DEVELOPING HPLC METHODS WHEN C18 COLUMNS DON'T WORK

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CHOOSING PHASES OTHER THAN C18

- **Why choose a C18 phase?**
  - Very versatile and retentive for RPLC with simple binary gradients.
  - Proven stability with wide range of mobile phase and additives.
  - Low drift and bleed with LC-MS and other detectors.
  - Past success with C18 columns.
- **When to try another phase chemistry?**
  - Isomers or very similar analytes can’t be resolved.
  - Too much retention or selectivity with C18 for desired analysis time.
  - Polar analytes not well retained with low or no organic modifier.
  - Polar analytes not well resolved even if retained.
  - A C18 method already in use is not rugged enough (revalidate).
- **Multiple column and solvent screening is recommended for resolving difficult mixtures and achieving rugged system performance.**

FACTORS CONTROLLING HPLC/UHPLC RESOLUTION*

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha-1}{\alpha}$$

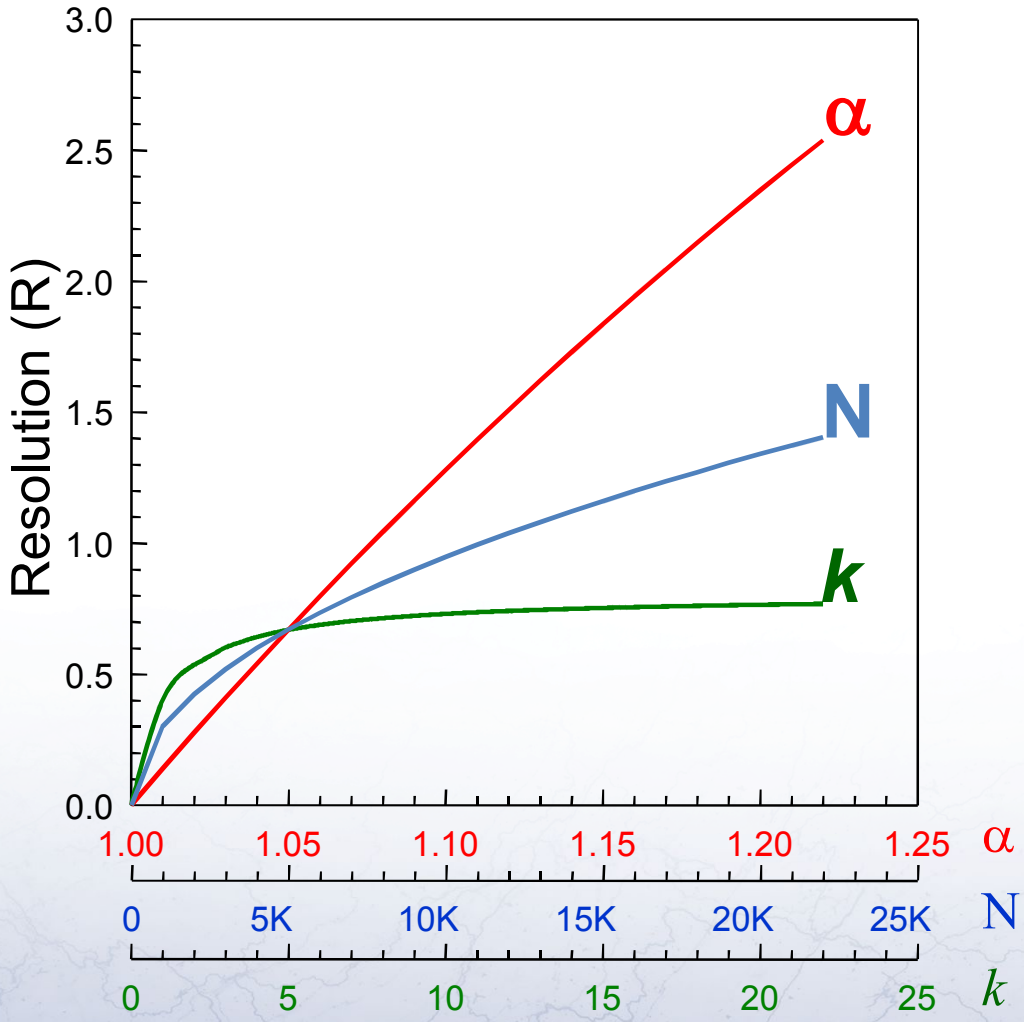
$N = 16\,(t_R/w_b)^2$ or

$N = 5.5\,(t_R/w_{1/2})^2$

$k = (t_R - t_0)/t_0$

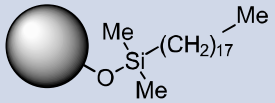
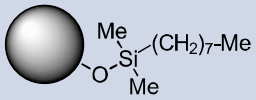
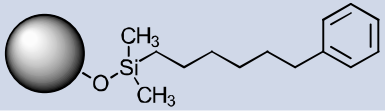
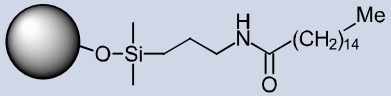
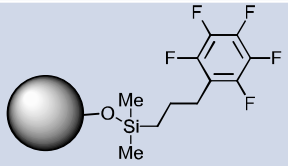
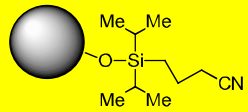
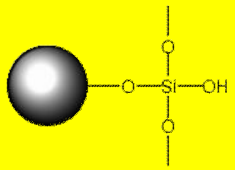
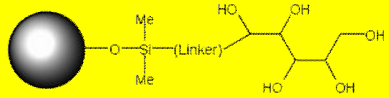
$\alpha = k_2/k_1$

All factors are important, but selectivity is considered the most effective term to change.



* Yun Mao, PhD Dissertation, Prof. Peter Carr, University of Minnesota, 2001.

HALO® PHASES FOR SMALL MOLECULE SEPARATION

	Phase	USP	Particle Sizes (µm)	Structure
Industry Big Five	**C18**	L1	2 2.7 5	
	C8	L7	2 2.7 5	
	Phenyl-Hexyl	L11	2 2.7 5	
	RP-Amide	L60	2 2.7 5	
	PFP	L43	2 2.7 5	
Phases For Special Situations	**ES-CN**	L10	2 2.7 5	
	HILIC (silica)	L3	2 2.7 5	
	Penta-HILIC	L95	2 2.7 5	

FEATURES AND APPLICATIONS OF HALO PHASES

HALO Column	Features and Benefits	Best Applications
C18, C8	Excellent performance for broad range of analyte polarities	Analytes differing by an aliphatic or aromatic group
Phenyl-Hexyl	Complementary selectivity to alkyl phases Enhanced selectivity for aromatic compounds	Aromatic molecules with electron-withdrawing groups (NO_2, COOH, COOR, halogens), heterocycles, benzodiazepines, highly aqueous conditions
RP-Amide	Complementary selectivity to alkyl phases Enhanced stability for minimum bleed and long life	Acidic and basic analytes, heterocycles, proton donors and acceptors, highly aqueous conditions
PFP	Complementary selectivity to alkyl phases Enhanced selectivity for stereoisomers Can be used in RPLC and HILIC modes	Basic analytes at low pH, stereoisomers, steroids, taxanes, substituted aromatics, highly aqueous conditions
ES-CN	Complementary selectivity to alkyl phases More retention for polar analytes and much less retention for non-polar analytes	Aromatic molecules with electron-withdrawing groups (NO_2, COOH, COOR, halogens), heterocycles, benzodiazepines, highly aqueous conditions
HILIC	Can be used in HILIC and normal-phase modes	Enhanced sensitivity and peak shape for LC-MS, analyses of basic analytes in HILIC mode, normal-phase analysis with non-aqueous mobile phases
Penta-HILIC	Ideal for separation of highly polar compounds that are not retained in RPLC	Polar acids, bases and zwitterions that are not retained or are poorly retained with RPLC

VARIABLES THAT AFFECT SELECTIVITY FOR RPLC METHOD DEVELOPMENT

Column & Instrument System

- **Column (dictated by method)**
  - **Stationary phase**
  - Particle morphology
  - Particle composition
  - Particle pore size
- **Instrument (not dictated)**
  - Delay/Dwell volume

Methods will be easier to reproduce between labs if selectivity comes from the stationary phase with simple mobile phases. Operational variables contribute more to error because they are cumulative. Keep mobile phases as simple as possible.

Operational (Mobile Phase)

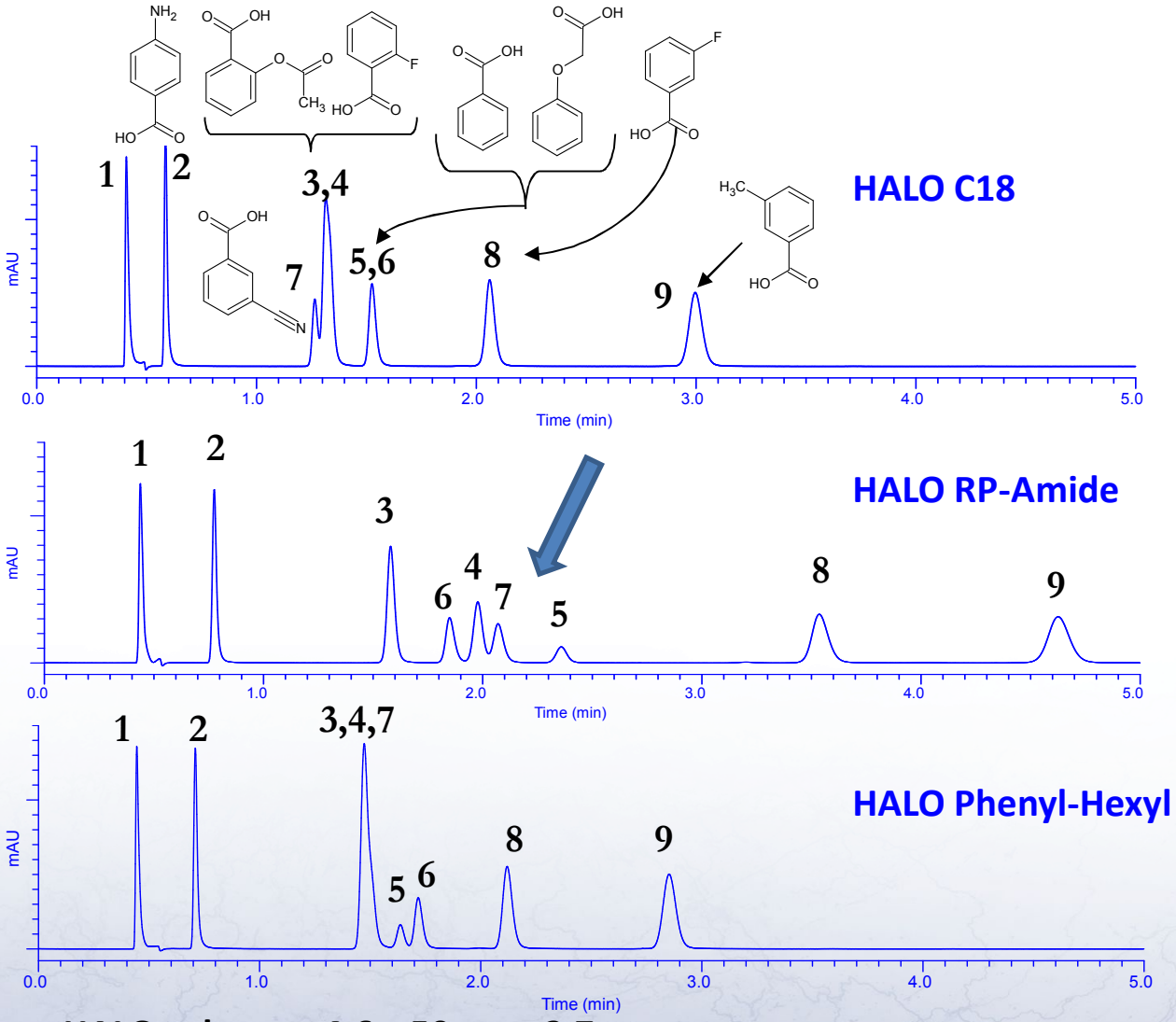
- **Mobile phase pH**
- **Organic modifier choice**
  - CH_3CN, CH_3OH, CH_3CN/CH_3OH blend, IPA, etc.
- **% Organic (isocratic) or gradient slope (gradient)**
- **Column temperature**
  - affects pH and ionization
- **Additive type**
  - TFA, H_3PO_4, phosphate, formate, acetate, NH_4OH, etc.
- **Additive concentration**
  - ionic strength, ionization suppression

RPLC SELECTIVITY RANKING *VS* HALO C18 AT DIFFERENT PH VALUES[1-2]

Phases Most Different from HALO C18	pH 2.8	pH 7
Most similar	C8	C8
	Phenyl-Hexyl	PFP
	ES-CN	Phenyl-Hexyl
	RP-Amide	ES-CN
Most different	PFP	RP-Amide

pH impacts charge on solutes that have acid-base properties and can also affect silica substrates. Silanol groups that are not covalently bonded will have neutral charge at low pH and negative charge at pH 7. Column rankings will also differ greatly between CH_3CN and CH_3OH and be very analyte dependent.

PHASE COMPARISON FOR CARBOXYLIC ACID SELECTIVITY



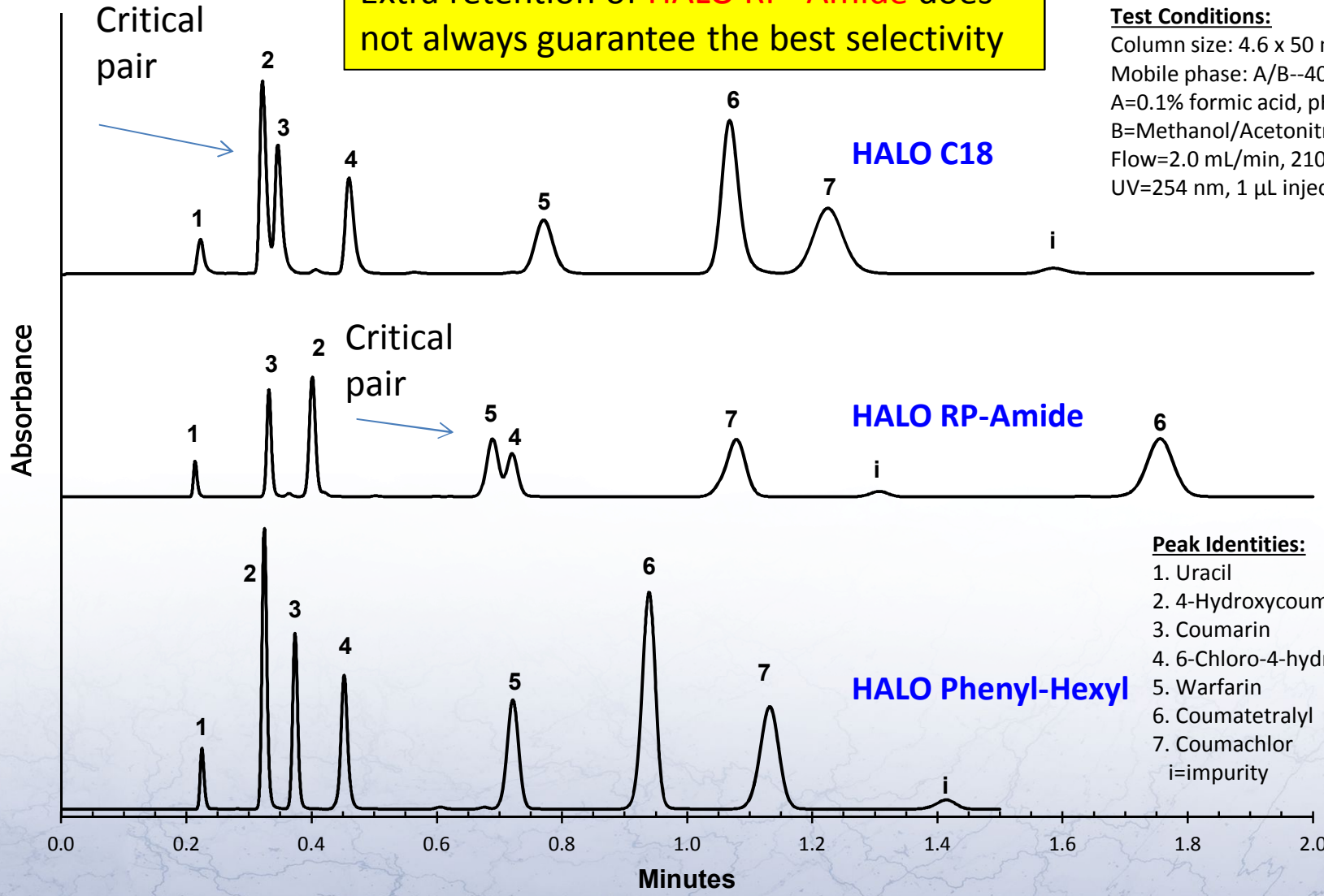
1. uracil
2. p-aminobenzoic acid
3. acetylsalicylic acid
4. 2-fluorobenzoic acid
5. benzoic acid
6. phenoxyacetic acid
7. 3-cyanobenzoic acid
8. 3-fluorobenzoic acid
9. m-toluic acid

H-bond acceptor (carbonyl) of HALO RP-Amide group attracts H-bond donor (acidic OH) and imparts more retention and better selectivity than either C18 or phenyl.

HALO columns, 4.6 x 50 mm, 2.7 µm
25:75 ACN/water with 0.1% formic acid, 25 °C, 1.0 mL/min

FAST SEPARATION OF ANTICOAGULANTS ON HALO FUSED-CORE PACKINGS

Extra retention of HALO RP- Amide does not always guarantee the best selectivity



Test Conditions:
Column size: 4.6 x 50 mm
Mobile phase: A/B--40/60
A=0.1% formic acid, pH=2.6
B=Methanol/Acetonitrile-50/50
Flow=2.0 mL/min, 210 Bar, 45 °C
UV=254 nm, 1 µL injection

Peak Identities:
1. Uracil
2. 4-Hydroxycoumarin
3. Coumarin
4. 6-Chloro-4-hydroxycoumarin
5. Warfarin
6. Coumatetralyl
7. Coumachlor

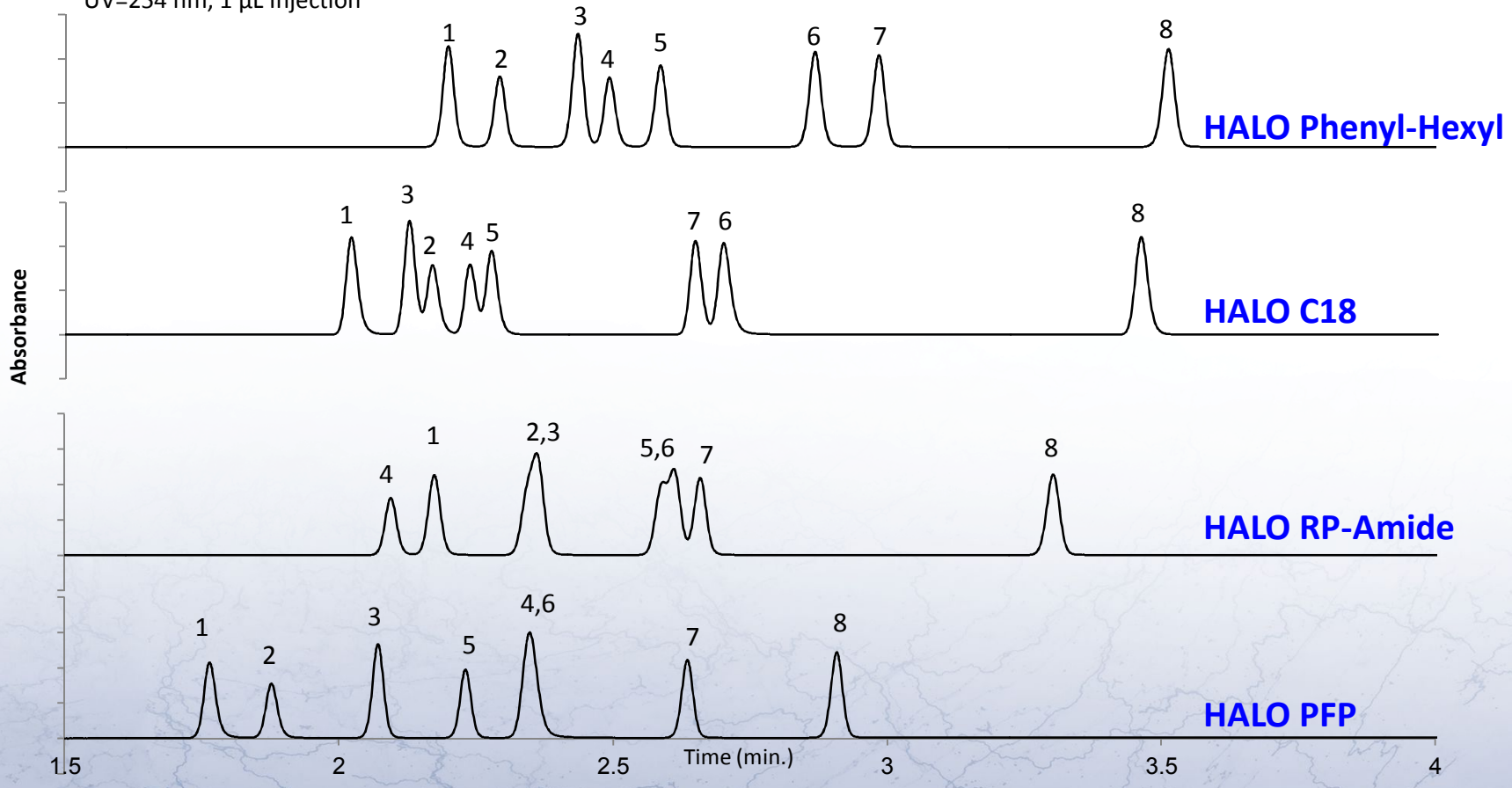
i=impurity

PHASE COMPARISON FOR BENZODIAZEPINES

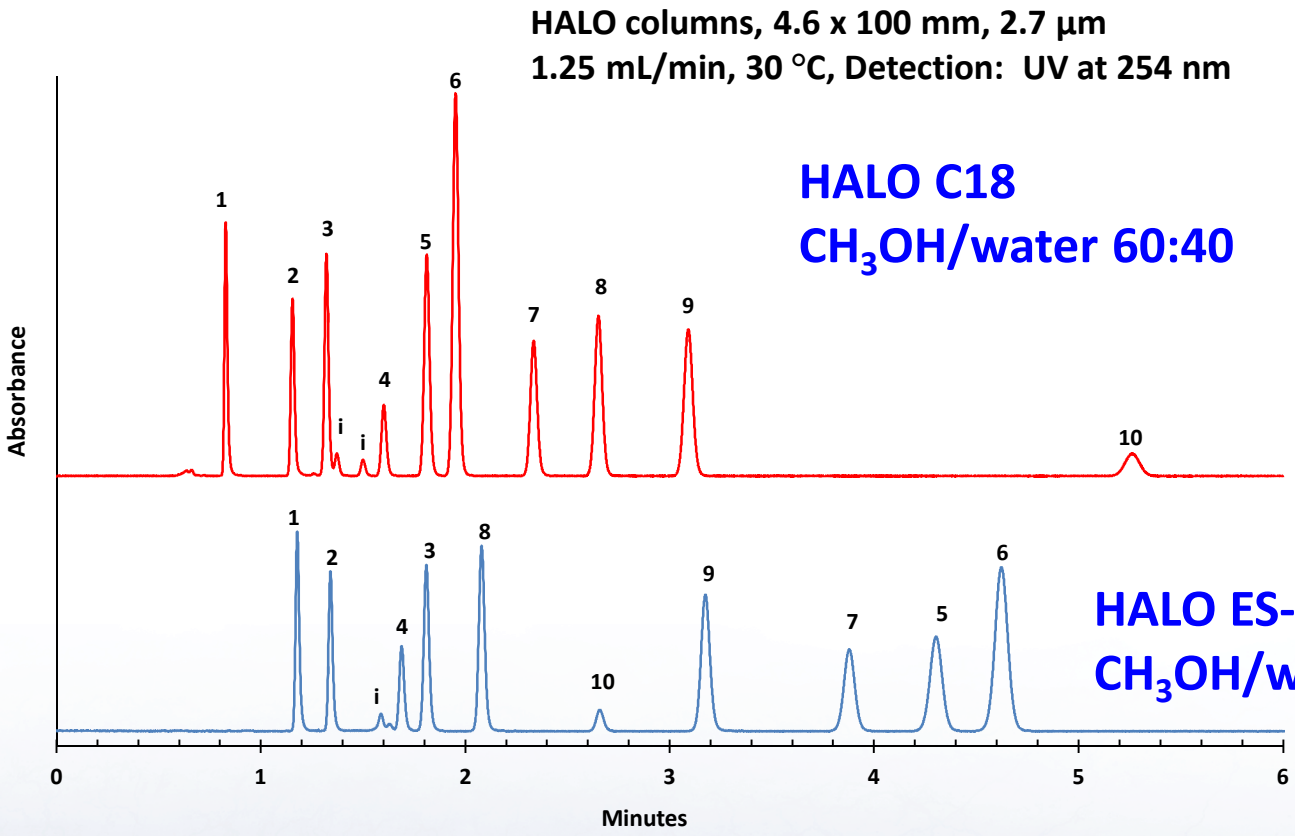
Test Conditions:
Column size: 4.6 x 50 mm
A = 25 mM Ammonium Acetate
B = Acetonitrile
Flow rate = 1.5 mL/min.
Gradient = 34–63 %B in 3.5 min.
Pressure = 200 bar
Temperature = 35 °C
UV=254 nm, 1 µL Injection

Peak Identities:
1. Oxazepam
2. Lorazepam
3. Nitrazepam
4. Alprazolam
5. Clonazepam
6. Temazepam
7. Flunitrazepam
8. Diazepam

HALO Phenyl phases are π -bases (electron-rich) and attract heterocyclic rings and other rings that are π acids (electron-poor) by shape-selective π-π interaction.



CYANO PHASES OFFER ALTERNATIVE SELECTIVITY AND INCREASED RETENTION FOR POLAR ANALYTES



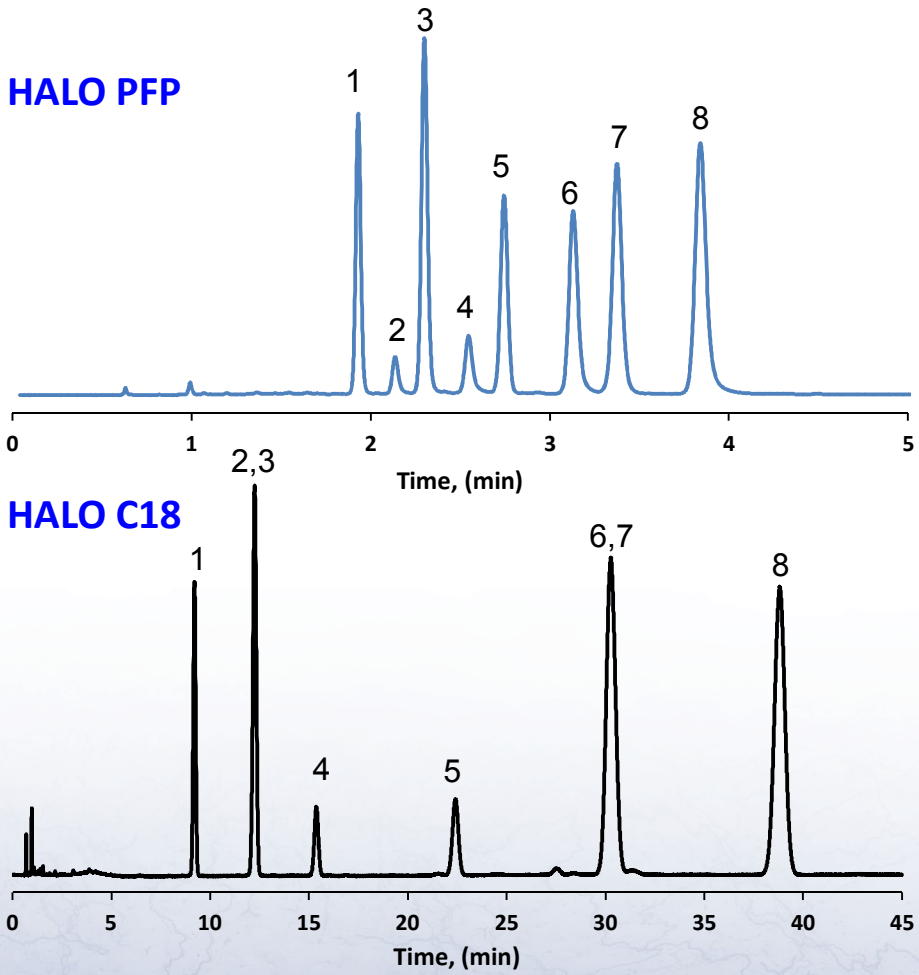
PEAK IDENTITIES:

1. Resorcinol
2. Benzyl alcohol
3. Phenylacetonitrile
4. 1-Indanol
5. 3,4-Dinitrotoluene
6. 2,3-Dinitrotoluene
7. 2,4-Dinitrotoluene
8. Anisole
9. 1-Chloro-4-nitrobenzene
10. Toluene

i = impurity

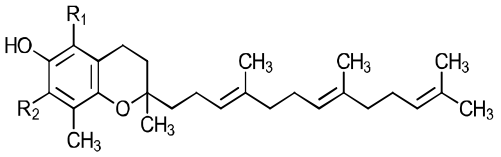
HALO Cyano phases interact by mild hydrophobic attraction plus dipole-dipole and dipole-aromatic forces and should be more popular in methods. Cyano requires only 10% less organic for equivalent retention, is very orthogonal to C18 for polar compounds, and is quite stable.

FASTER SEPARATION AND BETTER RESOLUTION USING HALO PFP COLUMN VS. HALO C18

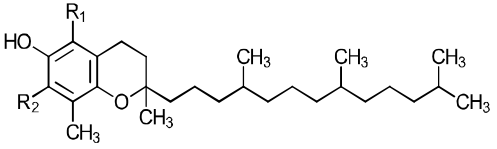


PEAK IDENTITIES:

1. δ-tocotrienol
2. β-tocotrienol
3. γ-tocotrienol
4. α-tocotrienol
5. δ-tocopherol
6. β-tocopherol
7. γ-tocopherol
8. α-tocopherol



Tocotrienol



Tocopherol

HALO PFP phases are π-acids and can attract solutes by π- π and several other mechanisms. HALO C18 has excess retention and selectivity overall, but cannot distinguish between isomer peaks 2,3 and 6,7.

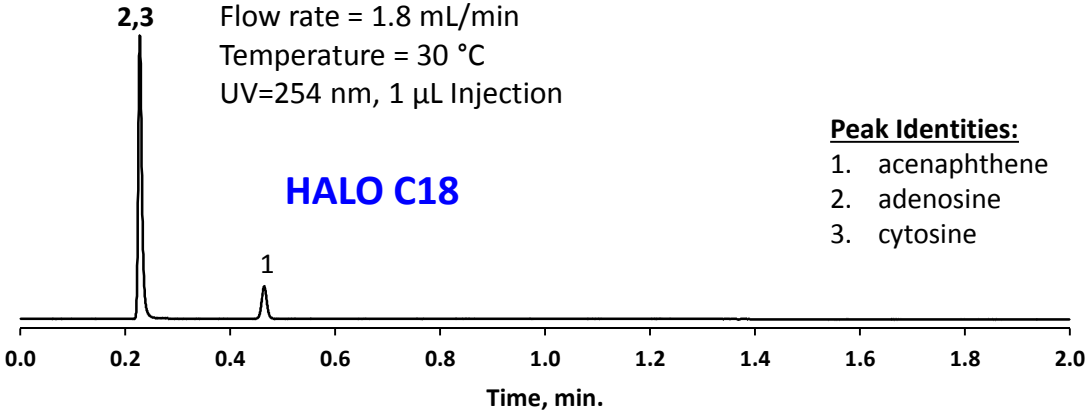
HALO columns, 4.6 x 150 mm, 2.7 µm
90:10 CH_3OH/water, 25°C, 1.5 mL/min, Detection: Fluorescence: Excitation, 296 nm; Emission, 325 nm

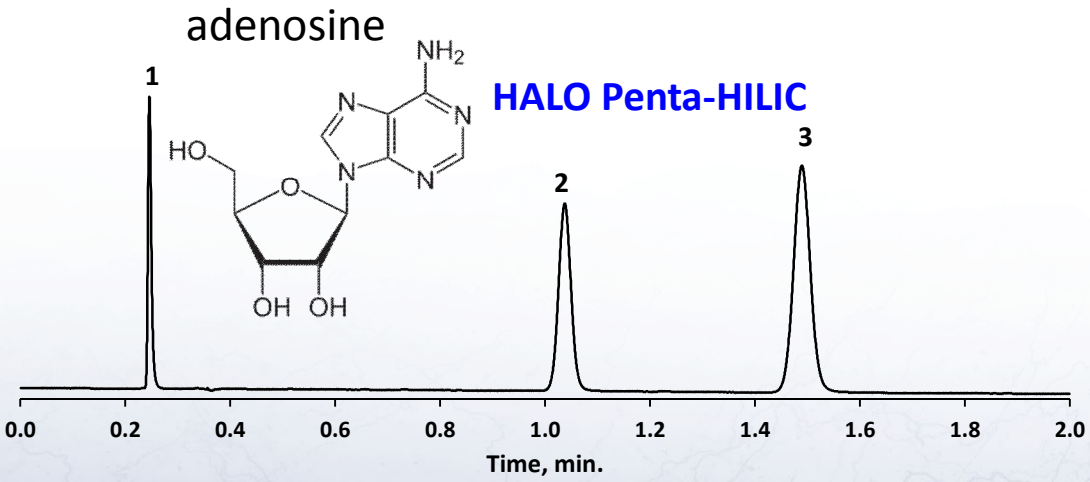
PHASE COMPARISON USING HILIC MODE

Nucleosides and nucleobases are often used as void markers for C18 columns, but they can be easily retained by Halo Penta-HILIC. Nucleosides, glycosides and other sugar-related compounds are ideal for Halo HILIC and Penta-HILIC. If RP and HILIC both show resolution, compare peak shape, equilibration, loading, and other factors.

Test Conditions:
Column = 4.6 x 50 mm
Mobile Phase = 90/10 Acetonitrile/ 0.1 M ammonium formate buffer, pH = 3.0
Flow rate = 1.8 mL/min
Temperature = 30 °C
UV=254 nm, 1 µL Injection

Peak Identities:
1. acenaphthene
2. adenosine
3. cytosine





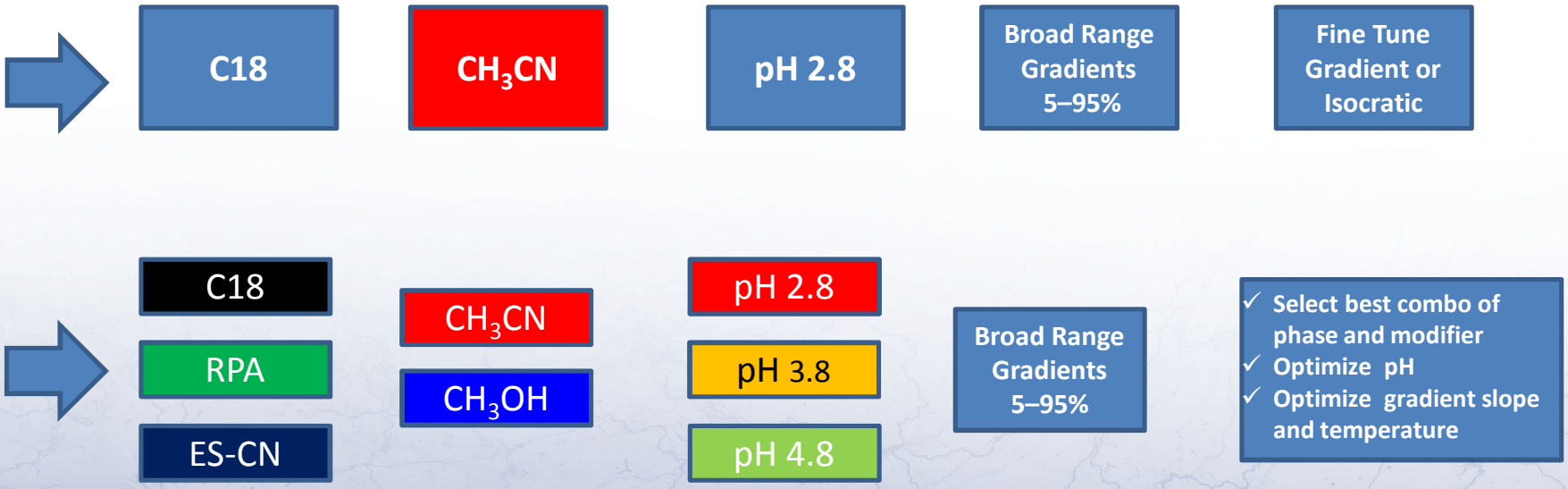
DEVELOPING AN RPLC SEPARATION METHOD

Objective: to develop a good separation with the desired R_s and the necessary robustness for the desired purpose

- Method needs vary from relatively simple to very demanding
  - Simple: one lab for only several days or weeks
  - Moderate: one lab for several months
  - Demanding: method applied in multiple labs country-wide or world-wide and used for 5-10 years or more
- Sample complexity (known analytes) impacts method development time
  - < 5-6
  - < 10
  - > 10
- Robustness and ruggedness required for demanding methods
  - Robust- insensitive to small changes in variables
  - Rugged- reproducible during use by many labs, instruments, analysts, etc.

METHOD DEVELOPMENT CAN VARY FROM RELATIVELY SIMPLE TO QUITE COMPLEX

- Separations that are simple often need little or no column screening so you can develop a separation quickly by varying solvent strength at low pH. Separations that are more difficult may not require a different phase, but you should compare different organic modifiers at multiple pH values.
- Demanding methods and more complex samples (e.g., stability-indicating method), usually require screening multiple phases, multiple organic modifiers, and several pH values to generate the most rugged separation.



APPLICATION OF MULTIPLE PHASES FOR STABILITY INDICATING METHOD DEVELOPMENT

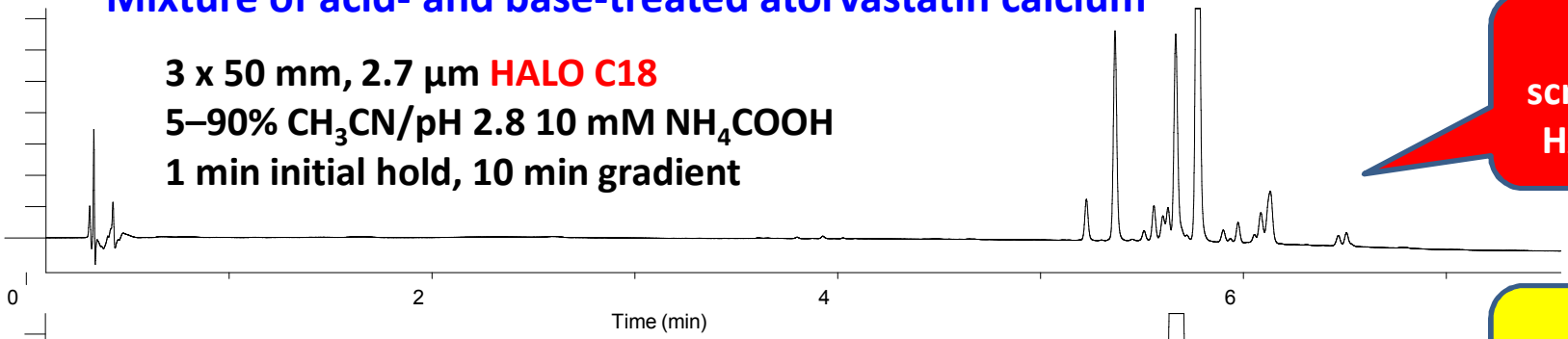
- Atorvastatin Calcium
  10 mg active/310 mg tablet
- Generate HCl-degraded and NaOH-degraded samples
- Pool acid- and base-treated samples together
- Compare five different HALO phases using both CH_3CN and CH_3OH at one pH (2.8, ammonium formate)
- Compare results and identify best option(s) for further development and optimization
- Use 3 x 50 mm, 2.7 µm HALO geometry

- Initially screened HALO C18 column using broad gradient with CH_3CN (locate primary drug)
- Fine tuned to narrower ranges
- Compared all phases using narrower range using both CH_3CN and CH_3OH

BROAD RANGE GRADIENT WITH C18 IS USEFUL TO ESTABLISH HYDROPHOBICITY OF COMPLEX SAMPLES

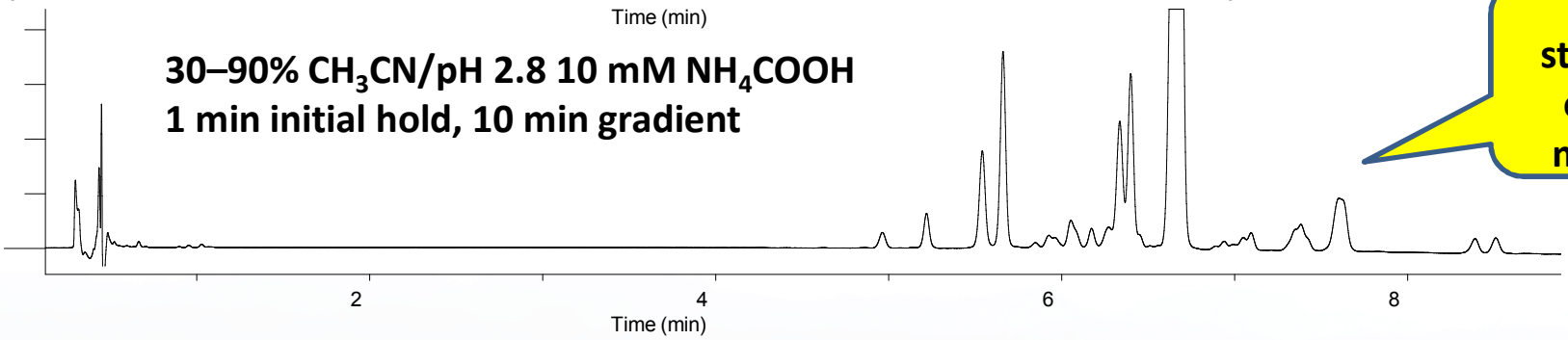
Mixture of acid- and base-treated atorvastatin calcium

3 x 50 mm, 2.7 µm HALO C18
5–90% CH_3CN/pH 2.8 10 mM NH_4COOH
1 min initial hold, 10 min gradient



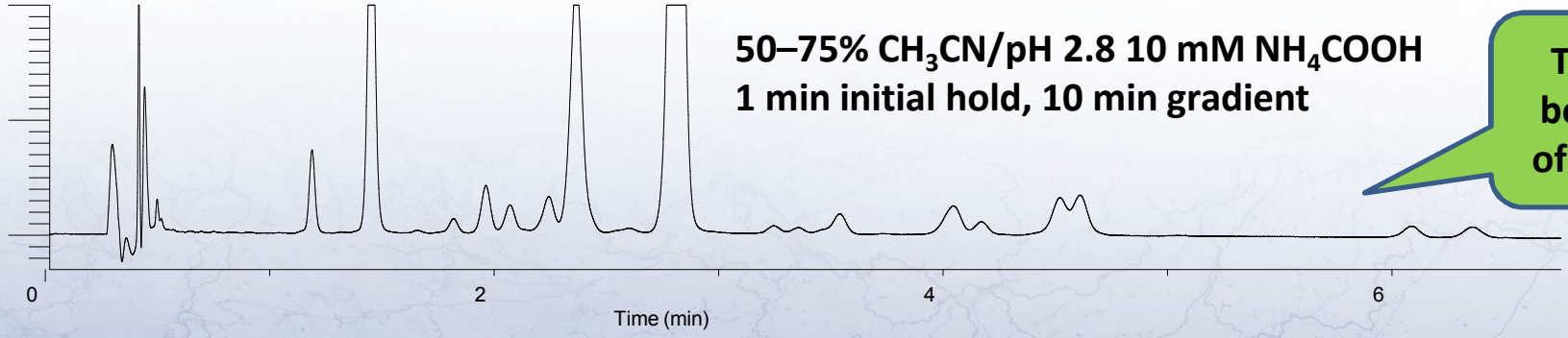
Initial screen with HALO C18

30–90% CH_3CN/pH 2.8 10 mM NH_4COOH
1 min initial hold, 10 min gradient

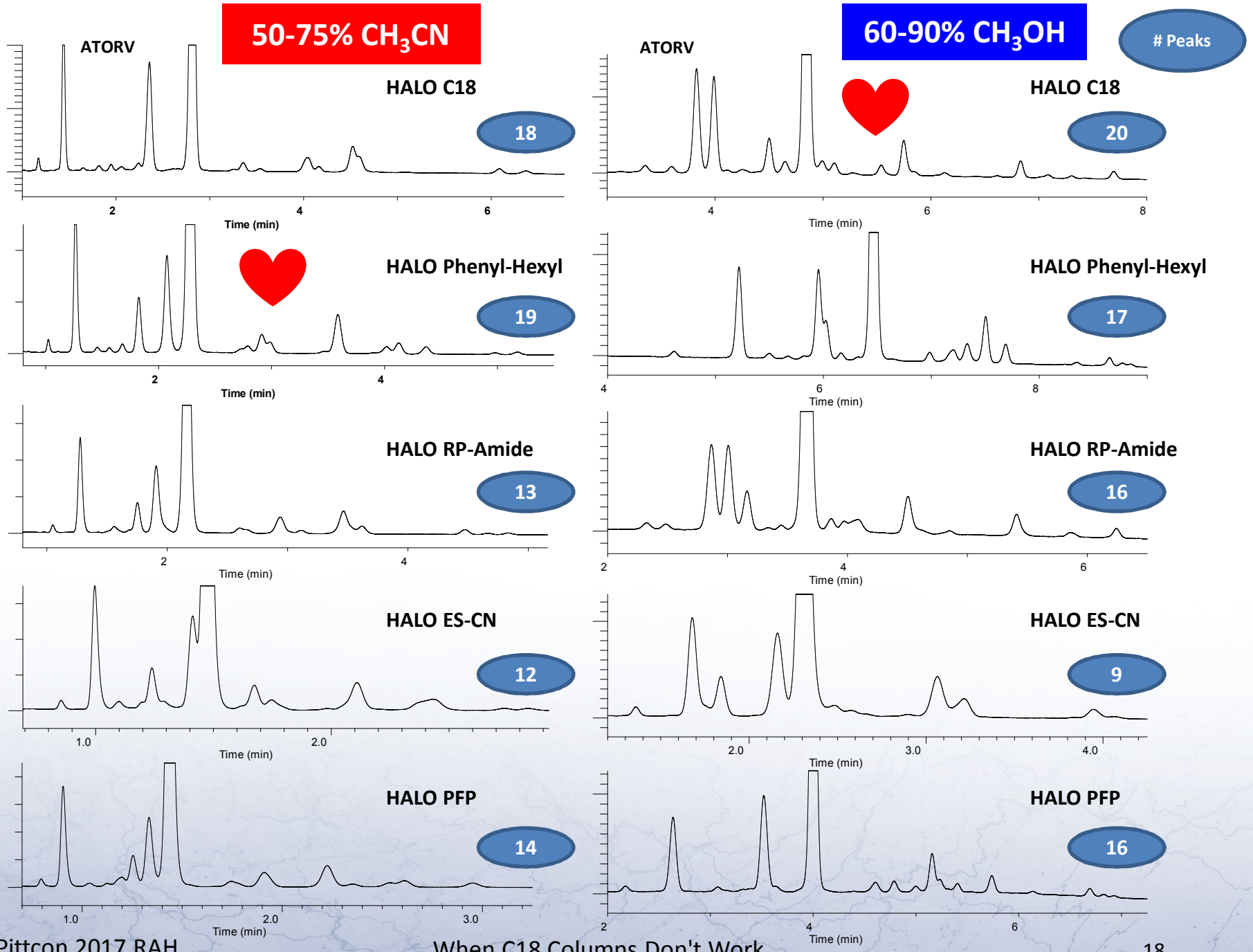


Raise starting % organic modifier

50–75% CH_3CN/pH 2.8 10 mM NH_4COOH
1 min initial hold, 10 min gradient

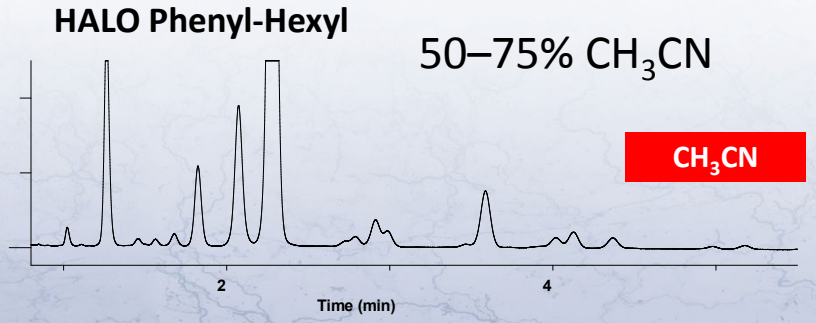


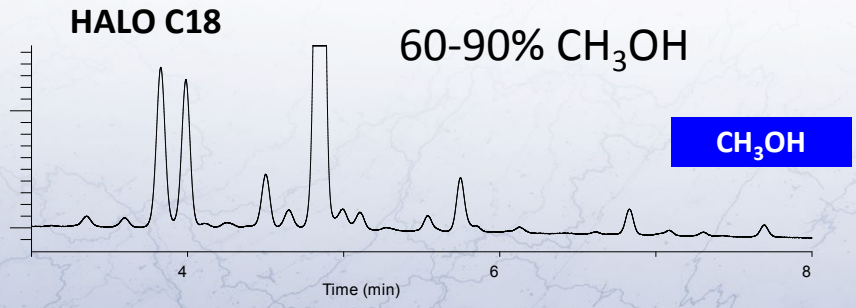
Truncate both ends of gradient


50-75% CH_3CN
60-90% CH_3OH
Peaks
ATORV
HALO C18
18
ATORV
HALO C18
20
Time (min)
HALO Phenyl-Hexyl
19
HALO Phenyl-Hexyl
17
HALO RP-Amide
13
HALO RP-Amide
16
HALO ES-CN
12
HALO ES-CN
9
HALO PFP
14
HALO PFP
16

HOW DO YOU CHOOSE WHICH COMBINATION TO DEVELOP AND OPTIMIZE FURTHER?

- Compare chromatograms for number of peaks observed.
- Compare shapes for detected peaks.
- Select most attractive phase/modifier combination(s) on points of merit:
  - # peaks separated
  - minimum R_s for critical peak pair
  - shortest analysis time
  - most peaks with acceptable USP T_f
- If no clear winner emerges, run several gradients having differing slopes.
  - For example, 10 minutes and 25 minutes for Phenyl-Hexyl (50–75% ACN) and C18 (60-90% CH_3OH).
  - Assess whether either combination stands out vs. criteria
- Compare separation and pressure on longer columns with higher efficiency.
- Check location of impurities for easier integration.





SUMMARY AND RECOMMENDATIONS

- C18 columns are widely preferred in HPLC methods and may be satisfactory for most simple methods, but a C18 phase may not be the best solution for complex samples in demanding methods.
- Polar analytes can be separated better on phases that have greater polarity than C18, but selection of preferred phases and operating conditions to resolve complex mixtures may require column screening.
- A development strategy for demanding situations should be holistic and document all system parameters that control selectivity. This is the reason behind testing method variables for robust behavior before the method is validated. A robust method is likely to be rugged in use.
- The best approach for HPLC method development is to systematically screen multiple stationary phases and operating parameters. Special software and instrumentation (column, solvent selection) is available to assist with automated screening, selection, and optimization. Shortcuts can lead to fragile methods.

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