, 3.0 x 150 mm

Isocratic Case Studies

What Changes are Possible?



L/dp = 150/.005 = 30,000

For -25-50%, L/dp can be 22,500-45,000

L/dp = 150/0.005 = 30,000 = criteria met

37% higher plates

L/dp = 100/.0027 = 37,037 = criteria met

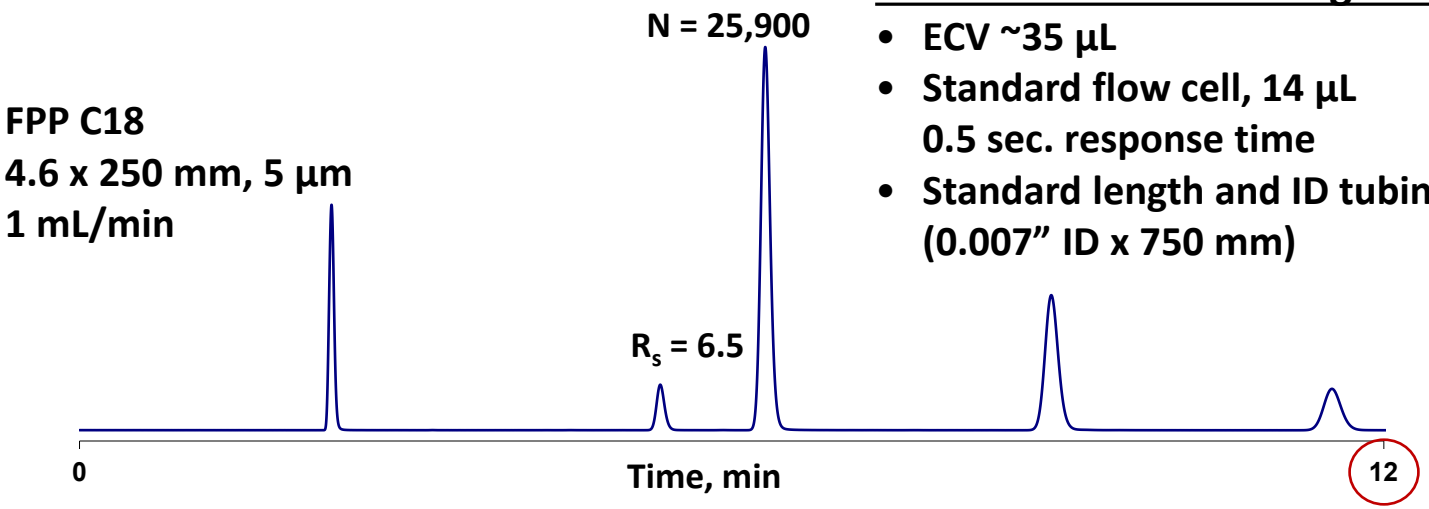
57% higher plates

L/dp = 50/0.002 = 25,000 = criteria met

3x times faster

41% higher plates

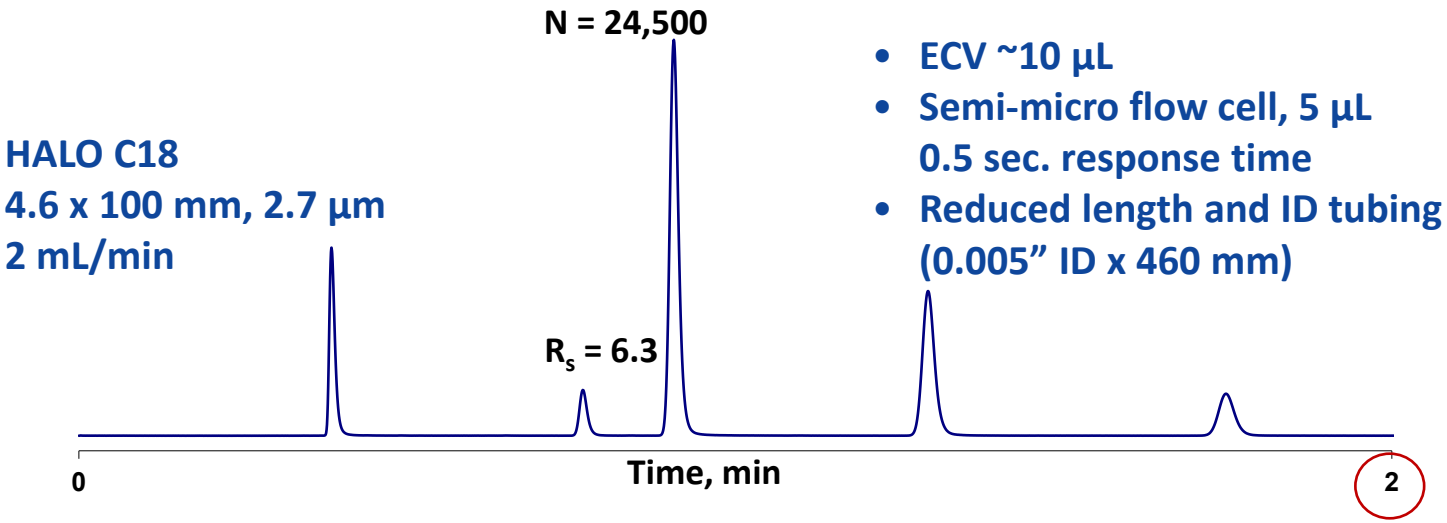
Increased Speed without Loss of Resolution



HPLC in Standard Configuration

- **ECV ~35 µL**
- **Standard flow cell, 14 µL**
  0.5 sec. response time
- **Standard length and ID tubing**
  (0.007" ID x 750 mm)

L/dp = 250/0.005 = 50,000
For -25-50%, L/dp can be 37,500-77,500
N = 25,900
For -25-50%, N can be 19,425-38,850



HPLC in Ultra-Low ECV Configuration

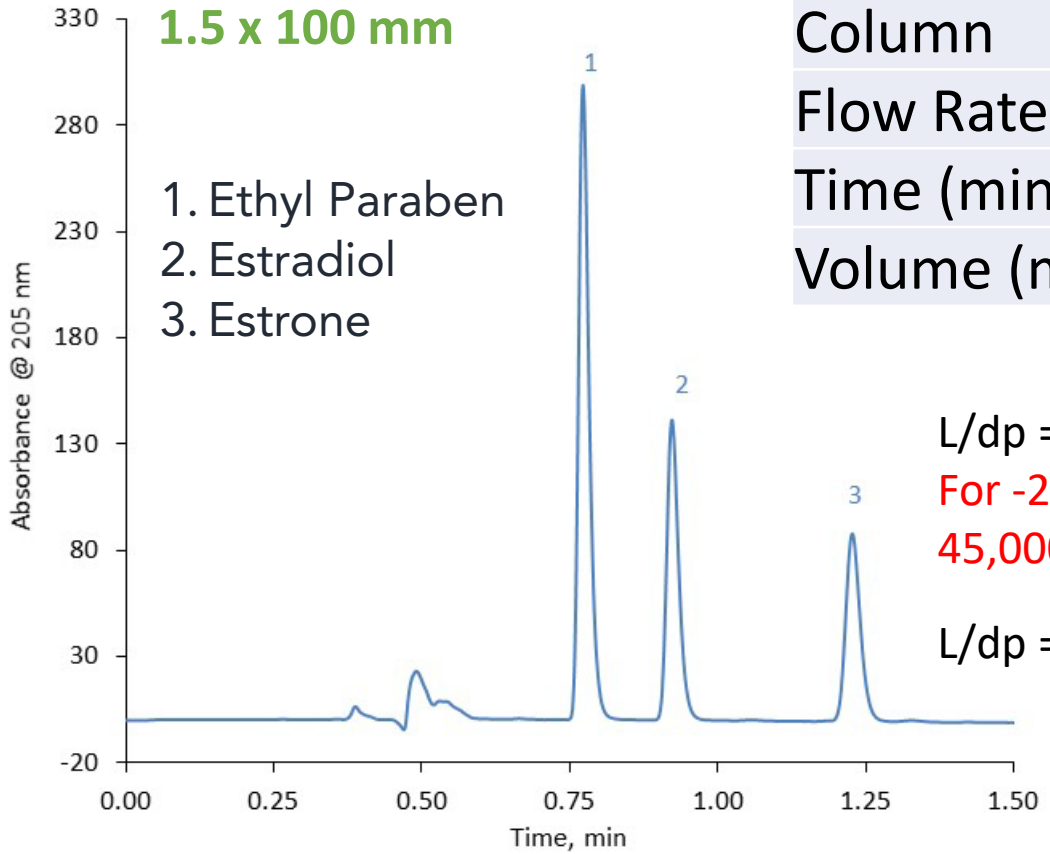
- **ECV ~10 µL**
- **Semi-micro flow cell, 5 µL**
  0.5 sec. response time
- **Reduced length and ID tubing**
  (0.005" ID x 460 mm)

L/dp = 100/0.0027 = 37,037
= criteria not met, but is met for plates
N = 24,500

6 times faster run time!

USP Monograph for Estradiol – Modified

HALO 90 Å C18, 2.7 µm
1.5 x 100 mm



	Original Method	Modified Method
Column	3.9 x 300 mm	1.5 x 100 mm
Flow Rate (mL/min)	1	0.2
Time (min)	8	1.5
Volume (mL)	8	0.3

L/dp = 300/0.01 = 30,000

For -25-50%, L/dp can be 22,500-45,000

L/dp = 100/0.0027 = 37,037

96% solvent savings!

Gradient Case Studies

While Making Changes, Take Notice!

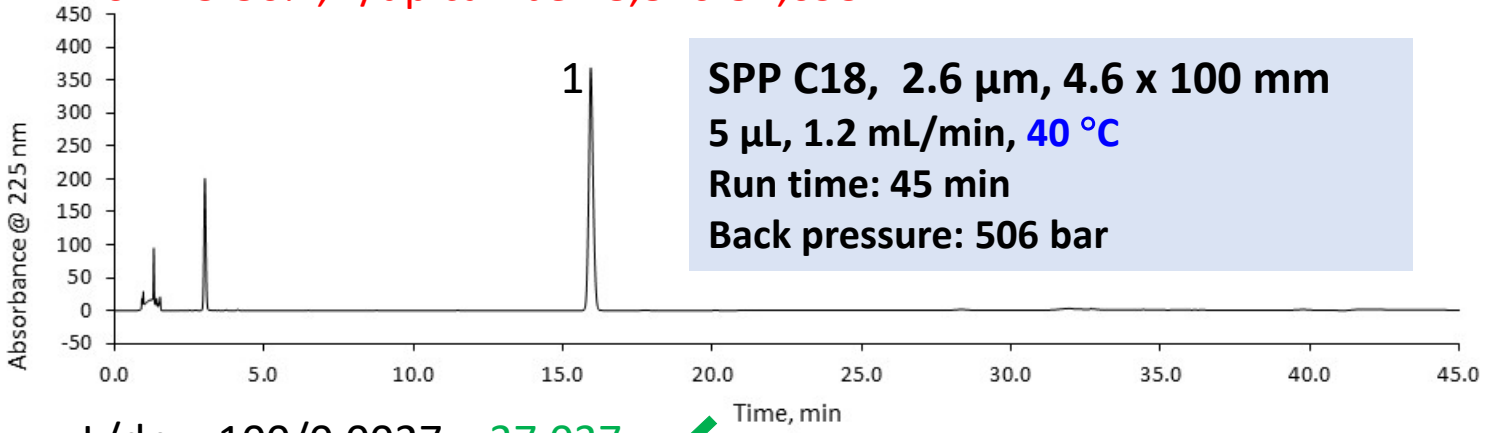
- Adjustment of conditions with gradient elution (HPLC) …is more critical than with isocratic (HPLC) …elution, since it may shift some peaks to a different step of the gradient …, potentially causing partial or complete coelution of adjacent peaks or peak inversion, and, thus leading to the incorrect assignment of peaks and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time.

- For some parameters the adjustments are explicitly defined in the monograph to ensure the system suitability.

USP Monograph for Cobamamide – Original

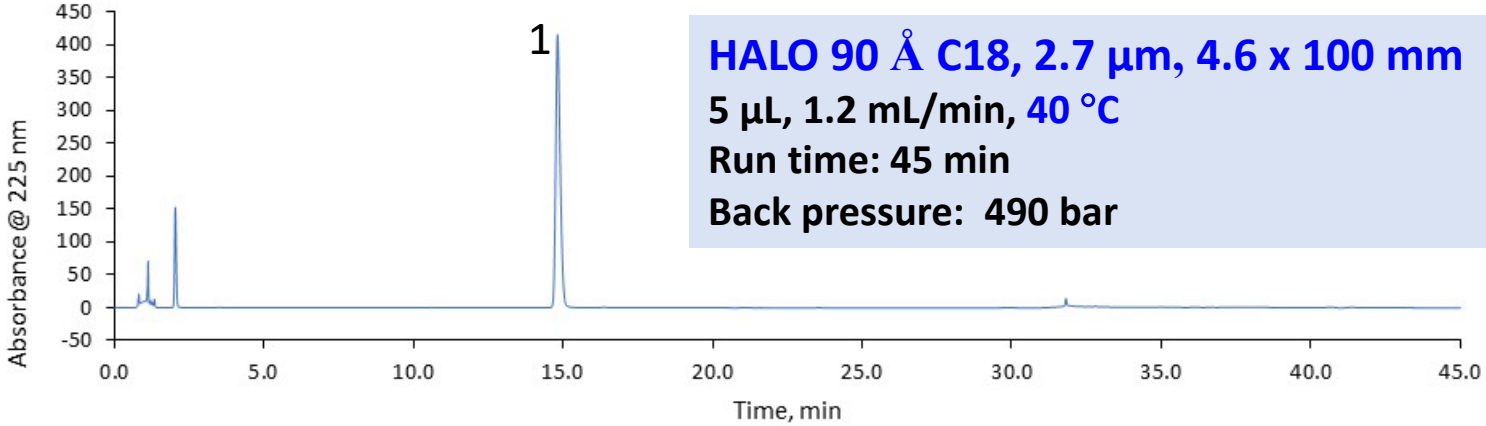
L/dp = 100/0.0026 = 38,462

For -25-50%, L/dp can be 28,846-57,693



SPP C18, 2.6 µm, 4.6 x 100 mm
5 µL, 1.2 mL/min, 40 °C
Run time: 45 min
Back pressure: 506 bar

L/dp = 100/0.0027 = 37,037 ✓

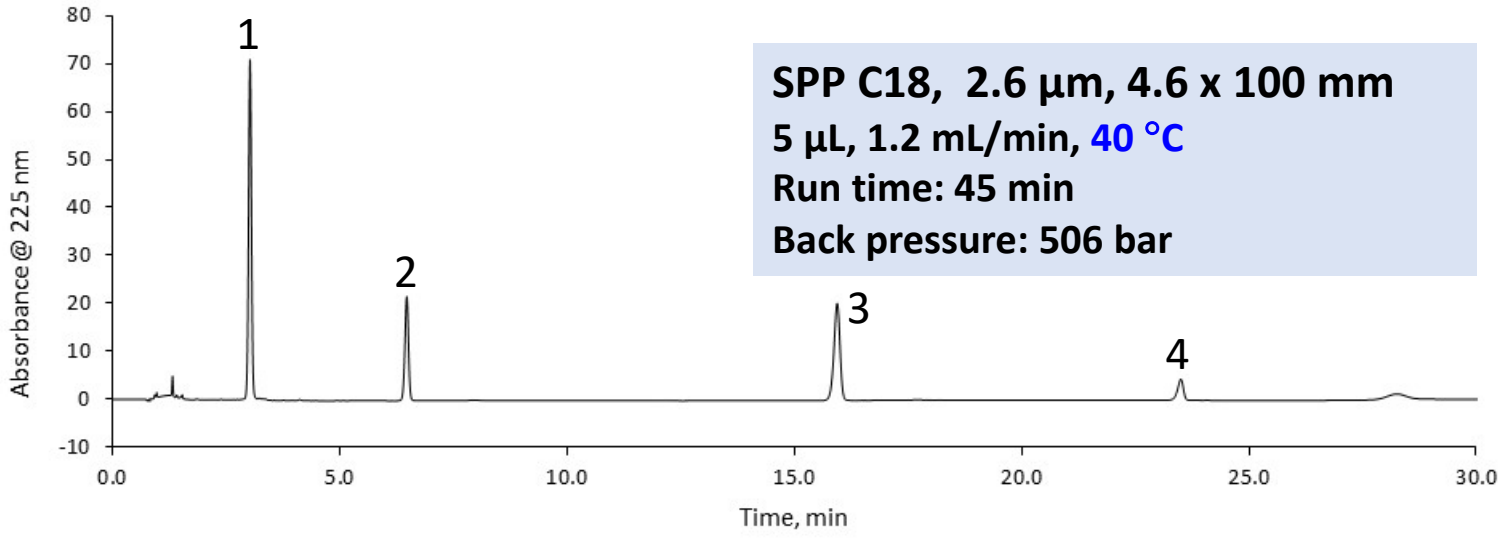


HALO 90 Å C18, 2.7 µm, 4.6 x 100 mm
5 µL, 1.2 mL/min, 40 °C
Run time: 45 min
Back pressure: 490 bar

- Cobamamide (labeled peak) is also known as adenosylcobalamin and is one of the biologically active forms of Vitamin B12
- Suitability requirements for cobamamide
  - Column efficiency: NLT 22,000 plates
  - Tailing factor: NMT 2.0

	Monograph Column	HALO C18
Efficiency	**Pass**	**Pass**
Tailing Factor	**Pass**	**Pass**

USP Monograph for Cobamamide – Original System Suitability



SPP C18, 2.6 µm, 4.6 x 100 mm
5 µL, 1.2 mL/min, 40 °C
Run time: 45 min
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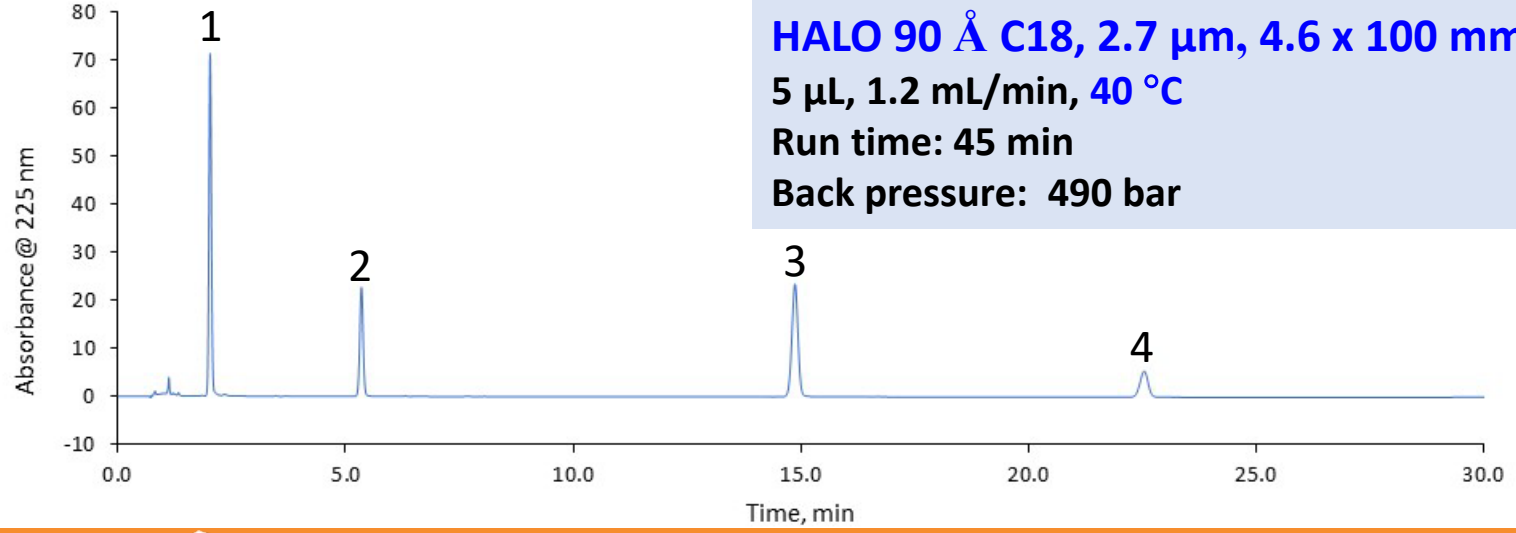


HALO 90 Å C18, 2.7 µm, 4.6 x 100 mm
5 µL, 1.2 mL/min, 40 °C
Run time: 45 min
Back pressure: 490 bar

Peak Identities
1. hydroxocobalamin chloride
2. cyanocobalamin
3. cobamamide
4. methylcobalamin

Elution order is identical on both columns

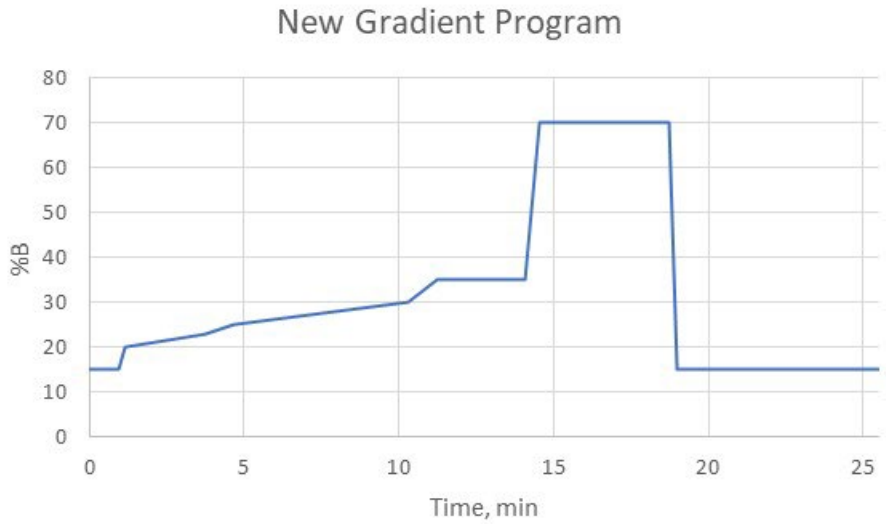
Modifying the Original Method

$$t_{G2} = t_{G1} \times (F_1/F_2)[(L_2 \times d_{c2}^2)/(L_1 \times d_{c1}^2)]$$

Flow rate 1 (F1)	1.2
Flow rate 2 (F2)	0.4
Length 1 (L1)	100
Length 2 (L2)	75
Column Diameter 1 (dc1)	4.6
Column Diameter 2 (dc2)	2.1

tG1	tG2	%B
0	0	15
2	0.94	15
2.5	1.17	20
8	3.75	23
10	4.69	25
22	10.32	30
24	11.25	35
30	14.07	35
31	14.54	70
40	18.76	70
40.5	18.99	15
45	21.10	15

- Flow rate increased to 0.4 mL/min instead of 0.25 mL/min (same linear velocity as 1.2 mL/min)
- Equilibration time increased by 4 minutes for 20 column volumes for a total run time of 25.5 min



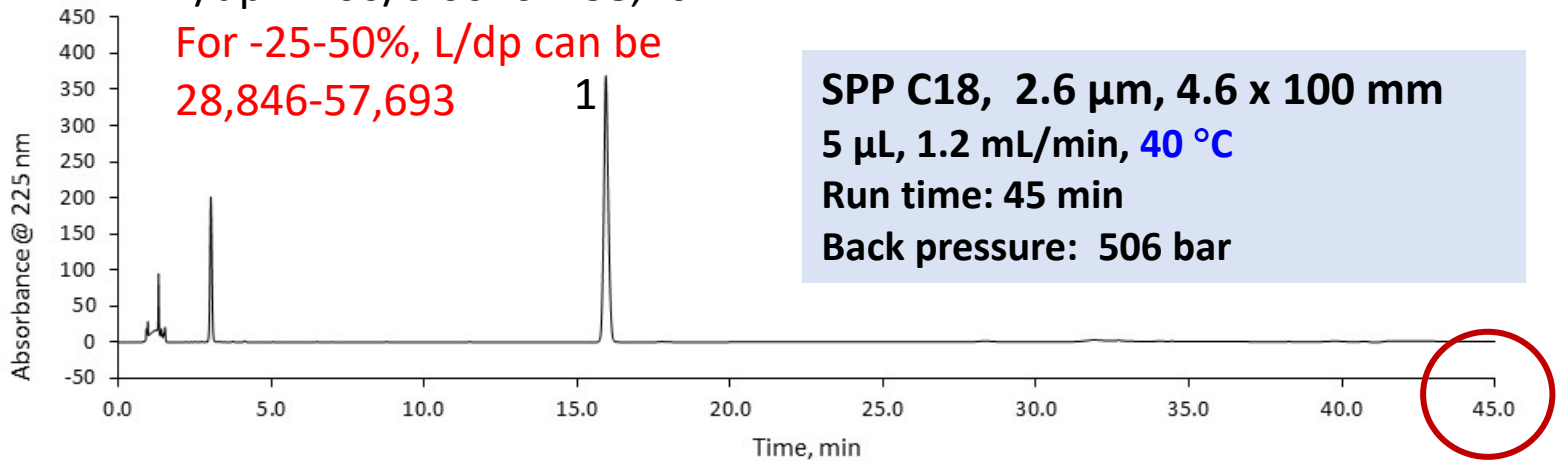
- Injection volume reduced to 0.8 µL from 5 µL by this equation:

$$V_{inj2} = V_{inj1} \times (L_2 d_{c2}^2)/(L_1 d_{c1}^2)$$

USP Monograph for Cobamamide – Modified

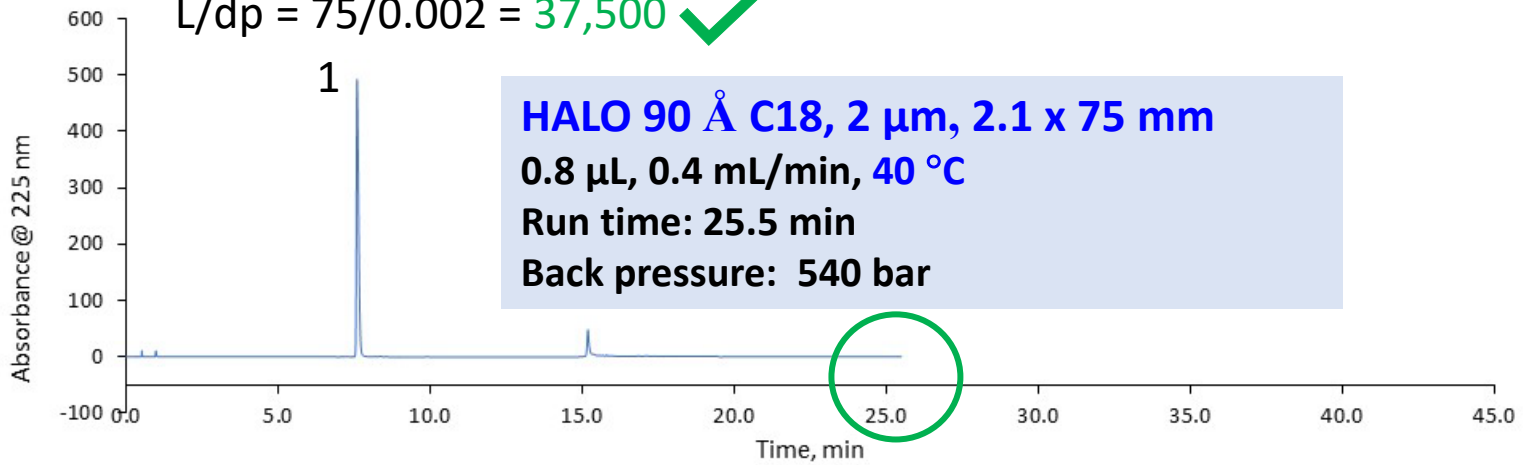
L/dp = 100/0.0026 = 38,462

For -25-50%, L/dp can be 28,846-57,693



SPP C18, 2.6 µm, 4.6 x 100 mm
5 µL, 1.2 mL/min, 40 °C
Run time: 45 min
Back pressure: 506 bar

L/dp = 75/0.002 = 37,500 ✓



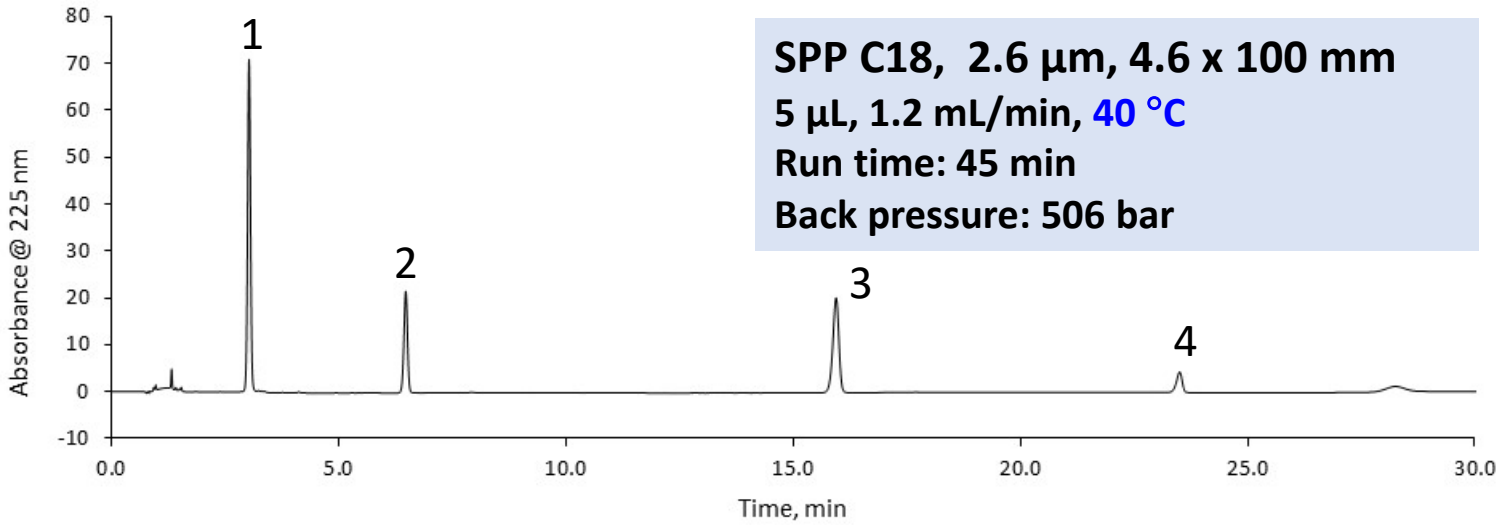
HALO 90 Å C18, 2 µm, 2.1 x 75 mm
0.8 µL, 0.4 mL/min, 40 °C
Run time: 25.5 min
Back pressure: 540 bar

Method was moved to a shorter SPP column with smaller i.d. and smaller particle size

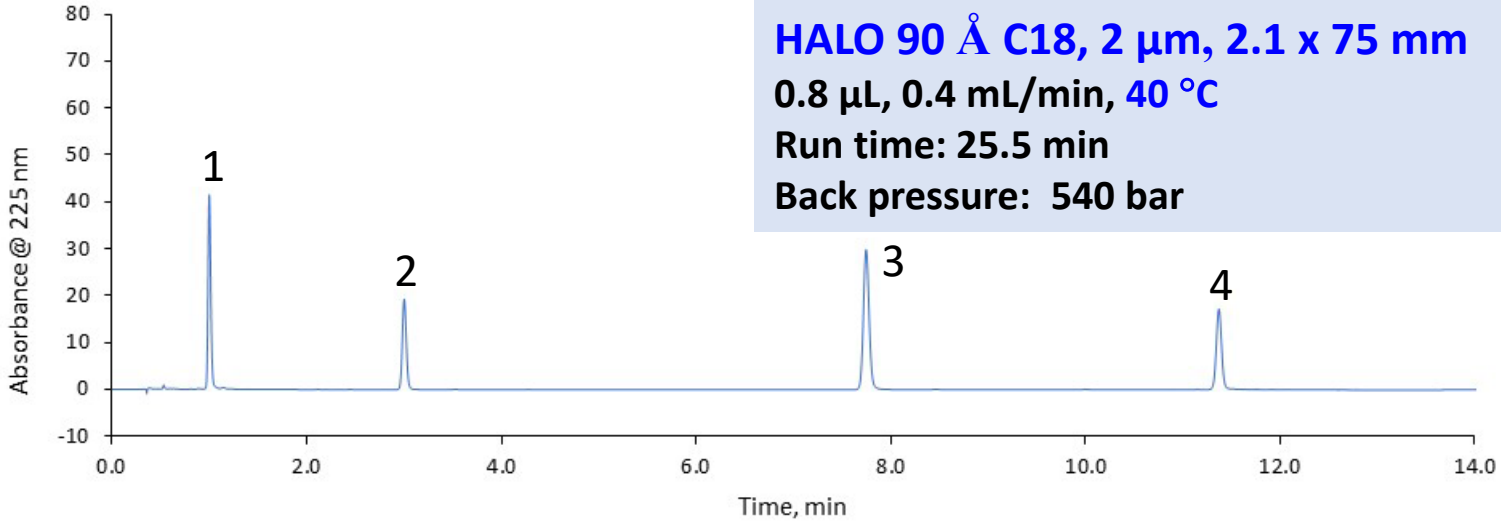
Total run time is 1.8 times faster!

54 mL/run compared to 10 mL/run which is 5.4 times less solvent used!

USP Monograph for Cobamamide – Modified System Suitability



SPP C18, 2.6 µm, 4.6 x 100 mm
5 µL, 1.2 mL/min, 40 °C
Run time: 45 min
Back pressure: 506 bar



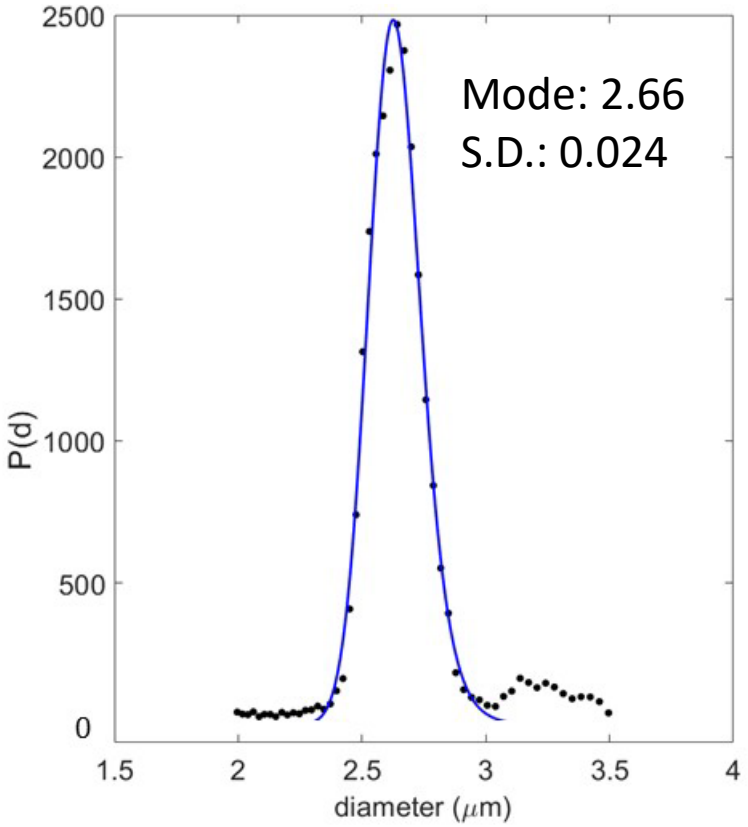
HALO 90 Å C18, 2 µm, 2.1 x 75 mm
0.8 µL, 0.4 mL/min, 40 °C
Run time: 25.5 min
Back pressure: 540 bar

Peak Identities
1. hydroxocobalamin chloride
2. cyanocobalamin
3. cobamamide
4. methylcobalamin

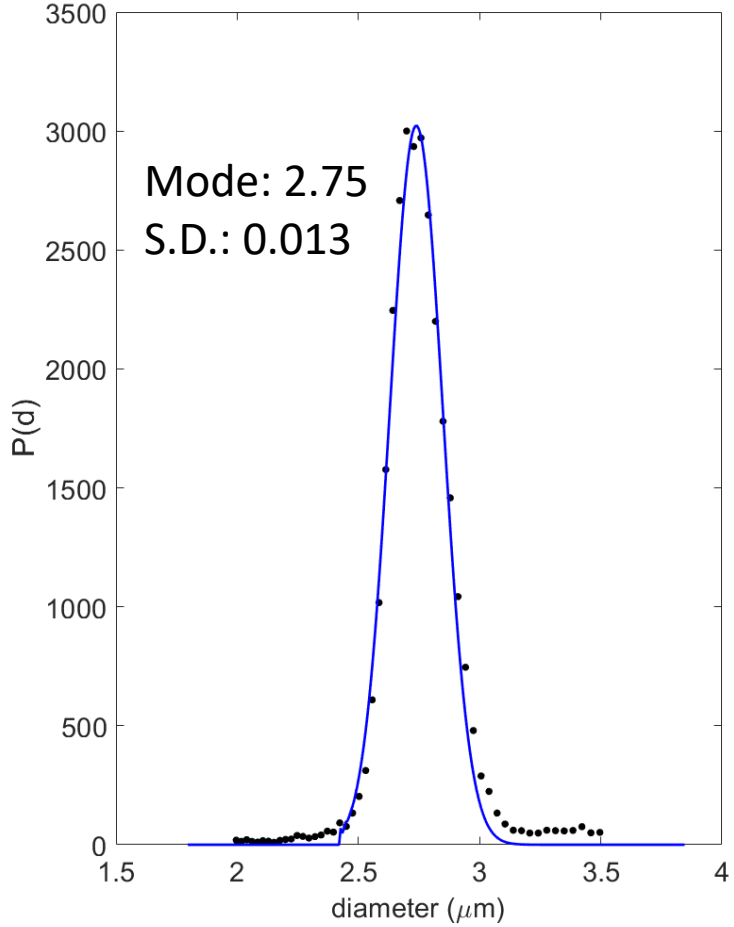
Elution order is identical on both columns

Particle Size Distribution Comparison

2.6 µm SPP

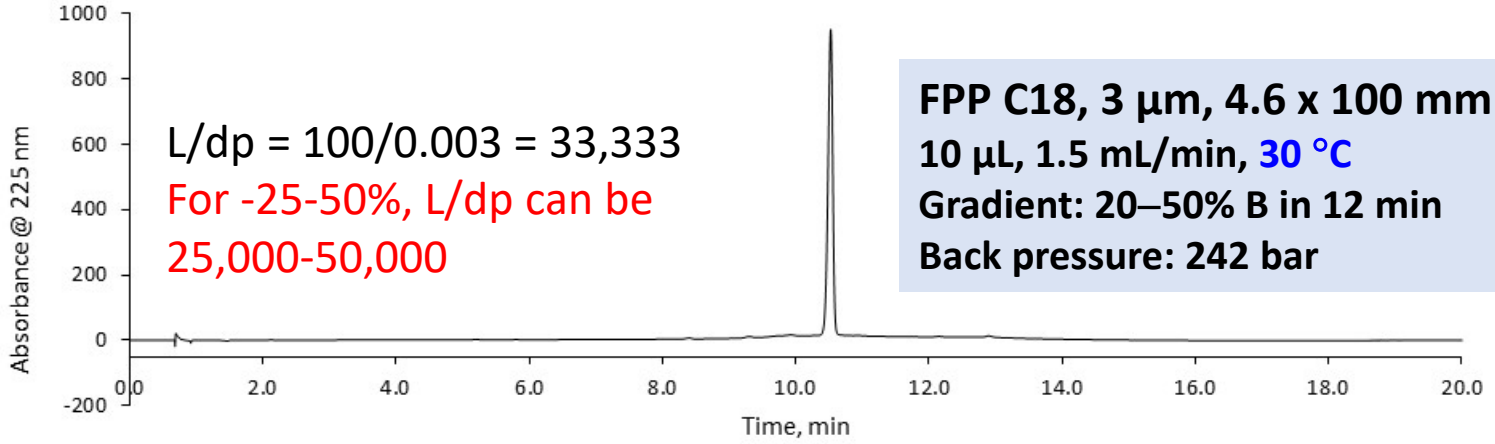


HALO® 2.7 µm

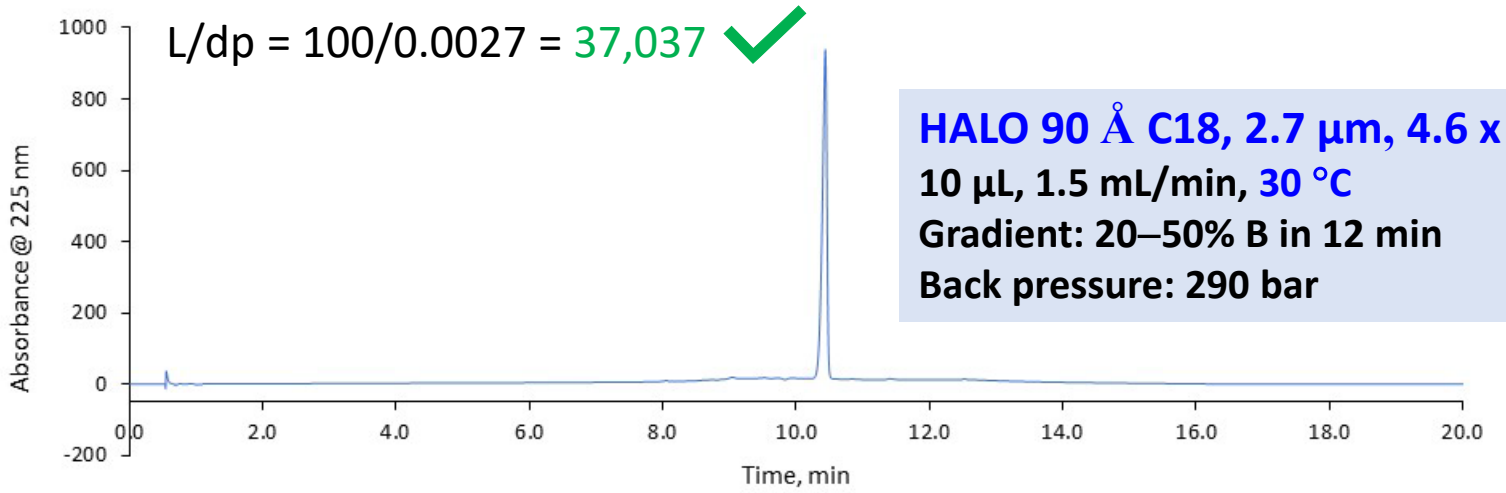


- No significant difference in particle size
- Slightly better standard deviation for HALO®

USP Monograph for Itraconazole – Original



FPP C18, 3 µm, 4.6 x 100 mm
10 µL, 1.5 mL/min, 30 °C
Gradient: 20–50% B in 12 min
Back pressure: 242 bar

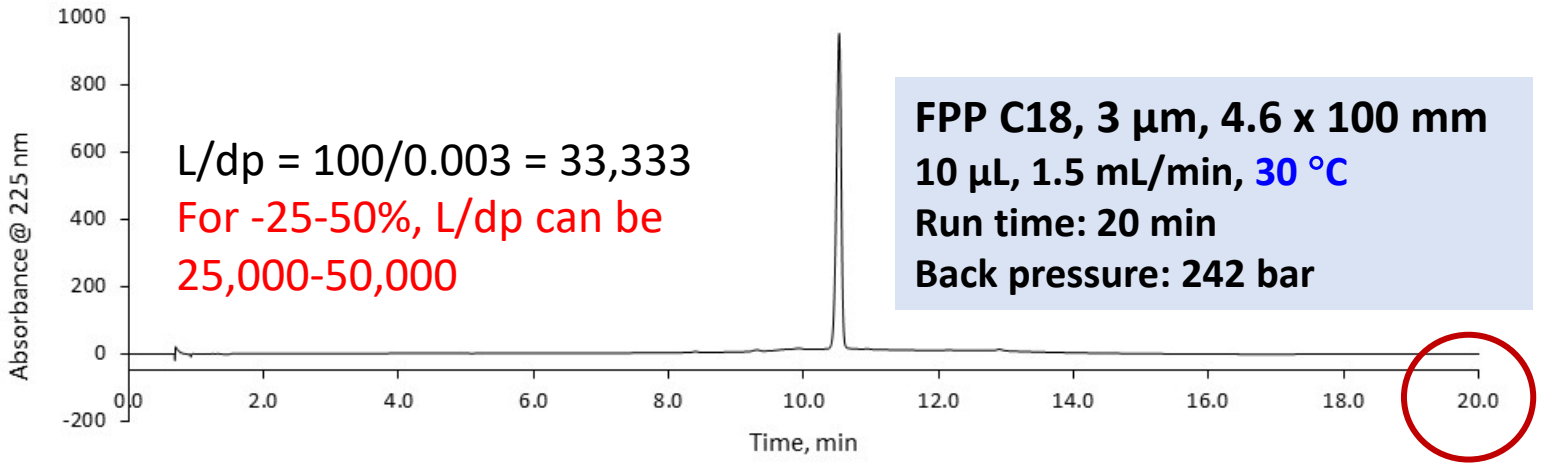


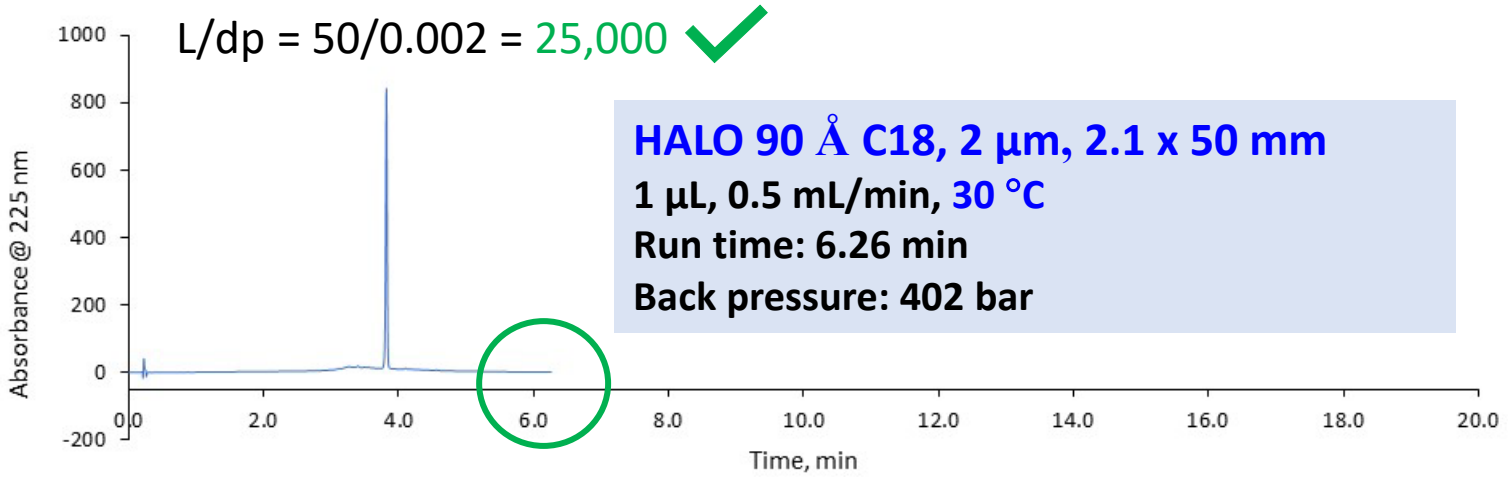
HALO 90 Å C18, 2.7 µm, 4.6 x 100 mm
10 µL, 1.5 mL/min, 30 °C
Gradient: 20–50% B in 12 min
Back pressure: 290 bar

- Itraconazole is an antifungal medication
- **Suitability Requirements**
  - Tailing factor: NMT 2.0
  - RSD: NMT 2.0%

	Monograph Column	HALO C18
Tailing Factor	**Pass**	**Pass**
RSD	**Pass**	**Pass**

USP Monograph for Itraconazole – Modified



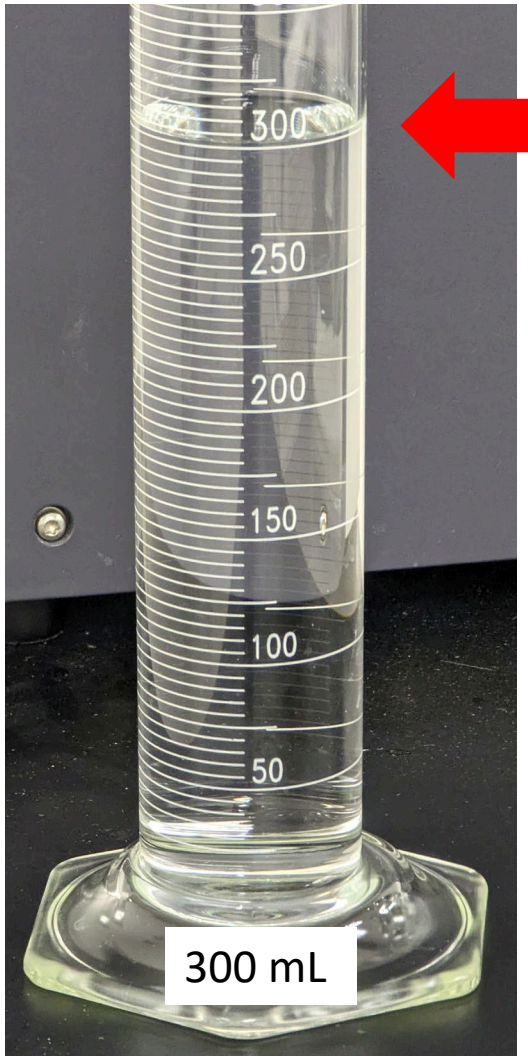


Method was moved to a shorter SPP column with smaller i.d. and smaller particle size

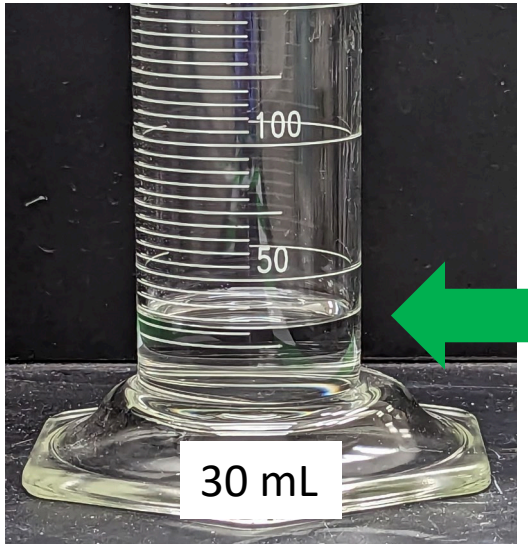
Total run time is > 3 times faster with the HALO® column!

USP Monograph for Itraconazole: Mobile Phase Reduction

For 10 injections:



compared to



10X reduction in mobile phase used!

Summary

- HALO® columns are made for ruggedness with high efficiency and high speed separations.
- It is important to optimize the instruments in order to gain the most benefit from HALO® column technology.
- Following new USP guidelines for gradient method modernization enables both FPP and SPP methods to be improved for speed and mobile phase savings using HALO® Fused-Core® column technology.

Acknowledgements

- Advanced Materials Technology, Inc.
  - Peter Pellegrinelli
  - Conner McHale
  - Dr. Taylor Harmon
  - Ben Libert
  - William Miles
  - Dr. Chuping Luo
  - Dan Messick
  - Melissa Ash
  - Stephanie Rosenberg

Questions

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