Optimizing Particle Pores for HPLC Separation of Verapamil and other Mid-Size Molecules

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Outline

- Background
  - Why consider the HPLC column pore environment?
    - Some key studies on optimizing pore size
    - Previous work using 160Å pores for 5000-15000 Da separations
    - Mid-size molecules = 500-5,000 Da
- Results and discussion:
  - Preliminary results with small peptide MWT of ~3,000 Da
  - Current study with a 'midsize' molecule: verapamil MWT of ~500 Da
  - Columns studied: C18 and C30: 90Å and 160Å
  - Exploiting mid-size separations with 160Å:
    - pharmaceutical, food and natural products separations

Conclusion: pore-size optimization important for 'mid-size' solutes separations

Why Consider the HPLC Column Pore Environment?

Resolution (R_s) gains through Pore Size Optimization:

- Correct choice of packing material's microstructure and pore size impact all three aspects of R_s efficiency, selectivity and retention
  - Increased selectivity by utility of polymeric bonded phases/phases with rigid structures that require larger pores for best results
  - Improved efficiency, especially at higher mobile phase velocities, resulting from reduced resistance to molecular diffusion for larger molecules
  - Improved retention due to greater access for larger molecules to interior surfaces of packing material where majority of surface area and bonded phase are present

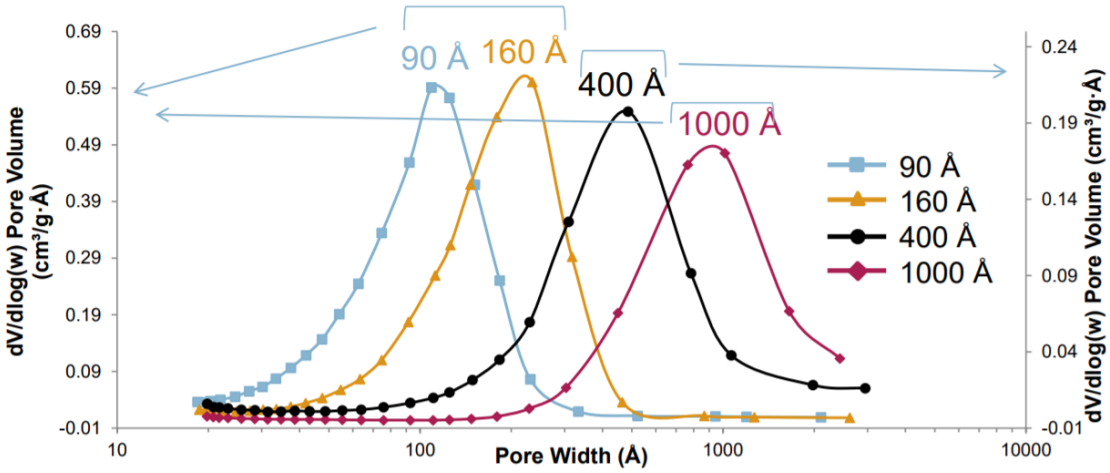
Why Consider the HPLC Column Pore Environment?

- Bonded phases with larger size or rigid structural features
  - may provide special selectivity for separating isomers/similar structured molecules
  - may block entrances to pores on typical small-pore silicas
- Smaller pores could limit access to pore structures due to physical hindrance (partial exclusion) of mid-size molecules:
  - limits retention (majority of surface area and bonded phase exists within the particle).
  - increase efficiency if the pore size is increased, especially when operating at above optimum flow rates, due to faster mass transfer.

Pore-size is important and often overlooked by analysts

HALO® Superficially Porous Particle Characteristics

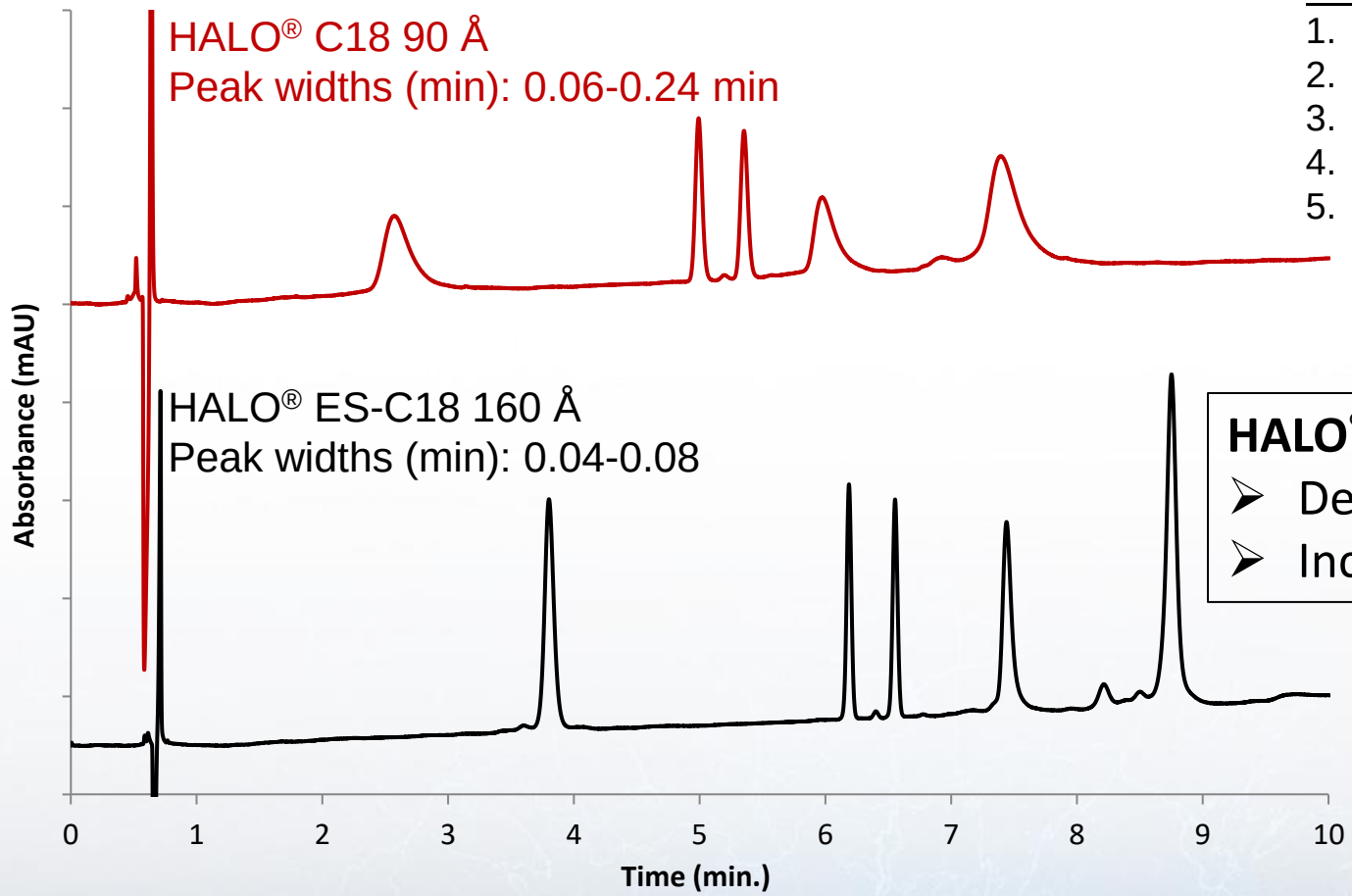
Pore Sizes of HALO® Silica for Different Molecules



Molecule	Molecule Diameter	Recommended Minimum HALO® Pore Size
Small drugs	5-20 Å	90 Å
Small peptides	20-30 Å	160 Å
Large peptides	30-50 Å	160 Å
Small proteins	50-100 Å	400 Å
Large proteins	100-200 Å	1000 Å

https://www.advanced-materials-tech.com/wp-content/uploads/prod_files/Add_Technical/AMT-UTH-Pore%20Size_BioClass_04_020.pdf

Previous work: 160 Å for Medium MWT analytes
Small Peptides 5000-15,000 Da



Peaks:

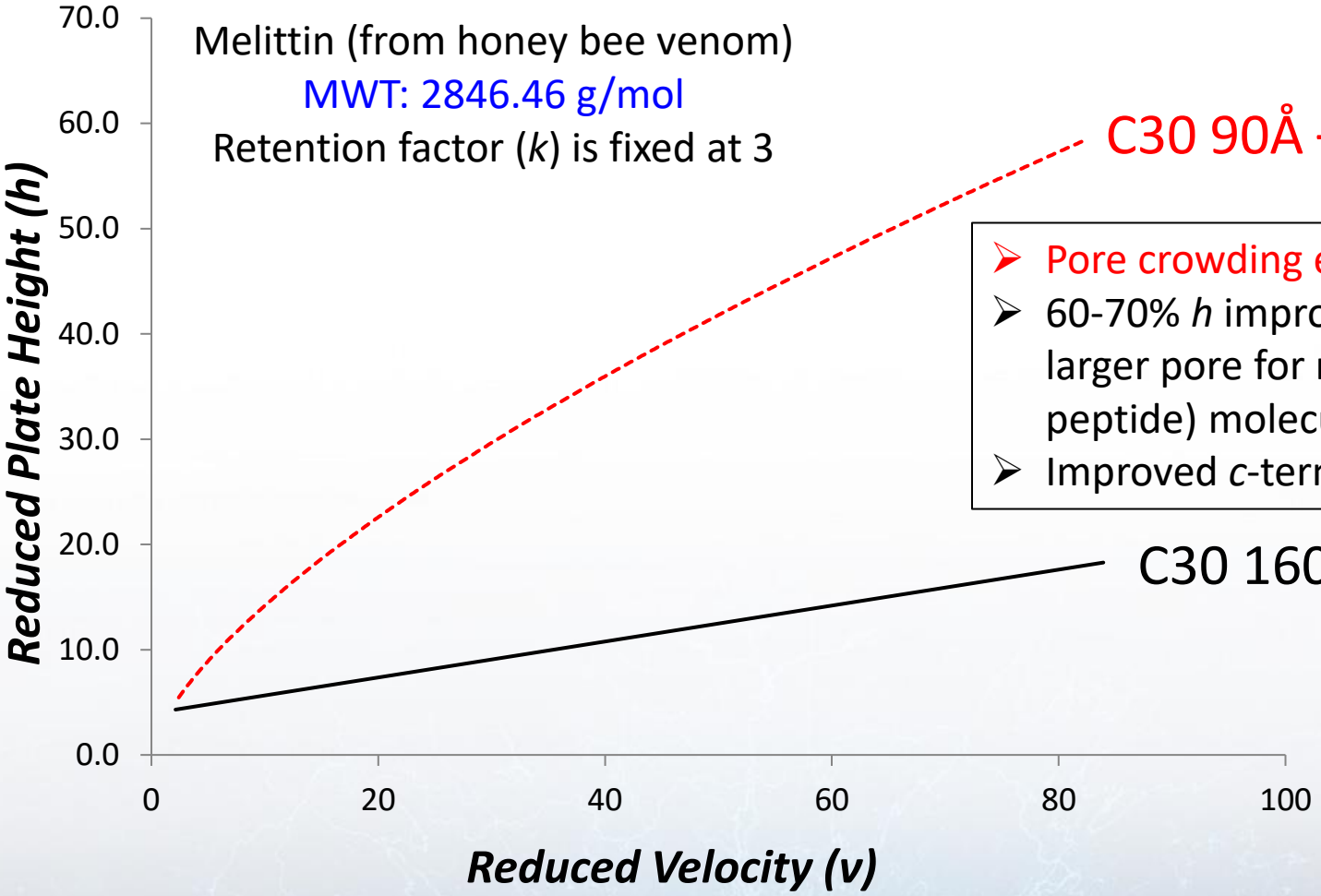
1. Ribonuclease A (13,700 g/mol)
2. Bovine Insulin (5733 g/mol)
3. Human Insulin (5808 g/mol)
4. Cytochrome *c* (12,400 g/mol)
5. Lysozyme (14,300 g/mol)

HALO® ES-C18 160 Å

- Decreased peak width
- Increased peak capacity

Columns: 2.7 µm d_p, 100 mm x 4.6 mm HALO C18 90 Å pores and HALO ES-C18 160 Å pores;
Mobile phase: A: water/0.1% trifluoroacetic acid; B: acetonitrile/0.1% trifluoroacetic acid;
Gradient: 25–42% B in 10 min; flow rate: 1.5 mL/min; temperature: 30 °C; detection: 215 nm.

Preliminary study: 160 Å for a mid-size solute

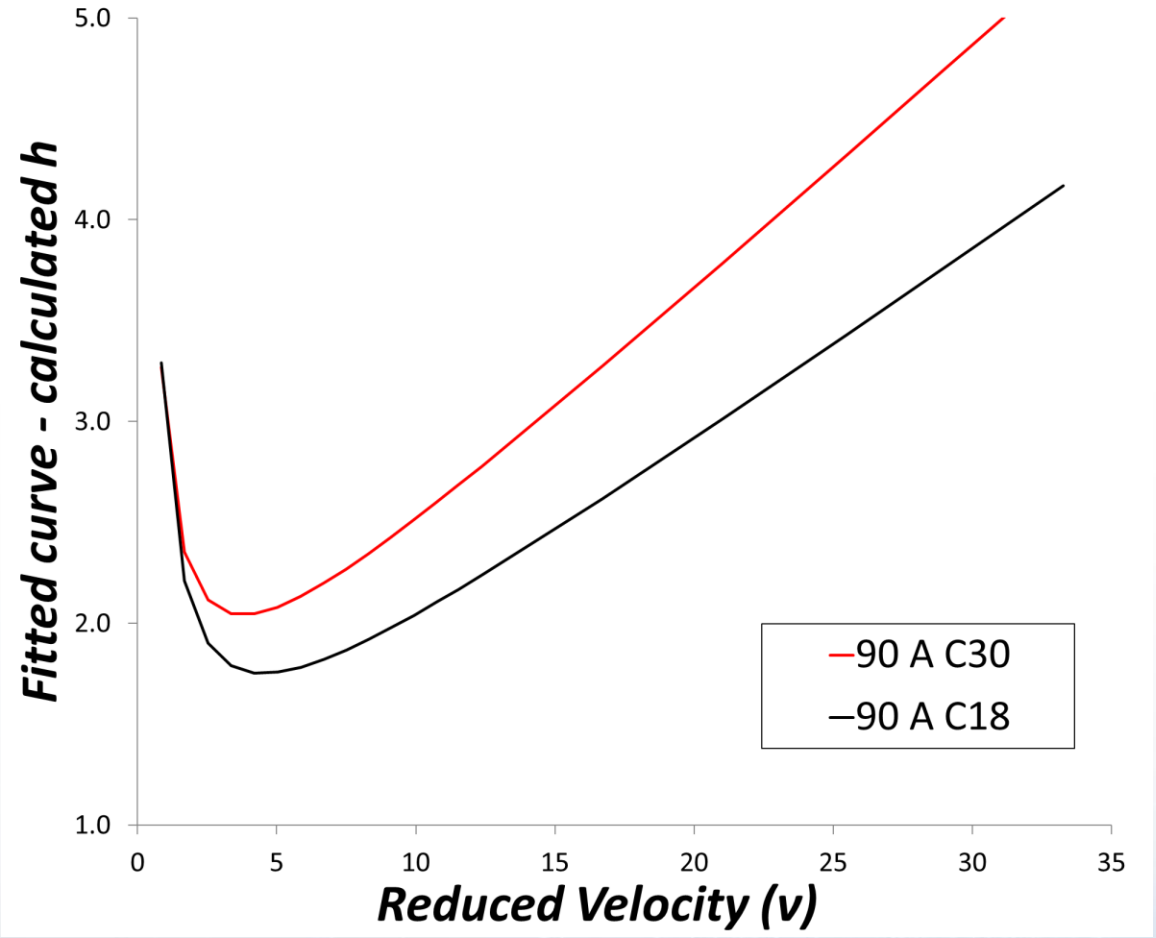


Column: C30 90Å and 160Å, 2.7 µm dp, 50 mm x 4.6 mm
Mobile phase: A: water/0.1% trifluoroacetic acid; B: acetonitrile/0.1% trifluoroacetic acid; k = 3
Temperature: 40 °C;
Detection: 215 nm.

‘Mid-size’ solute van Deemter Comparisons

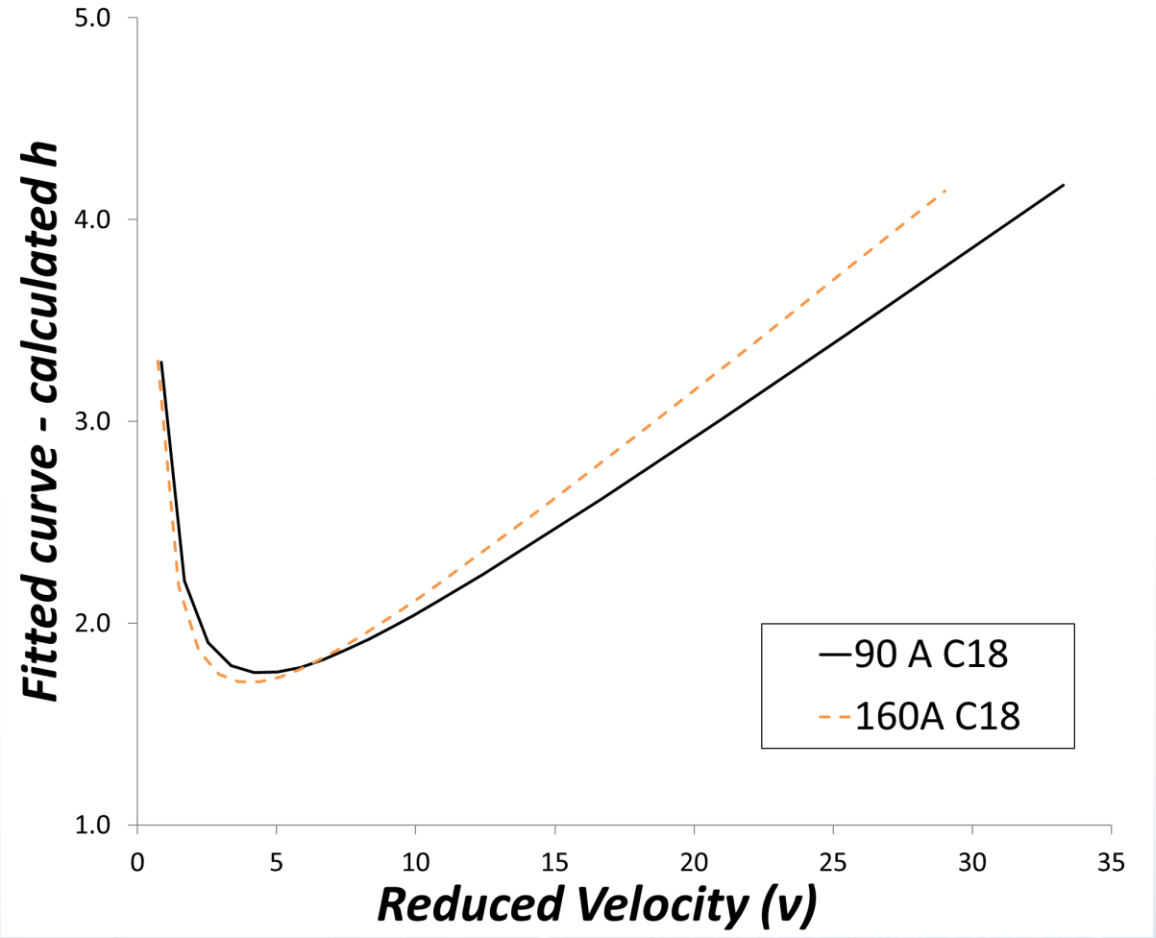
- Verapamil 454.6 g/mol
- Injection volume of 0.5 µL (0.4 mg/mL in 0.1% TFA in water)
- Column format: 4.6 x 50 mm, 2.7 um d_p – 90 / 160 Å
- Ligands: C18 and C30
- Isocratic Mobile Phase: 0.1% TFA in Water/acetonitrile isocratic, gravimetrically prepared, pumped via single line, ACN adjusted to give k=3
- Column thermostat temperature: 40°C
- Wilke-Chang diffusion estimate (D_m) (cm^2/s)
  - Verapamil 454.6 g/mol 6.3-6.4 e^{-6}
  - Naphthalene 128 g/mol 1.40 e^{-05}

Verapamil 90 Å: C18 vs. C30



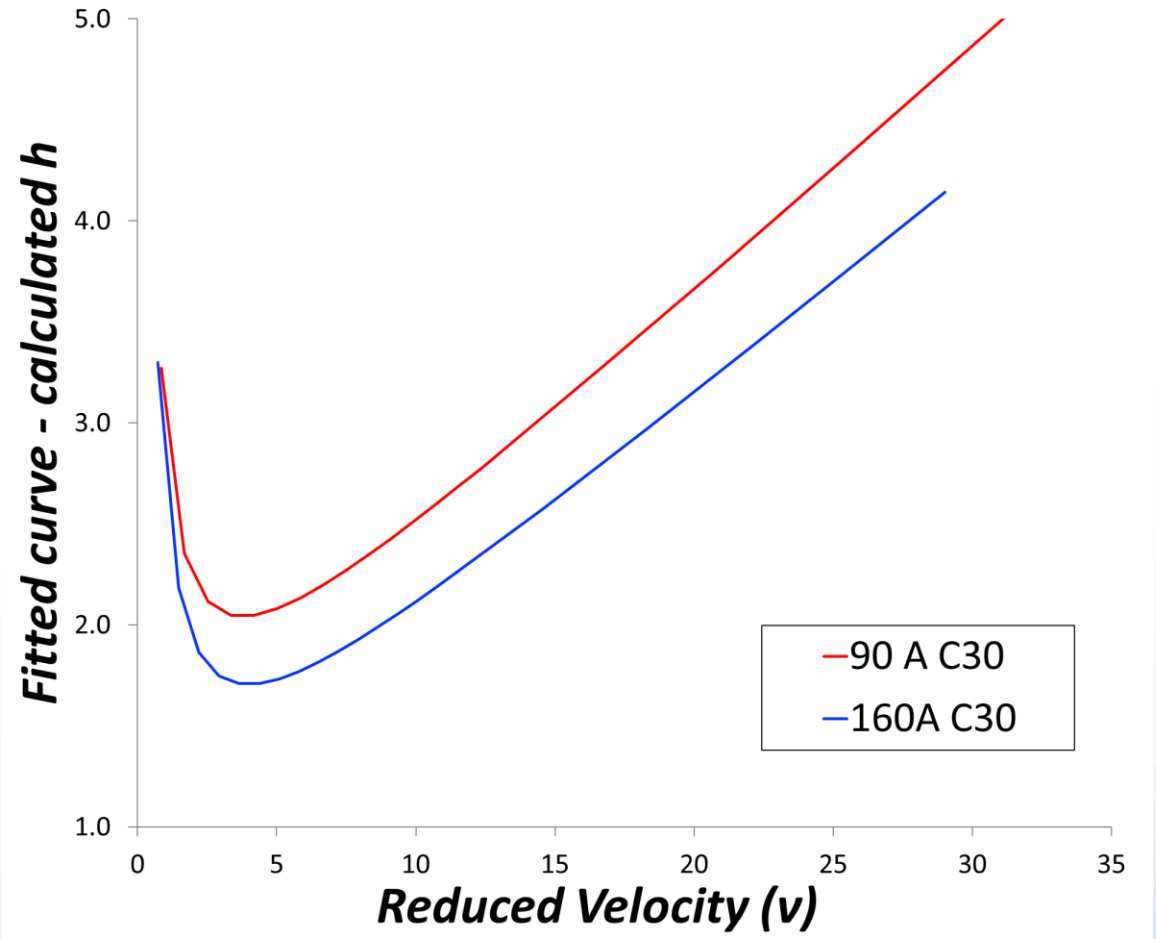
- C18 clearly had an advantage compared to C30 on the same pore size with lower reduced plate heights

Verapamil C18: 90 Å vs. 160 Å



- Performance is comparable at optimum
- Indication of very little crowding.
- *c*-term difference not significant.
- Good column performance can be supplied with same phase selectivity and different pore sizes.

Verapamil C30: 90 Å and 160 Å



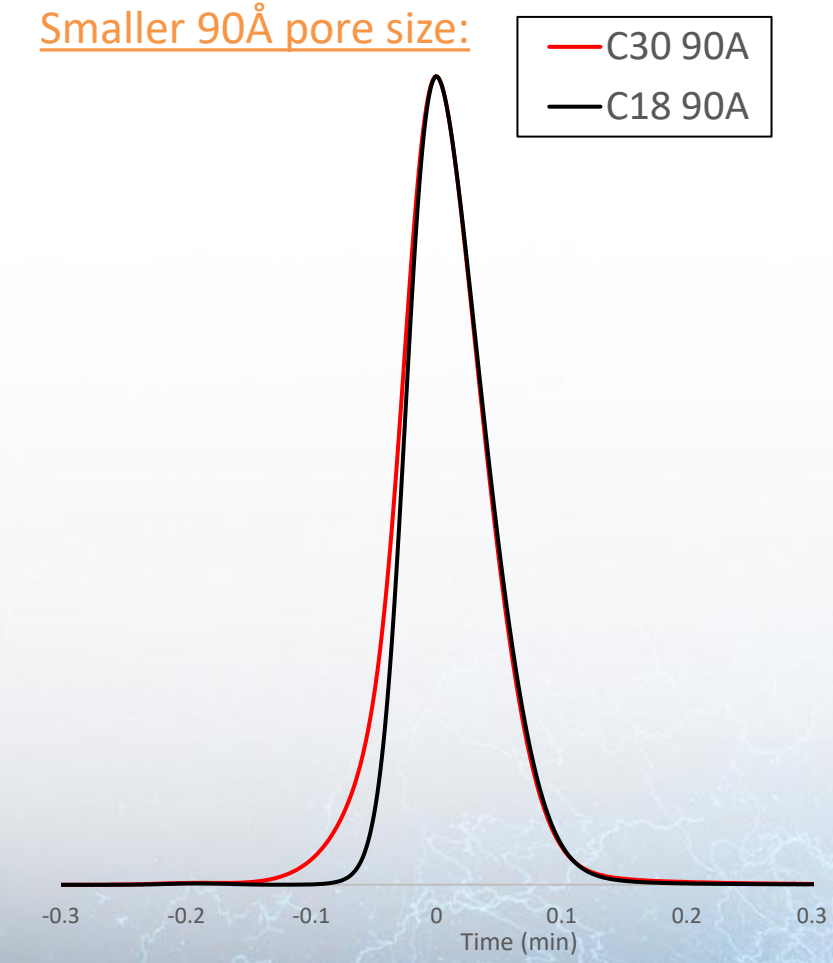
C30 phase:

- Larger pore decreased reduced plate heights for bulky C30 phase
- See the impact of pore crowding in 90A
- Overall C30 160Å lowest *h*

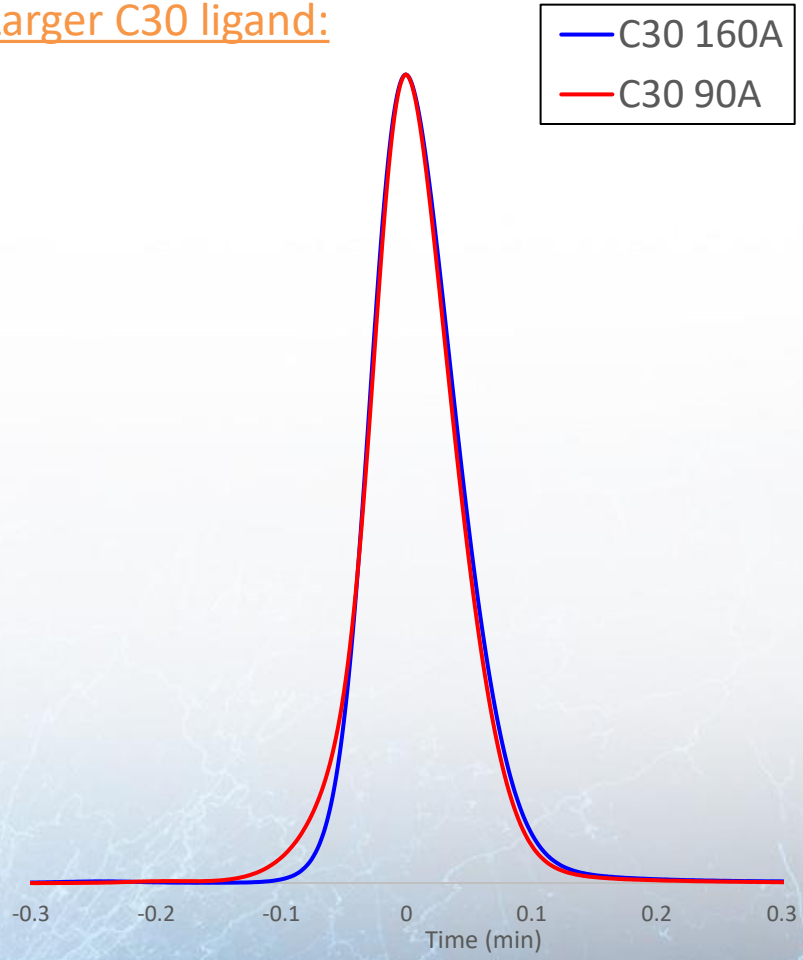
Verapamil Peak Shape Comparisons Near *h*-min

- Impact of pore crowding for smaller pore size bonded with larger ligand
- Commercial products: best combinations for mid-sized molecules

Smaller 90Å pore size:



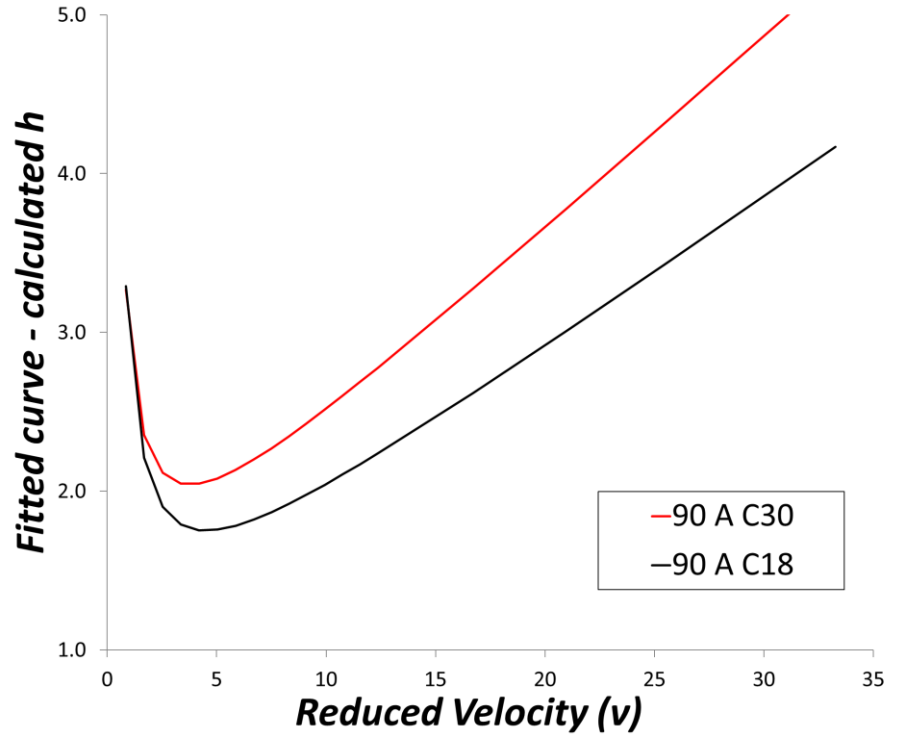
Larger C30 ligand:



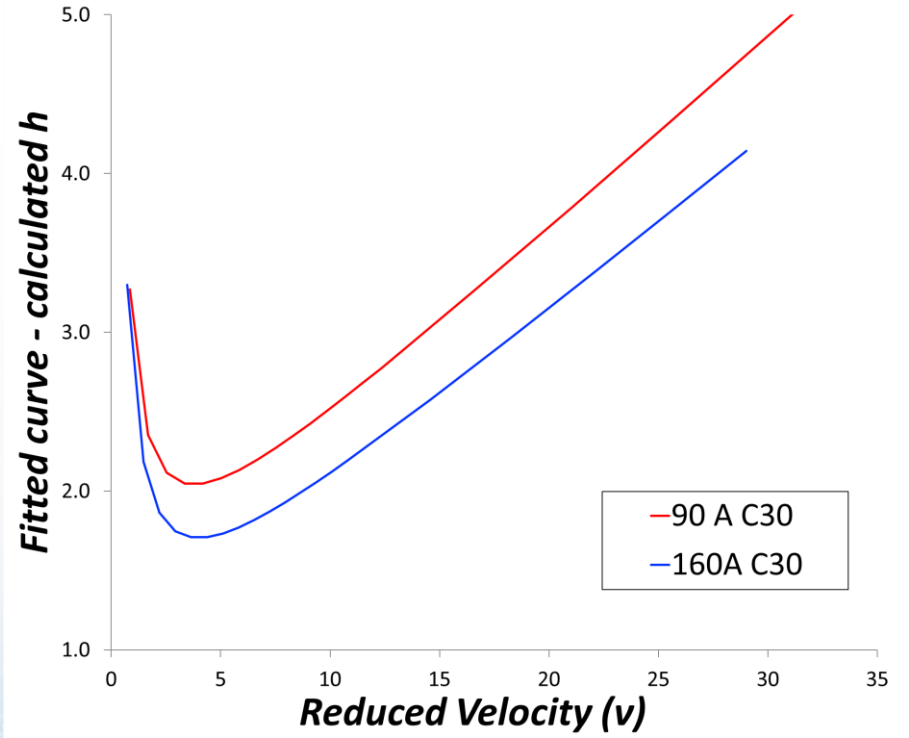
Verapamil van D Summary

- Impact of pore crowding for smaller pore size bonded with larger ligand
- Commercial products: best combinations for mid-sized molecules

Smaller 90Å pore size:

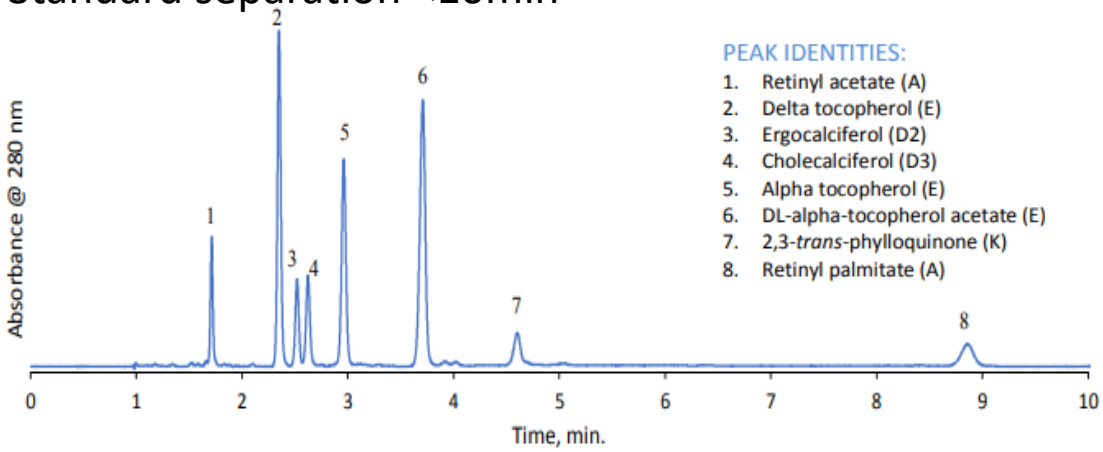


Larger C30 ligand:

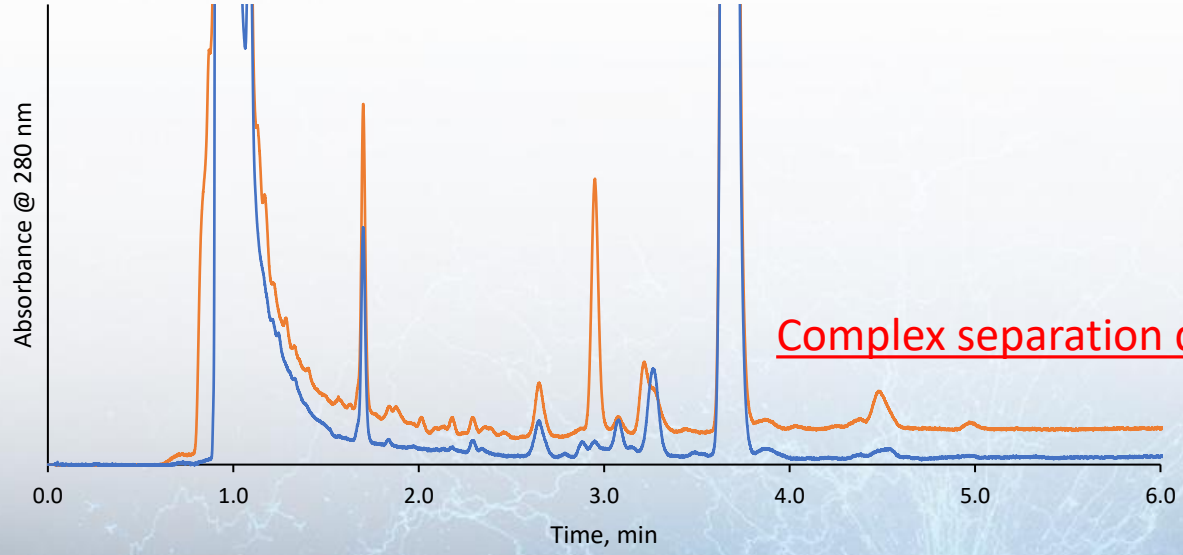


Mid-size Molecules Separations
Fat Soluble Vitamins

Standard separation <10min



Profiling two different multivitamin tablets <6 min

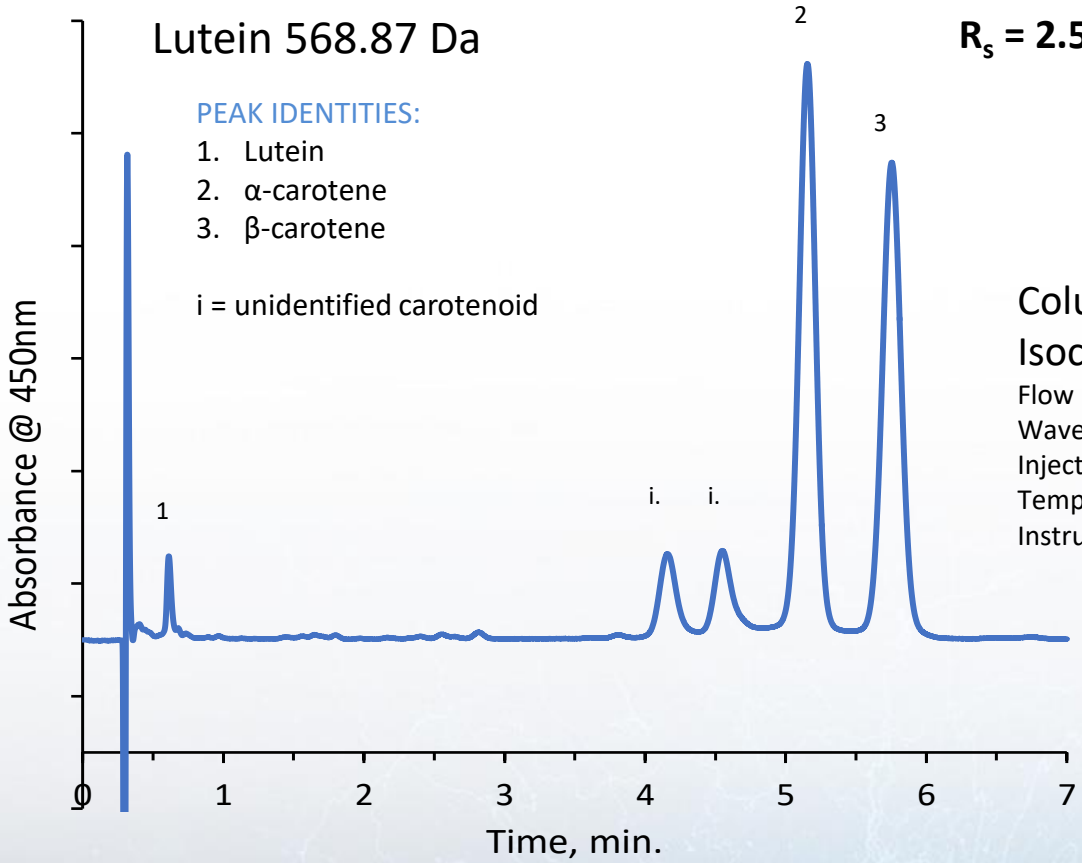


Complex separation due to sample matrix

Analytical Column: HALO 160 Å C30, 2.7 µm, 4.6 x 150 mm
Part Number: 92114-730
Isocratic: Methanol
Flow Rate: 1.5 mL/min
Pressure: 262 bar
Temperature: 30 °C
Injection Volume: 2.0 µL
Sample Solvent: Methanol
Detection: 280 nm, PDA
LC System: Shimadzu Nexera X2

Mid-size Molecules Separations
Carotenoids Extracted from Carrot Juice

Baseline separation of α and β-carotene with HALO® C30 < 6.5 min

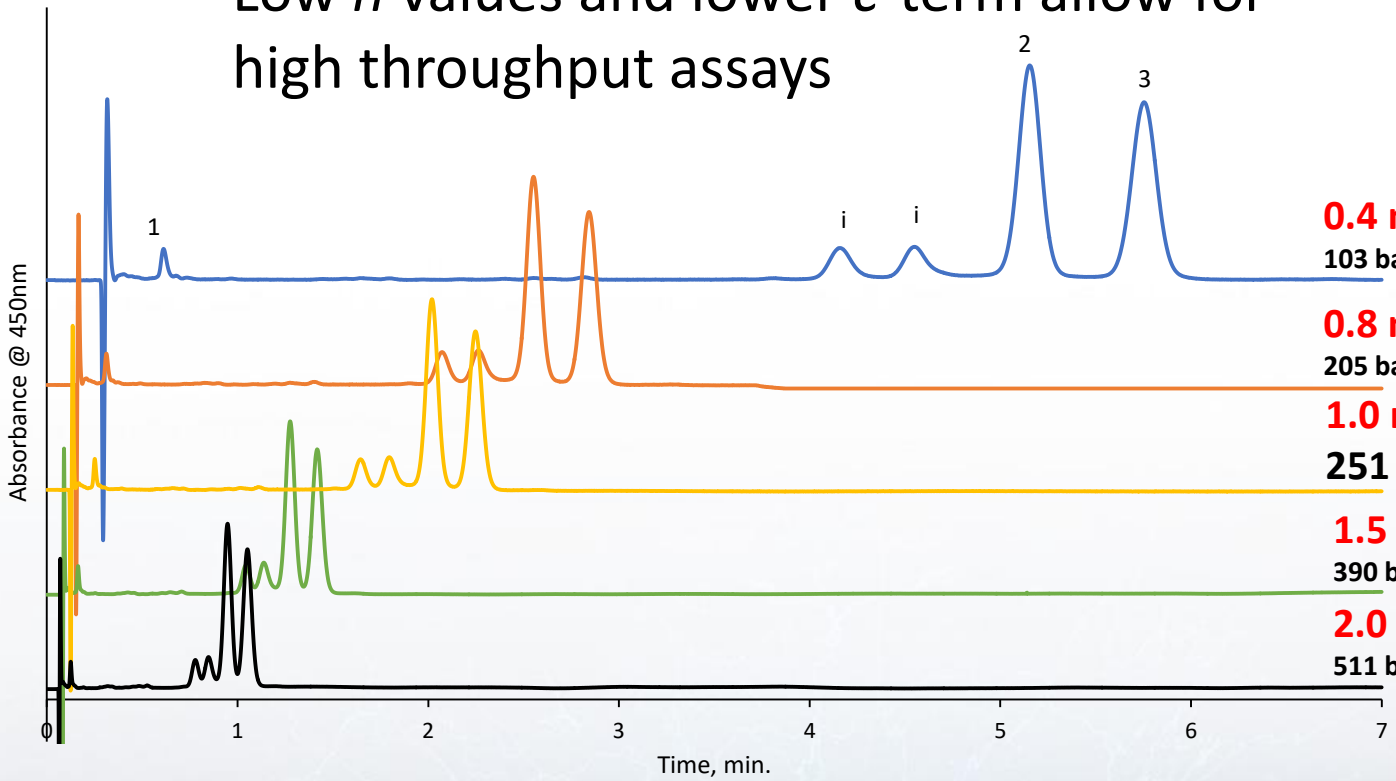


R_s = **2.54**

Column: HALO 160 Å C30, 2.7 µm, 2.1 x 50 mm
Isocratic: 100% methanol
Flow Rate: 0.4 mL/min
Wavelength: UV 450 nm, PDA
Injection: 2.5 µL
Temperature: 30 °C
Instrument: Shimadzu Nexera X2

Fast Mid-size Molecules Separations
Carotenoid MWT Range 540-570 Da

HALO 160 Å C30, 2.7 µm, 2.1 x 50 mm
Low *h* values and lower *c*-term allow for high throughput assays



R_s = 2.54

R_s = 1.99

R_s = 1.82

R_s = 1.49

R_s = 1.32

Column: HALO 160 Å C30, 2.7 µm, 2.1 x 50 mm
Isocratic: 100% methanol
Flow Rate: as indicated
Wavelength: UV 450 nm, PDA
Injection: 2.5 µL
Temperature: 30 °C
Instrument: Shimadzu Nexera X2

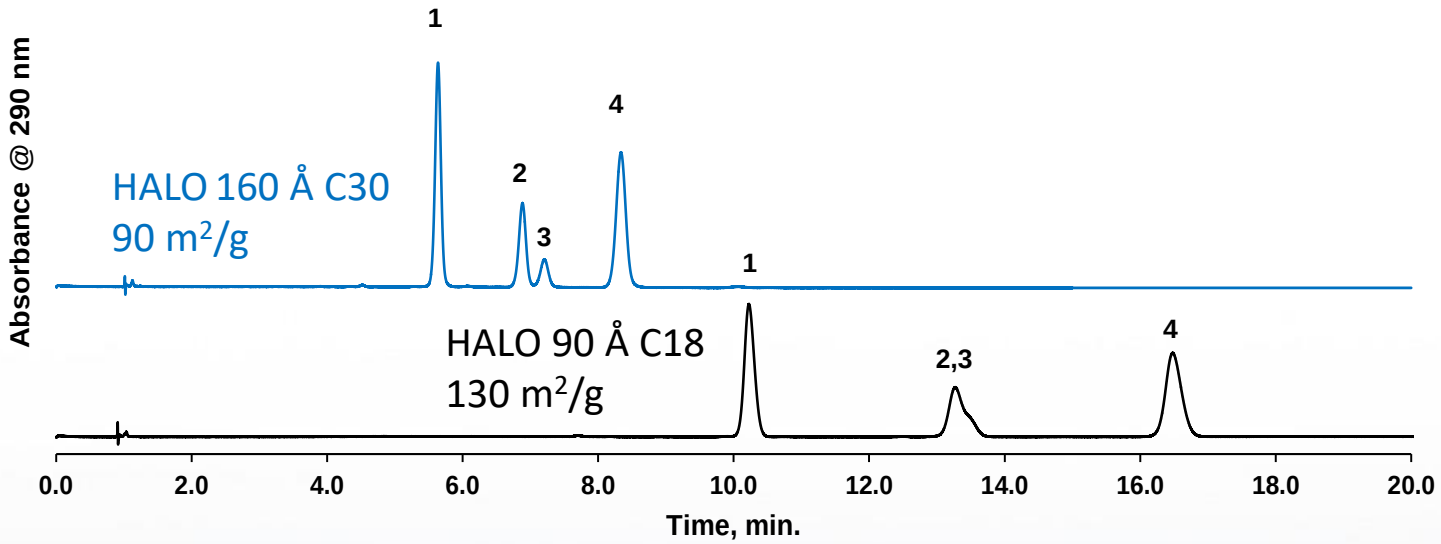
PEAK IDENTITIES:

1. Lutein
2. α-carotene
3. β-carotene

i = unidentified carotenoid

Mid-size Molecules Separations
Vitamin E: Tocopherols

Baseline resolution – shape selectivity with HALO® C30

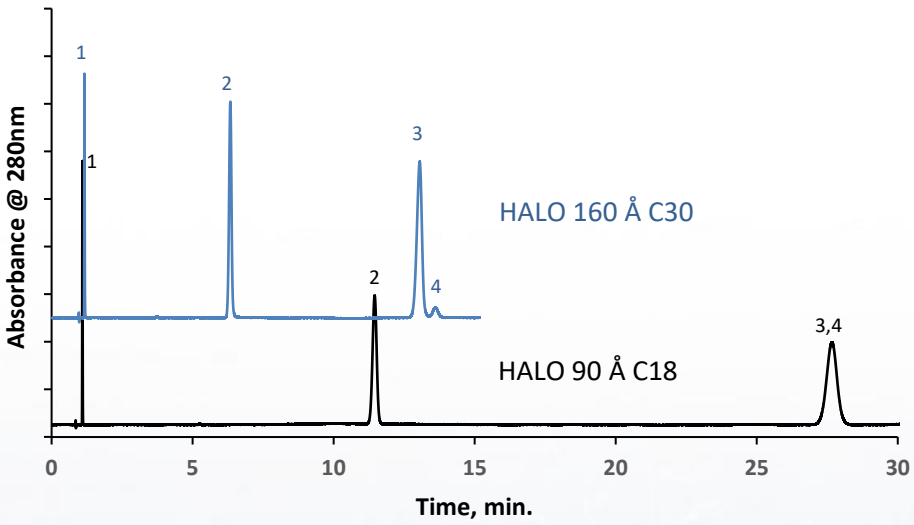


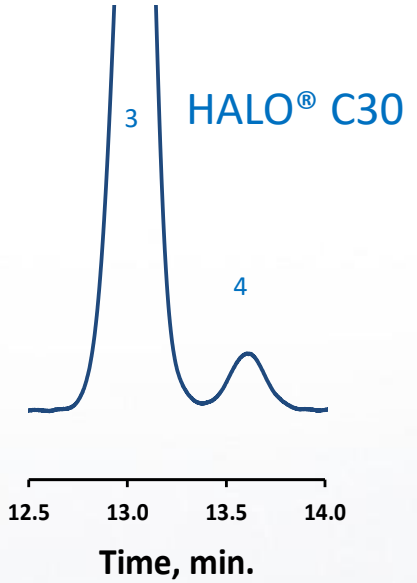
Peak	Tocopherol	R1	R2
1	Delta (δ)	H	H
2	Gamma (γ)	H	CH_3
3	Beta (β)	CH_3	H
4	Alpha (α)	CH_3	CH_3

Columns: HALO 160 Å C30 and HALO 90 Å C18 , 2.7 µm, 4.6 x 150 mm
Isocratic: 5/95 water/methanol
Flow Rate: 1.5 mL/min
Temperature: 10 °C
Injection Volume: 1.5 µL
LC System: Agilent 1200 SL
Detection: UV 290 nm, PDA

Mid-size Molecules Separations
Vitamin K

C30 160Å: improved resolution (selectivity and sharper peak widths), less retention - faster assay.

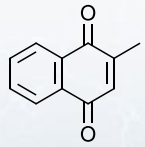




Column format: 2.7 µm, 4.6 x 150 mm
Isocratic: 5/95 water/methanol
Flow Rate: 1.5 mL/min
Temperature: 25 °C
Injection Volume: 1 µL
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm

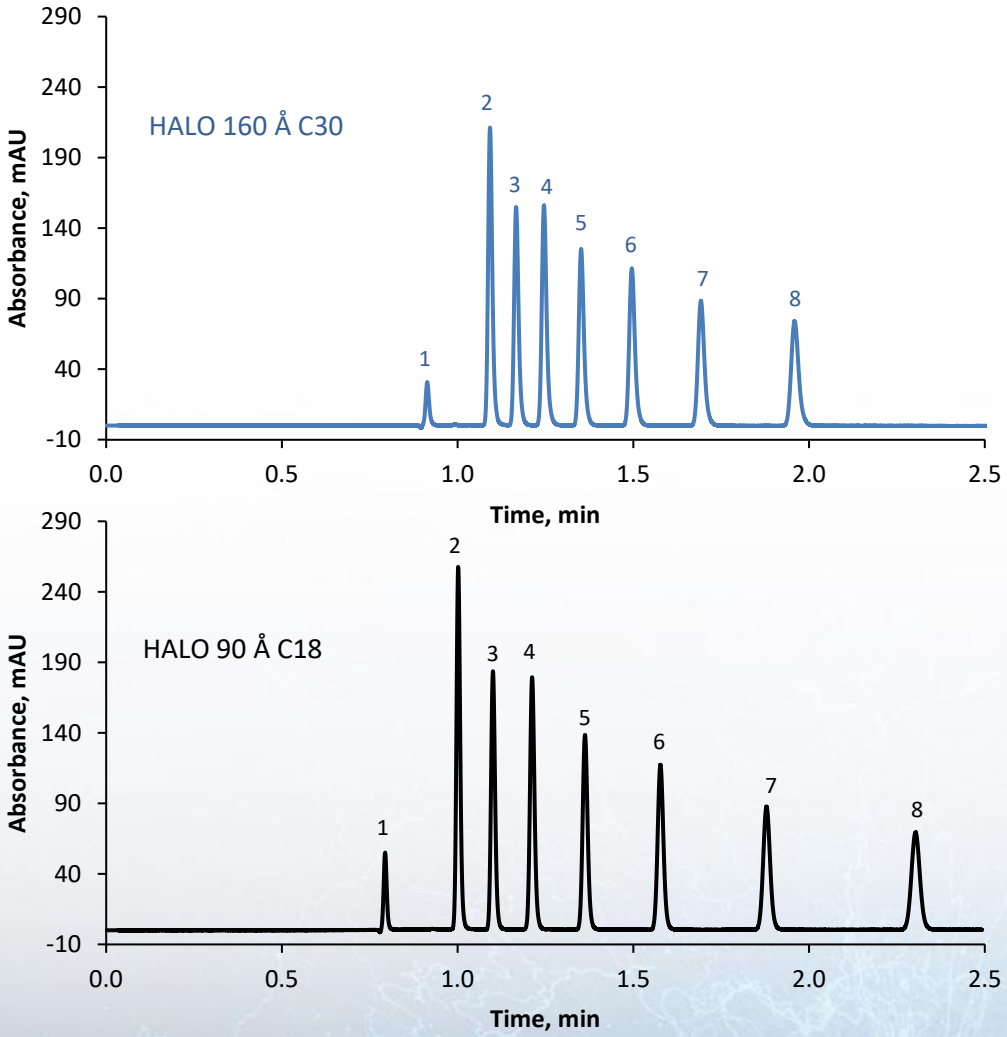
PEAK IDENTITIES:

1. Menadione (K3)
2. Menaquinone 4 (K2)
3. 2,3-*trans*-phylloquinone (K1)
4. *cis*-phylloquinone (K1)



Vitamin K3: Menadione

Small Molecule Separation of Alkylphenones <200 Da



- Small molecule separations still ok via C30 160Å
- Useful for Mid-size + small MWT separations
- Analysis speed can be increased with larger pore columns

Test conditions:
Column: 4.6 x 150 mm
Mobile phase: A = water, B = ACN, Isocratic 85% B
Flowrate: 1.5 mL/min
Temperature: 40 °C
UV wavelength: 254 nm

Conclusions

Conclusion: pore-size optimization important for ‘mid-size’ solutes separations

- van Deemter work verapamil ~450 Da best separated with longer alkyl chain and larger pore C30 160Å phase
- Exploit larger 160 Å pore advantages for mid-size analytes 500-5,000 Da
- Future work: pore-size optimization during method development requires similar phases available in a larger pore size

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