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Solutions within.

Troubleshooting Liquid Chromatographic Separations: Avoiding Common Issues through Informed Method Development Practices

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Objective

Most of the difficult problems encountered are a result of poor method development – not column or instrument failure

Lofty Goal: To provide HPLC troubleshooting tips by walking through the *entire* method development process

Sample of questions addressed:

- Why do we choose certain columns?
- Why do we run at a certain wavelength? Use ESI vs. APCI?
- Why 10 mM phosphate buffer? Why acetonitrile? Why pH 3?
- Why certain flow rates and sample volumes?
- What does temperature have to do with it?
- What are the best tools for a given separation?
- What are the limits based on detection?

Avoid living on the edge and KISS it!

Outline

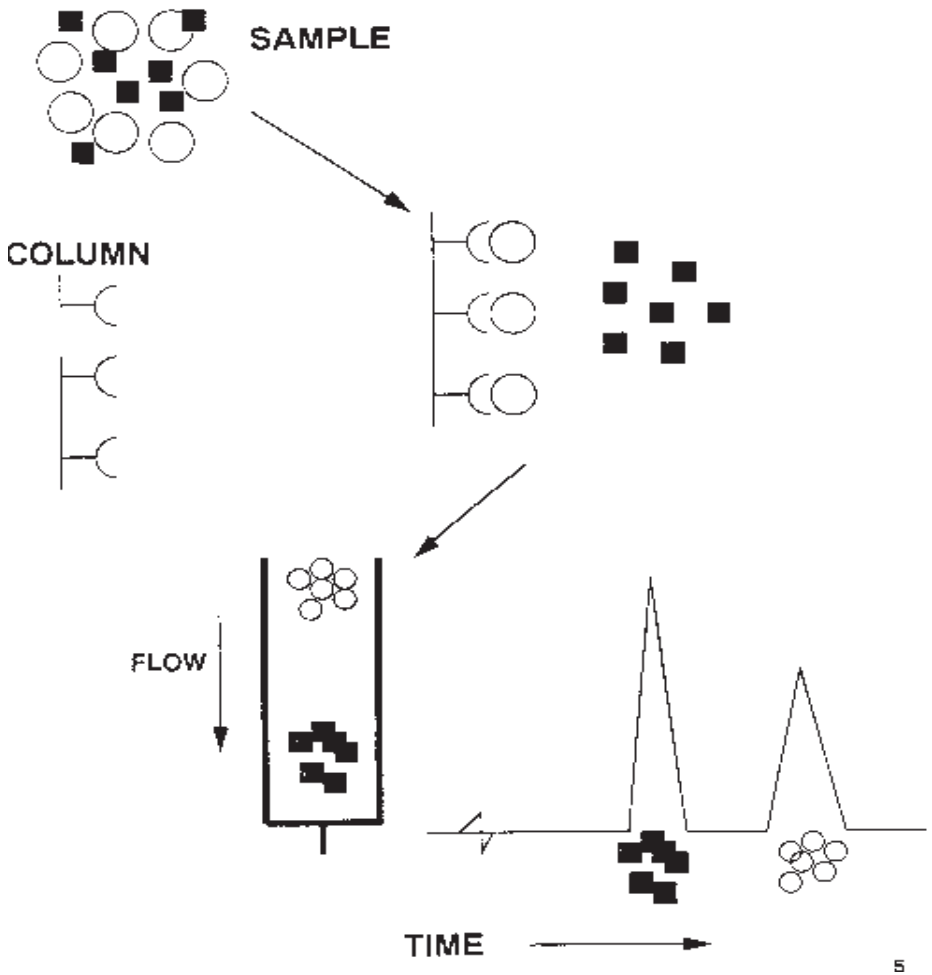
- Twelve most common problems encountered in HPLC
- Review common method development practices, potential pitfalls
- Discuss alternatives to “Force-Fed” C18 methods
  - Embedded polar group phases
  - Fluorinated aromatic phases
  - Relate back to the 12 common problems
- (maybe) some further issues

Introduction: The 12 Problems

- Poor resolution
- Poor peak shape
- Lack of retention of polar compounds
- Time to develop a method
- Separation of critical pairs
- High throughput
- Wide range of sample polarities
- Ruggedness/robustness
- Column stability
- Mass spec compatibility
- Low detection limits
- Scale-up to prep

Each problem can be circumvented by utilizing proper method development strategies and tools

Schematic of a Separation



- Chromatography is all about differential interactions of the analytes with the solvent and the stationary phase
- Our methods must be developed with an understanding of how these interactions work and how they are controlled
- Also about staying within the boundaries of physical parameters
  - solubility
  - chemical stability
  - detection compatibility

Method Development Scenario

- General Steps:
  - Understand the goals of the method: “Fit for Purpose”
    - Assay – need only retain and resolve a target analyte
    - Purity – need to retain and resolve all potential impurities
    - Quantification from dirty matrix – sample prep
  - Understand analyte properties
    - Ionization – $pK_{a,}$ acidic, basic
    - Solubility – logP/logD,
    - UV absorption/MS ionization – detection
  - The iterative process:
    - Choose a column
    - Choose a scouting mobile phase
    - Test results
    - Adapt conditions
    - Test results…..

Choice of Column and the 12 Problems

Resolution
- Retention, efficiency and selectivity

Peak shape
- Silanol activity

Retention of polar compounds
- Availability of polar and ionic interactions

Time to develop a method
- The right tool speeds the job

Separation of critical pairs
- Availability of needed interactions

High throughput
- Smaller dimension = faster analysis
- Superior selectivity = faster analysis
- UPLC/SP particles = flatter van D

Wide range of sample polarities
- Correct blend of interactions

Ruggedness/robustness
- Column stability

Mass spec compatibility
- Low column bleed

Low detection limits
- Efficiency

Scale-up to prep
- Availability of preparative dimensions and particle sizes

Resolution

Efficiency Retention Selectivity

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

N

- Increase column length
- Decrease particle size (N ~ $1/d_p$)

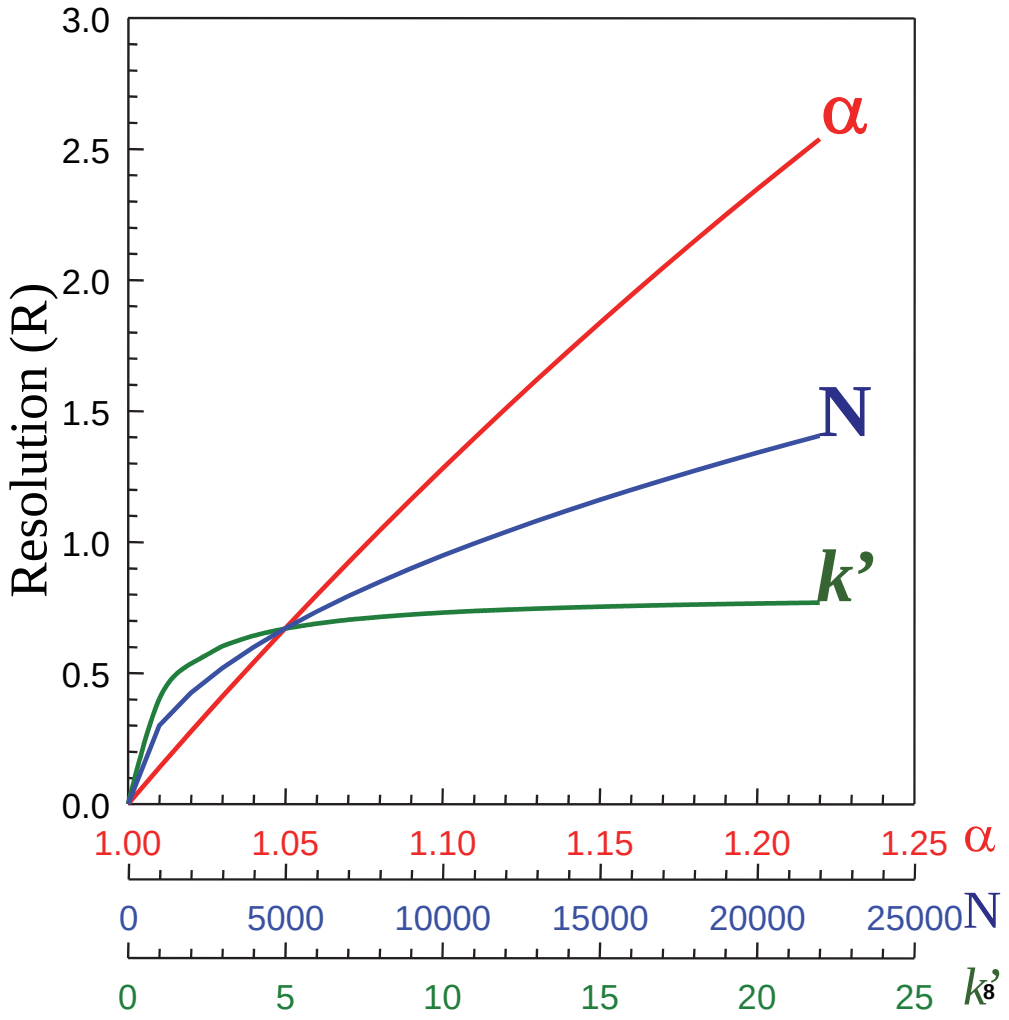
α

- Change stationary phase
- Change mobile phase solvent
- Change buffer pH

k

- Increase (weaker solvent)
- Decrease (stronger solvent)
- Invoke different interactions

Zhao, J.H. and P.W. Carr. Analytical Chemistry, 1999. 71(14): p. 2623-2632



Selectivity

- Interactions that contribute to retention and selectivity
- Stationary phase selectivity and method development practices
- What do we know about the different chemistries/how can we apply them intelligently to solve problems?
  - Embedded polar group (EPG) stationary phases
  - Aromatic Fluorinated Stationary Phases

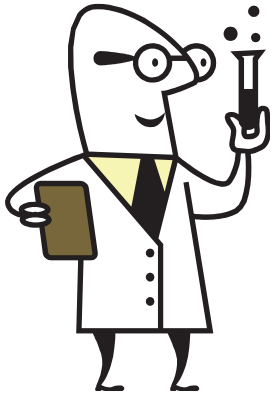
Selectivity (contd.)

Interactions that contribute to retention and selectivity

- Dispersive or hydrophobic interactions
- Polar interactions (hydrogen bonding and other dipole interactions)
- Ionic interactions

Dispersive interactions are commonly considered most important in reversed-phase chromatography

Polar and ionic interactions, however, are often responsible for a significant amount of retention and selectivity



Stationary Phase Selectivity and Method Development Practices

Common practice is to commence method development with a C18

Often a second C18 will be utilized if separation/retention not achieved

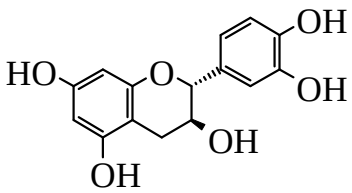
Two alternatives;

- Force-feed the C18 (ion-pair, high pH, exotic mobile phases)
- Use alternative stationary phase chemistries

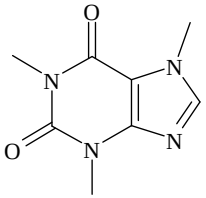
Forced C18 methods generally suffer robustness, reproducibility problems – very complicated, dynamic systems

Alternative stationary phases are generally not well understood, resulting in method development difficulties

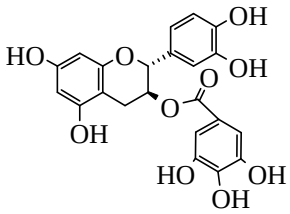
Caffeine and Green Tea Catechins



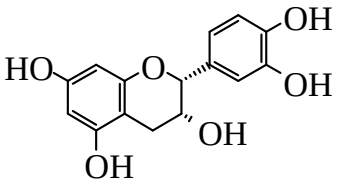
Catechin



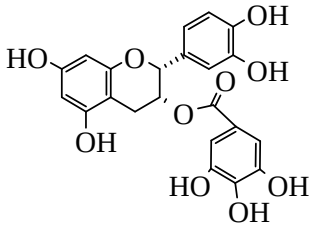
Caffeine



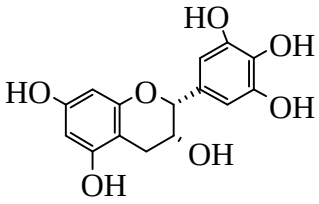
Catechin Gallate



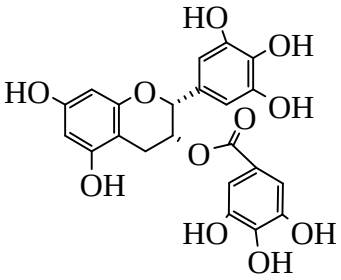
Epicatechin



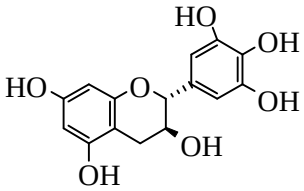
Epicatechin Gallate



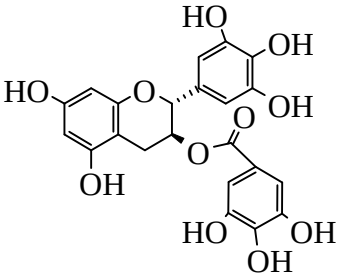
Epigallocatechin



Epigallocatechin Gallate



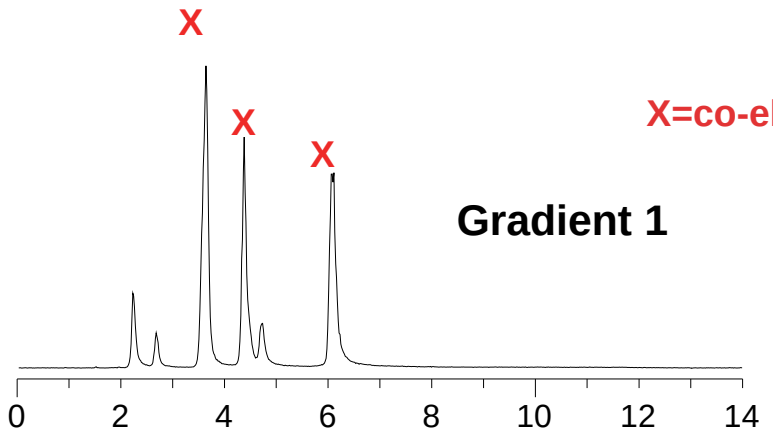
Gallocatechin

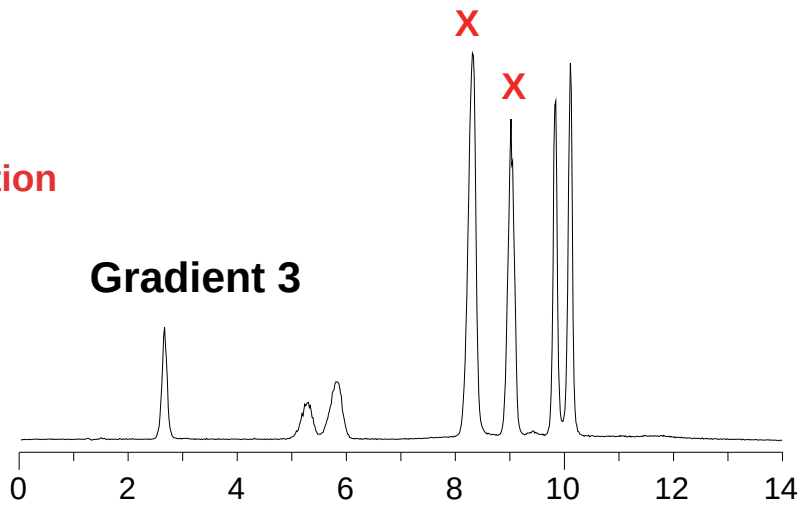


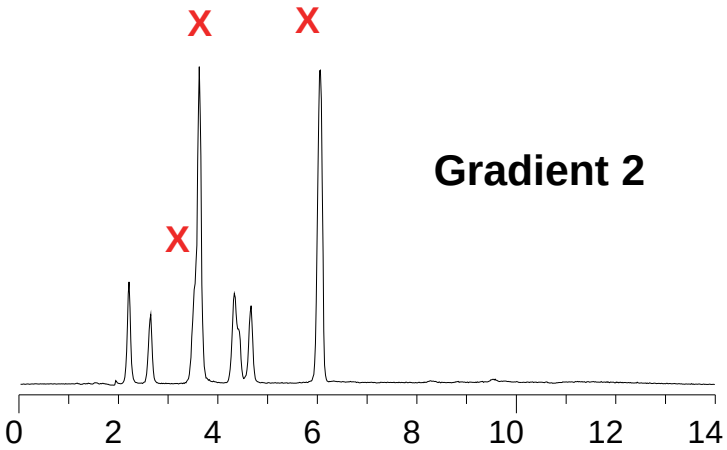
Gallocatechin Gallate

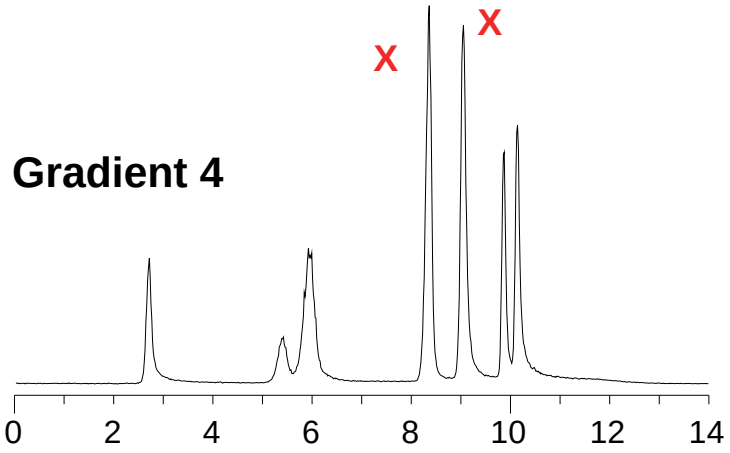
Lack of Selectivity for Catechins using C18 - Resolution

X=co-elution

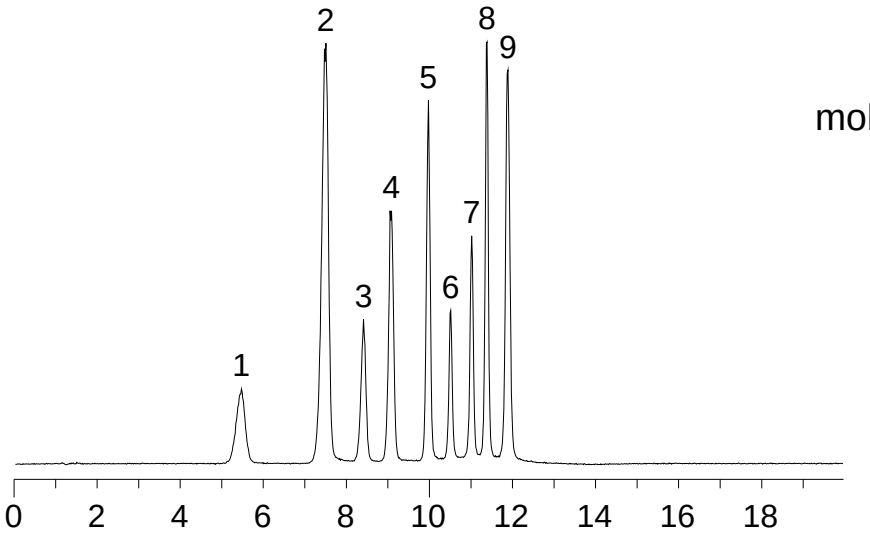








Successful Separation of Catechins and Caffeine using a Polar Embedded Phase



Peak ID and m/z ratio

1. Gallocatechin, m/z 305
2. Caffeine, m/z 195
3. Epigallocatechin, m/z 305
4. Catechin, m/z 289
5. Epicatechin, m/z 289
6. Epigallocatechin gallate, m/z 457
7. Gallocatechin gallate, m/z 457
8. Epicatechin gallate, m/z 441
9. Catechin gallate, m/z 441

column: Ascentis RP-Amide, 15 cm x 4.6 mm, 5 µm particles

mobile phase: (A) 10 mM ammonium formate, pH 3.0 with concentrated formic acid
(B) methanol

flow rate: 1 mL/min., split to the mass spectrometer

temp.: 35 °C

det.: MS, ESI (-) and (+) in selected ion recording (SIR) mode (combined)

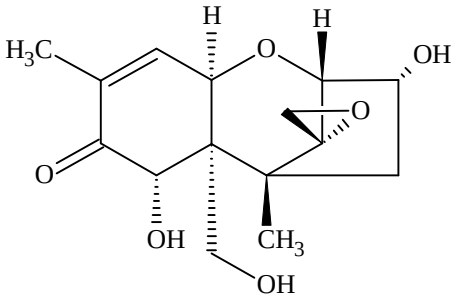
injection: 5 µL

sample: 10 µg/mL each in 80:20, 10 mM ammonium formate, pH 3.0 with concentrated formic acid:methanol

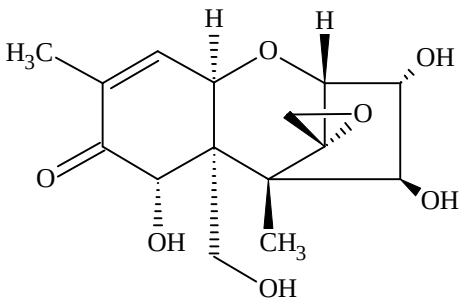
gradient:

Min	%A	%B
0	80	20
5	80	20
10	45	55
12	80	20

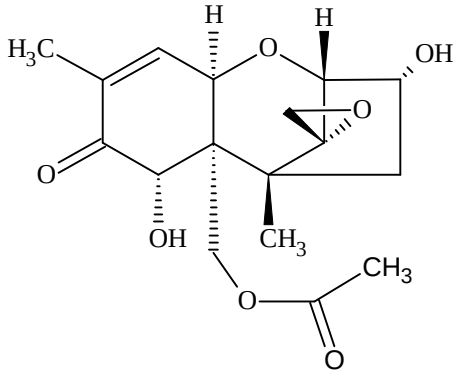
DON and Related Compounds



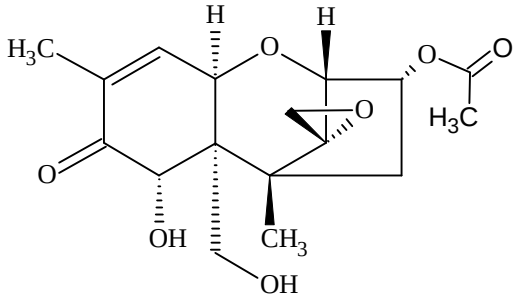
Deoxynivalenol



Nivalenol

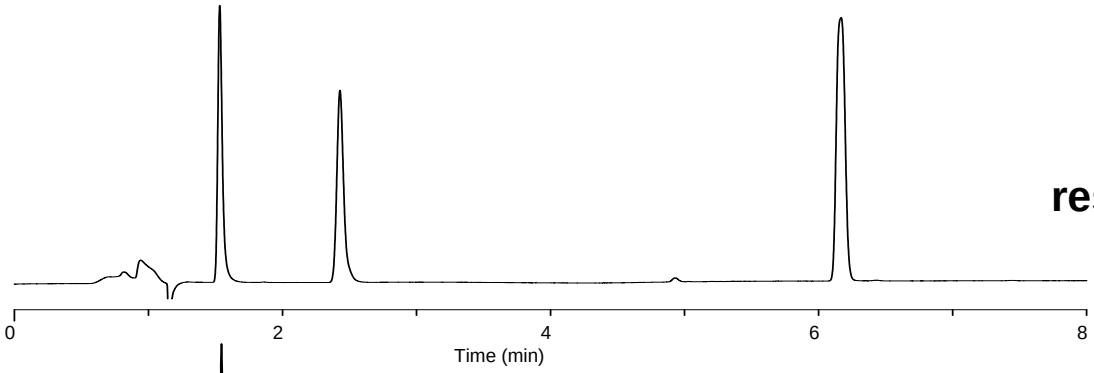


15-Acetyldeoxynivalenol

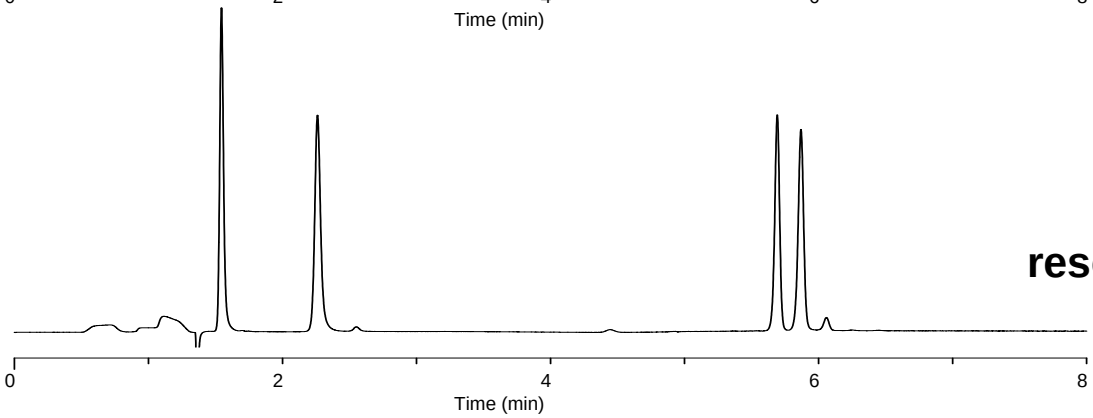


3-Acetyldeoxynivalenol

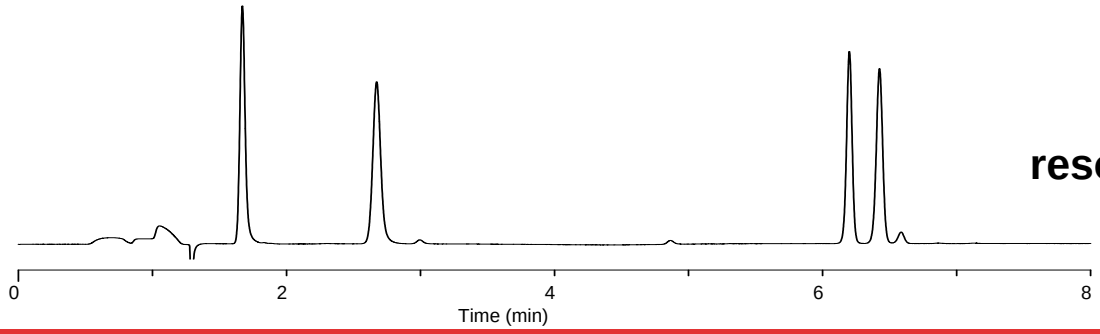
Resolution Comparison between C18 and Aromatic Stationary Phases



C18
resolution peaks 3 & 4 = 0



F5
resolution peaks 3 & 4 = 2.53



Phenyl-Hexyl
resolution peaks 3 & 4 = 2.77

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Hydrophobic Selectivity

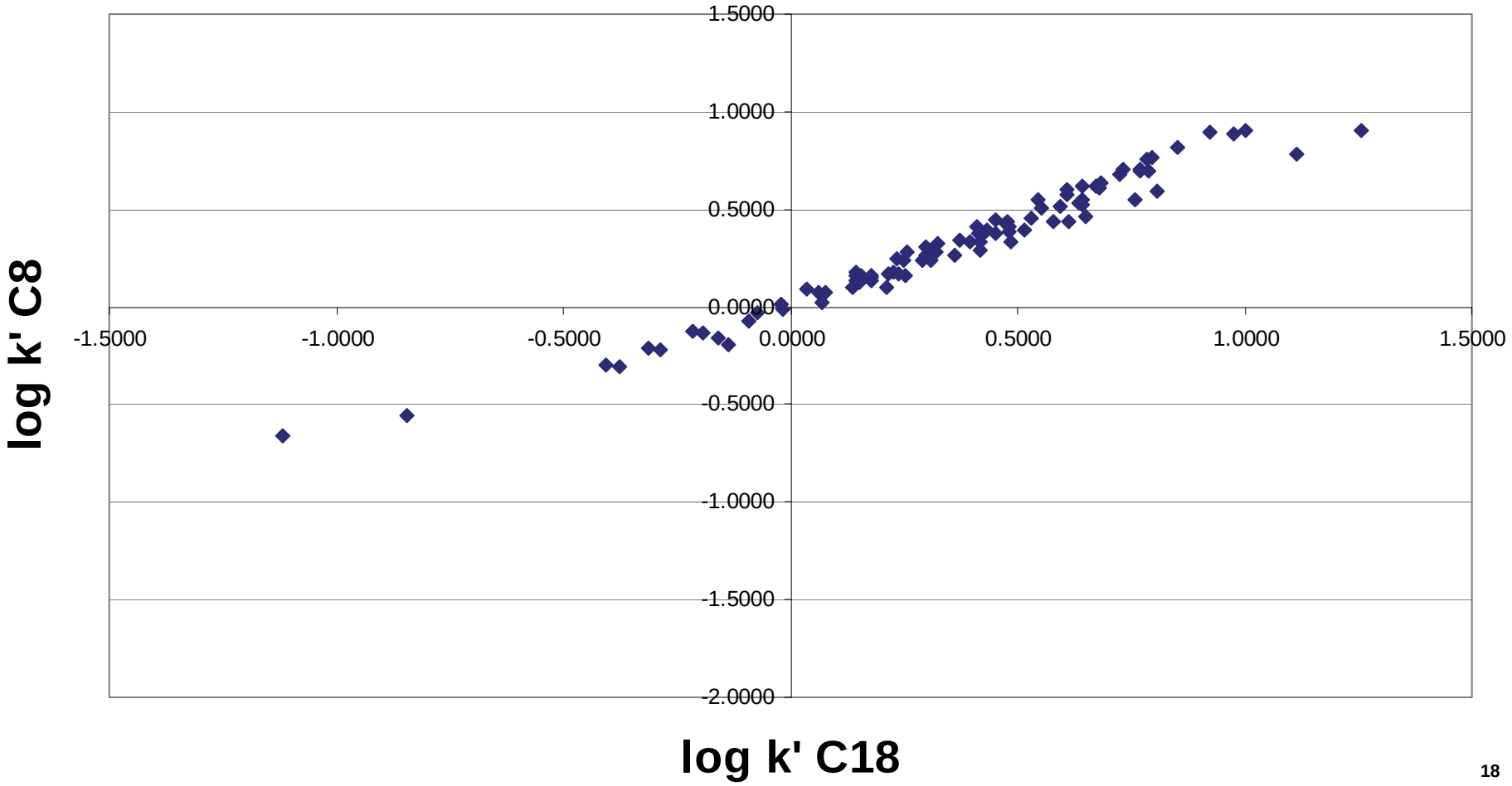
M.R. Euerby, P. Petersson, J. Chromatogr. A 994 (2003) 13.

Large difference in hydrophobicity

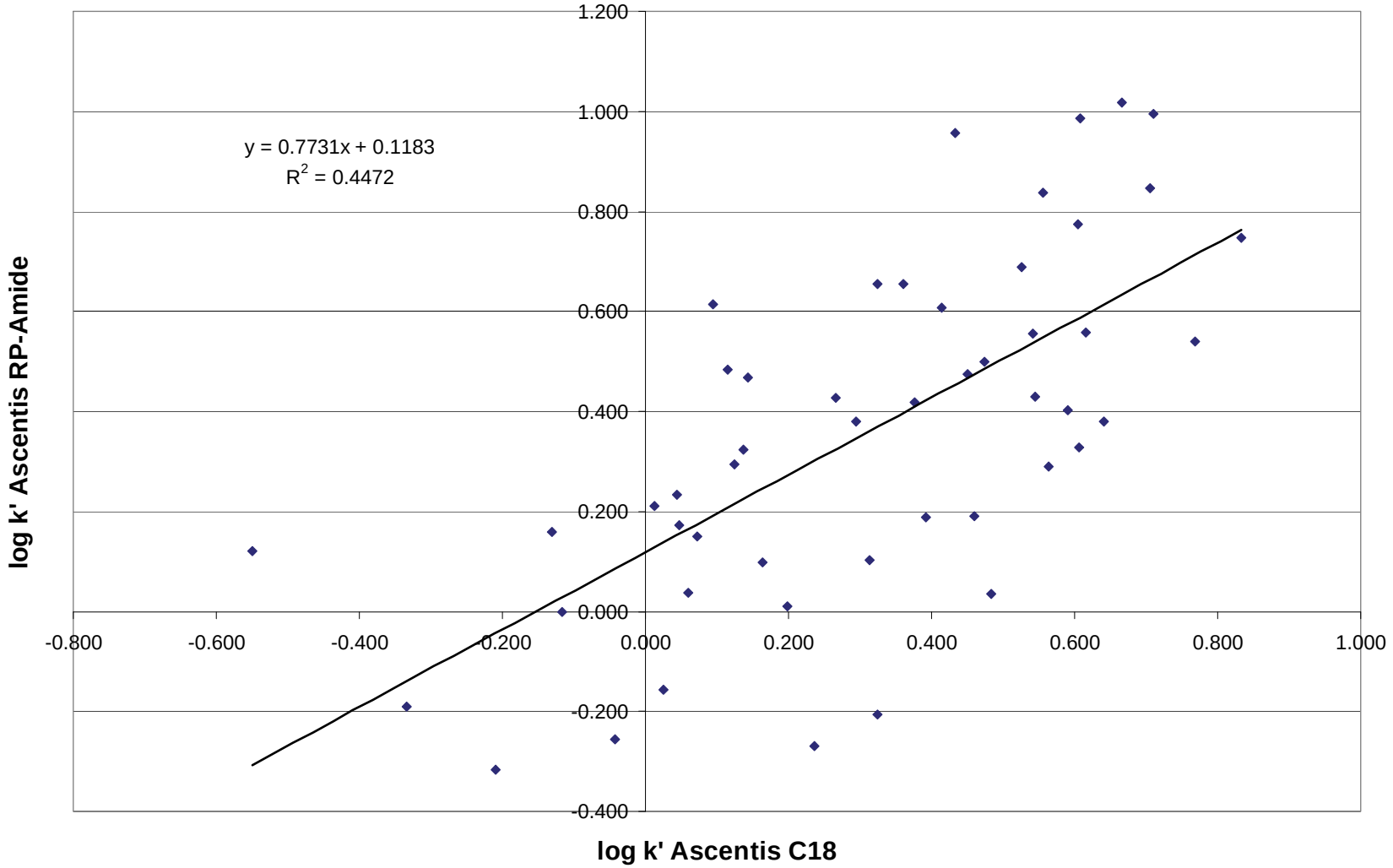
Little difference in selectivity

Column	k_{PB}	a_{CH2}	$a_{T/O}$	$a_{C/P}$	$a_{B/P\_7.6}$	$a_{B/P\_2.7}$
Ascentis C18	7.35	1.50	1.59	0.37	0.31	0.08
Discovery C18	3.32	1.48	1.51	0.39	0.28	0.10
Discovery HS C18	6.68	1.40	1.55	0.40	0.38	0.10
ACE	4.58	1.46	1.52	0.40	0.47	0.13
Luna (2)	6.34	1.47	1.23	0.41	0.26	0.06
Symmetry	6.51	1.46	1.49	0.41	0.68	0.01
Inertsil (3)	7.74	1.45	0.29	0.48	0.29	0.01

Selectivity Comparison Between Alkyl Stationary Phases



Selectivity Comparison Between EPG and C18 Stationary Phases



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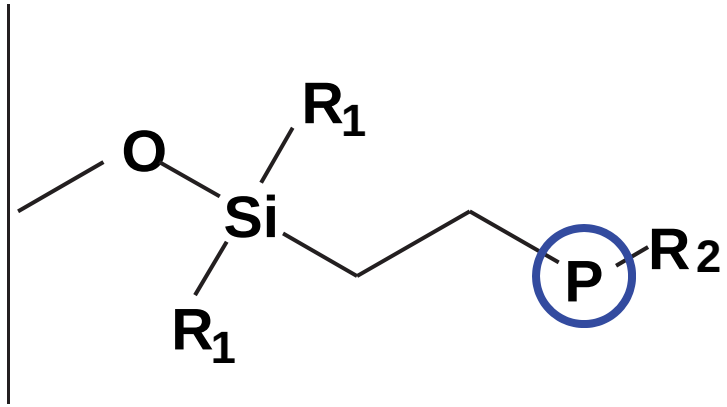
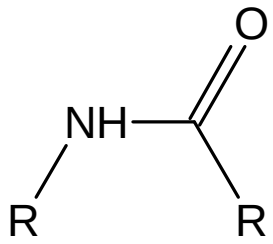
EPG Phases

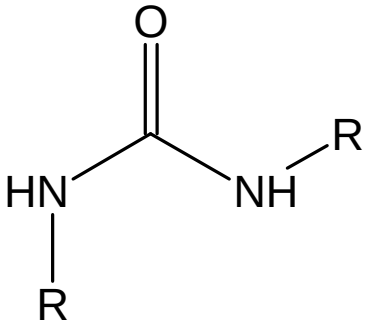


- Nitrogen-containing EPG phases
  - Also ether type phases

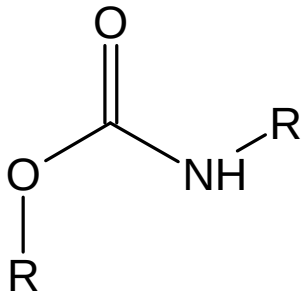
Where P can be:



Amide



Urea



Carbamate

Linear Solvation Energy Relationships (LSER)

The LSER model relates fundamental molecular solute descriptors to the free energy related to a phase transfer process.

Properties include dispersion and molecular volume (V), polarizability (S), electron lone pair interactions (E) and hydrogen bonding contributions to free energy (A and B).

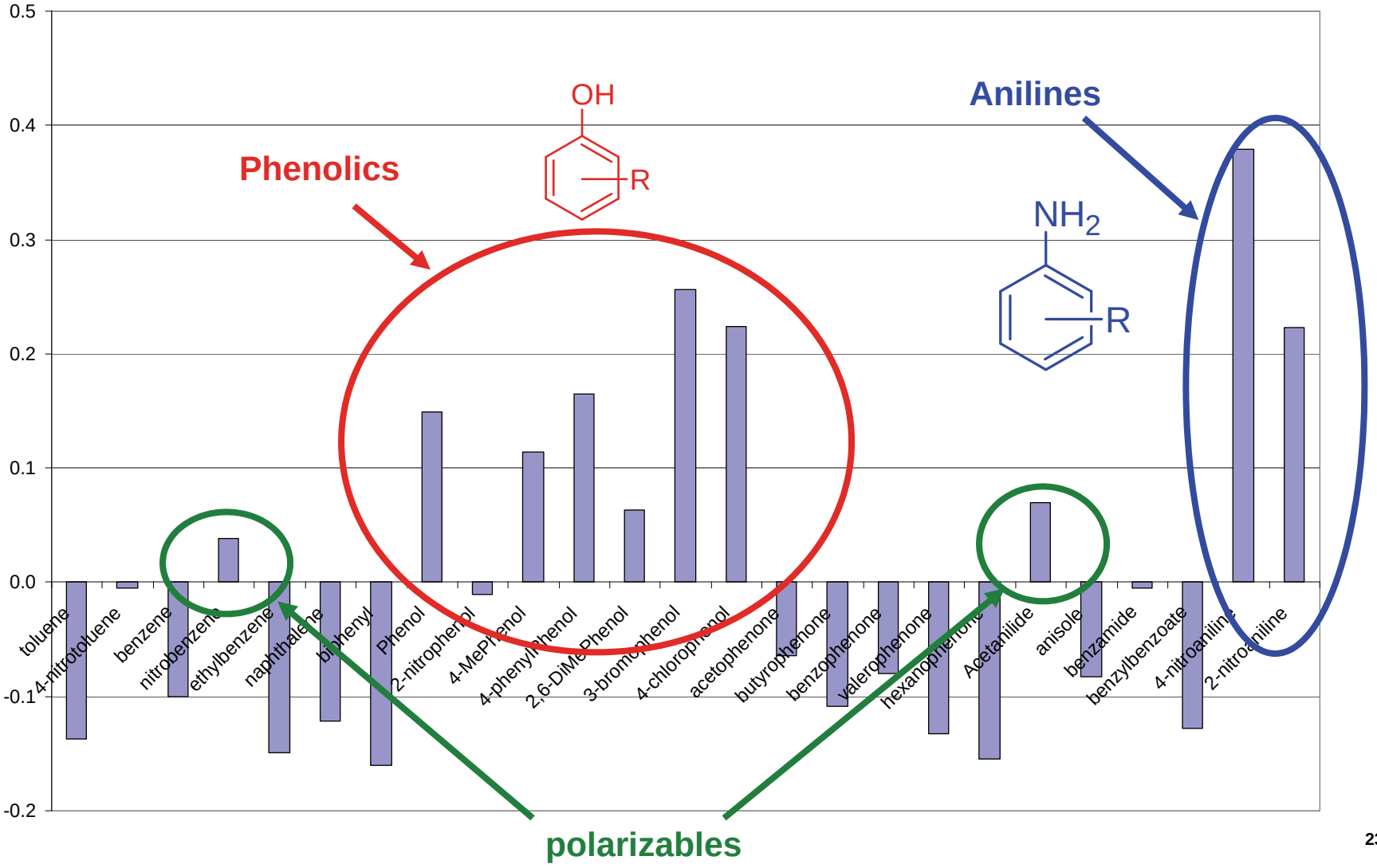
$$\log k' = c + eE + sS + aA + bB + vV$$

Multiple Linear Regression Analysis

Stationary Phase	Constant (c)	v	e	s	a	b
Ascentis RP-Amide	**-0.496±0.06**	**2.23±0.07**	**0.145±0.08**	**-0.385±0.07**	**0.068±0.05**	**-2.51±0.13**
Ascentis C18	**-0.421±0.09**	**2.30±0.11**	**0.267±0.11**	**-0.731±0.10**	**-0.256±0.08**	**-2.10±0.18**

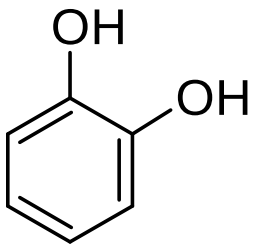
- The molar volume (*v*) and electron lone pair (*e*) descriptors are shown to be statistically the same
- The polarization (*s*), hydrogen bonding acceptor (*a*) and hydrogen bond donator (*b*) terms are statistically different

Difference in Retention
[log k´ (RP-Amide) – log k´ (C18)]

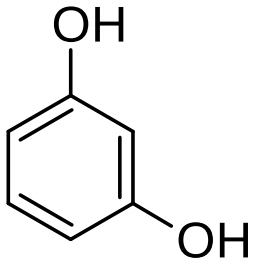


Example Application: Separation of Catechols and Resorcinols

Based on the LSER and classification studies, analytes that can *donate toward hydrogen bonding should provide differential retention on EPG phases compared to C18 phases.*



Catechol

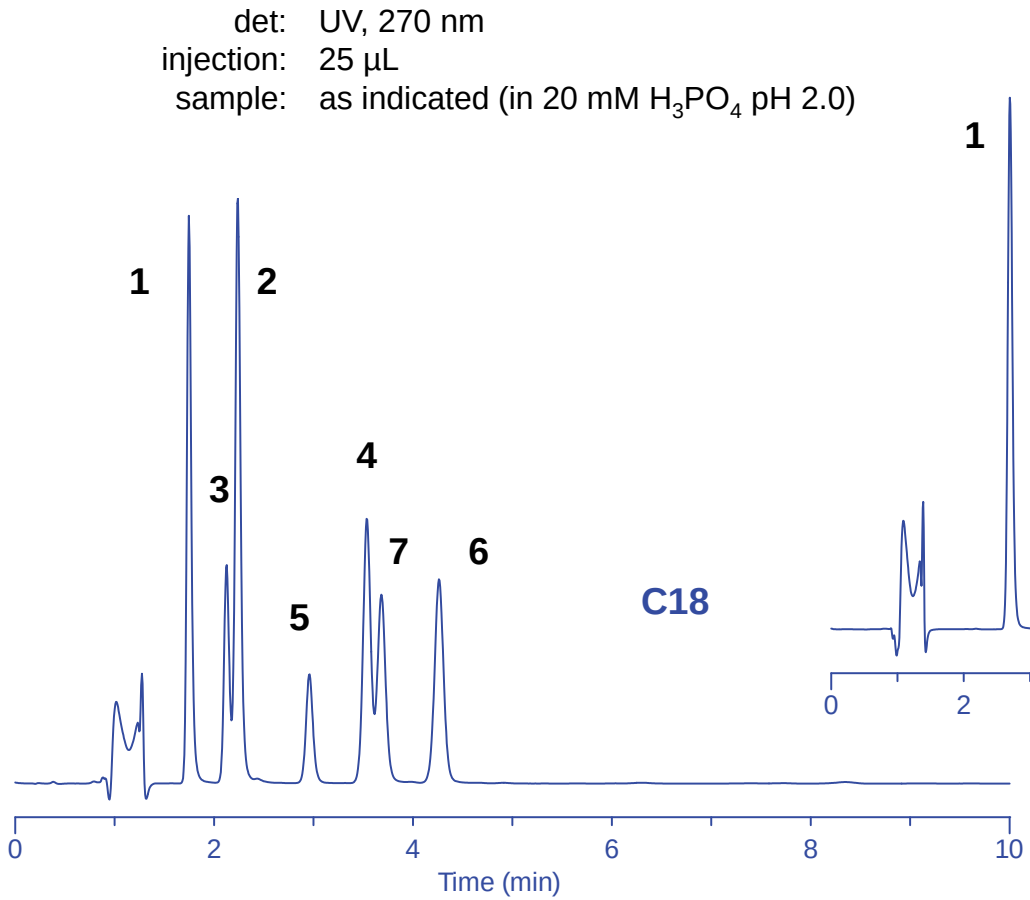


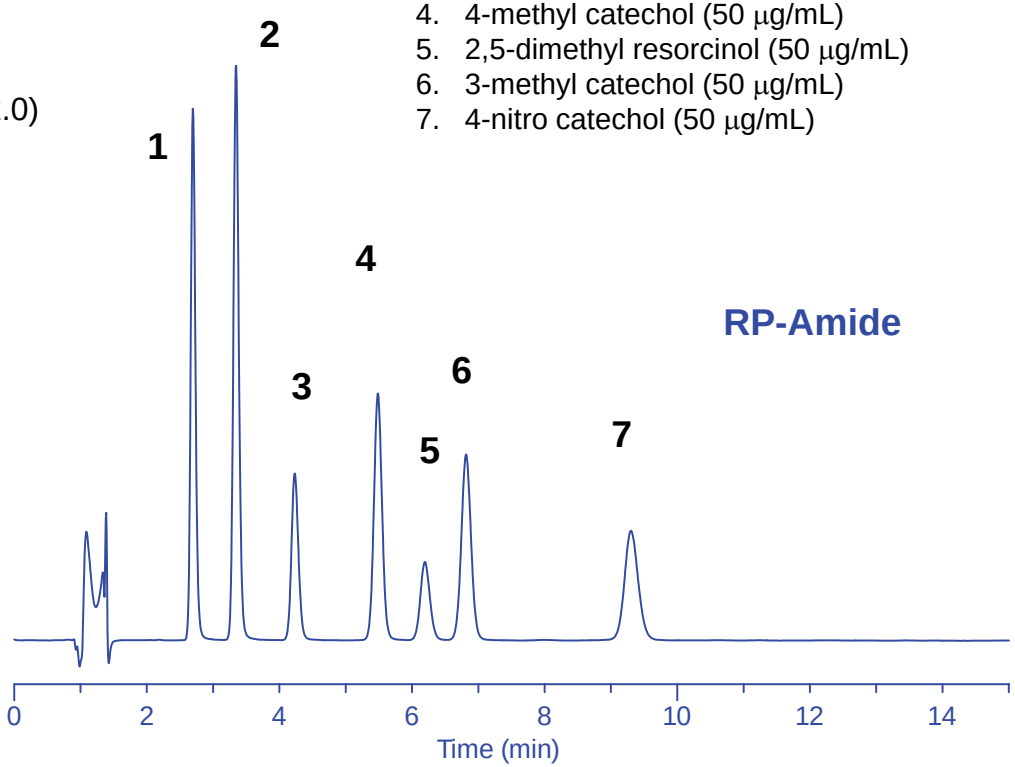
Resorcinol

Example Application: Separation of Catechols and Resorcinols

column:	Ascentis RP-Amide or C18, 15 cm x 4.6 mm, 5 µm particles
mobile phase:	75:25, 20 mM phosphoric acid (pH 2.0 unadjusted):CH_3CN
temp:	30 °C
flow rate:	1.5 mL/min
det:	UV, 270 nm
injection:	25 µL
sample:	as indicated (in 20 mM H_3PO_4 pH 2.0)

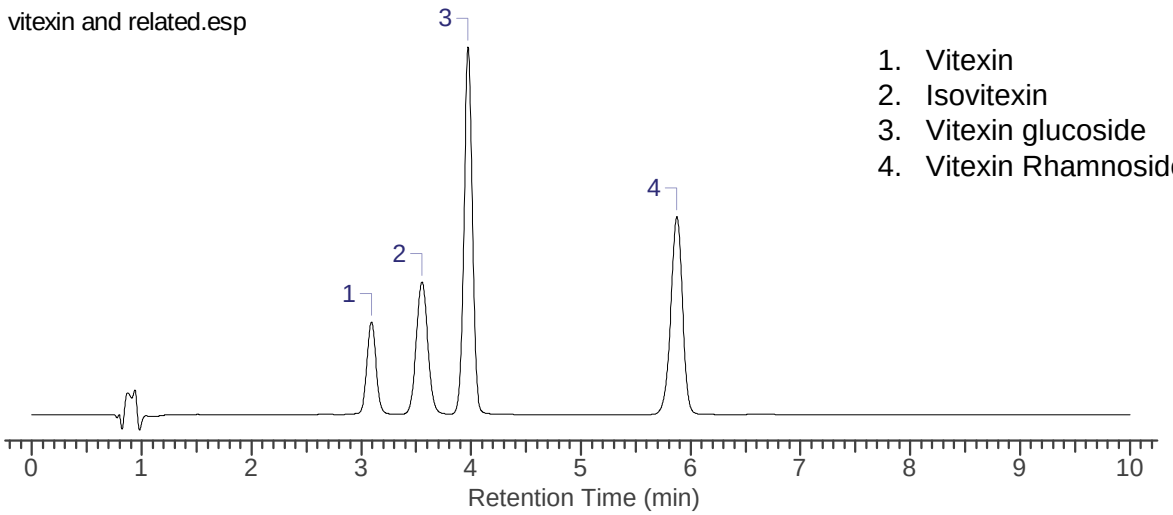
1. Resorcinol (50 µg/mL)
2. Catechol (50 µg/mL)
3. 2-methyl resorcinol (50 µg/mL)
4. 4-methyl catechol (50 µg/mL)
5. 2,5-dimethyl resorcinol (50 µg/mL)
6. 3-methyl catechol (50 µg/mL)
7. 4-nitro catechol (50 µg/mL)



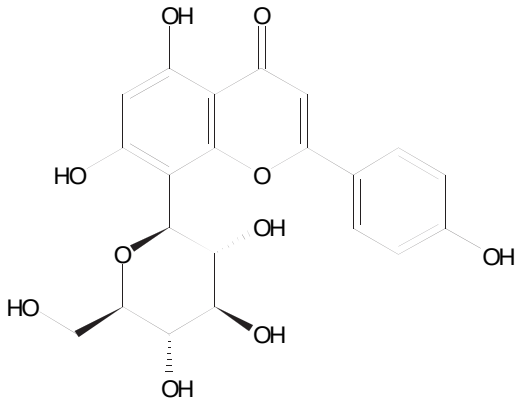


Vitexin and Related Compounds

column:	Ascentis RP-Amide or C18, 10 cm x 3 mm, 2.7 µm particles
mobile phase:	water/acetonitrile (85:15 v/v); 10 mM citric acid
temp:	55 °C
flow rate:	1.5 mL/min
injection:	2 µL



1. Vitexin
2. Isovitexin
3. Vitexin glucoside
4. Vitexin Rhamnoside



Euerby Column Classification Results

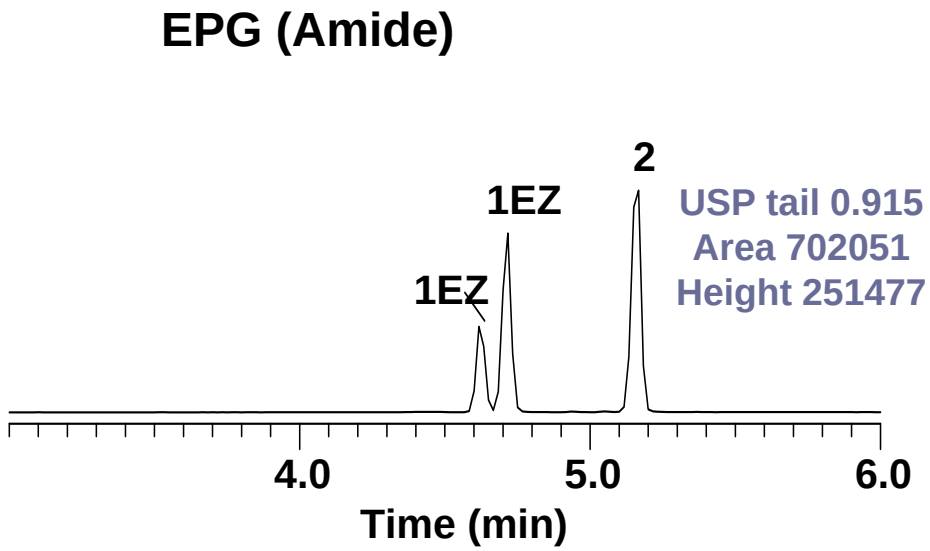
Column	k_{PB}	α_{CH2}	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{B/P}$ 2.7	$\alpha_{B/P}$ 7.6
Discovery C18 (Control)	2.92	1.42	1.52	0.37	0.08	0.28
Ascentis RP-Amide	5.23	1.48	1.61	0.24	0.05	0.25
Ascentis C18	7.35	1.50	1.59	0.37	0.08	0.31

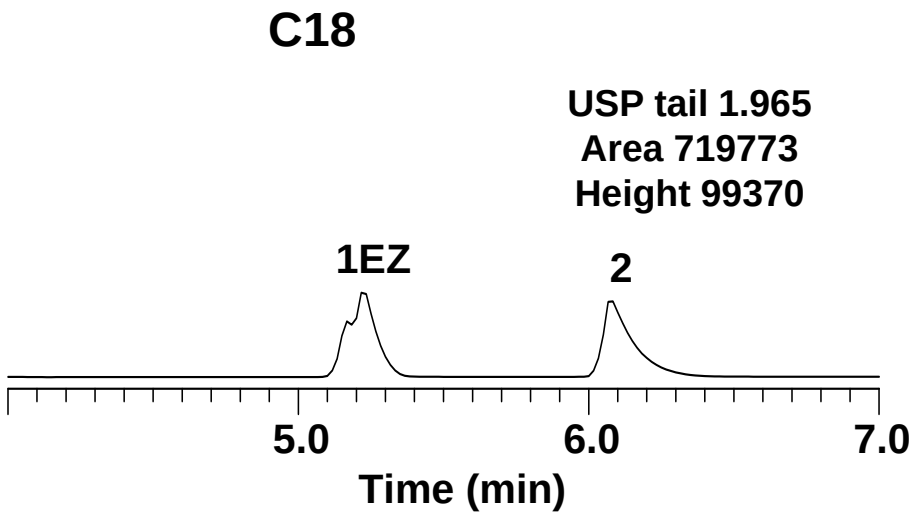
Ion-exchange capacity

Tamoxifen and Metabolites

- 0.1 % formic acid in water

Peak IDs
1. 4-hydroxytamoxifen (E and Z) isomers (50 µg/mL)
2. Tamoxifen (50 µg/mL)





- Peak heights is > 2.5 greater; lower LODs expected with EPG, the end of the peak becomes difficult to determine with C18. As a result, the baseline is therefore drawn in different places for multiple injections; resulting in error in measurement accuracy and precision are lost due to tailing peaks.

EPG Phases

Two key observations:

- Provides different selectivity as a result of hydrogen bond accepting ability
- Provides shielding of surface silanol interactions with basic analytes

Where to use them?

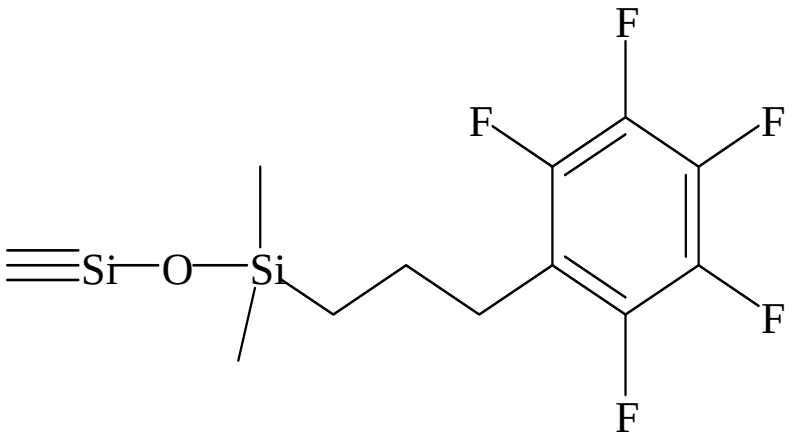
- First line for hydrophobic bases!
- Good alternative for moderately polar compounds as well as those that can donate toward a hydrogen bond
- Often find improved selectivity for positional isomers as compared to C18

Fluorinated Stationary Phases: Introduction

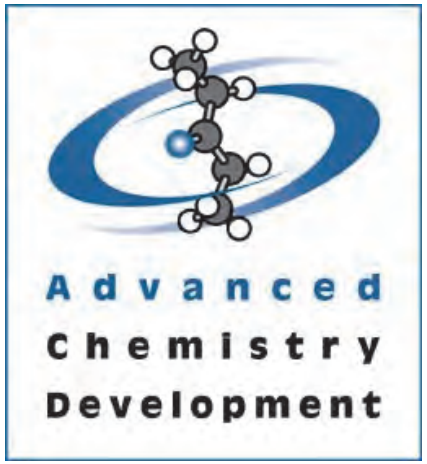
Fluorinated phases have also become a popular alternative to alkyl phases in recent years

Fluorinated phases such as pentafluorophenylpropyl-modified silica (PFPP/PFP or F5) offer the potential for:

- Dipole-dipole interactions
- π-π interactions
- Charge-transfer interactions
- Ionic interactions
- Rigidity imparts some steric selectivity



Shape Selectivity and IEX Differences



HPLC Column Selector

Build version: 08 February 2010

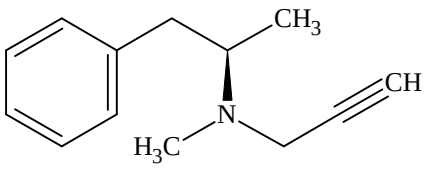
The Column Selector is based on an article by M.R. Euerby and P. Petersson published in *J. Chromatogr. A* **2003**, *994*, 13-36.

Single pair column comparison

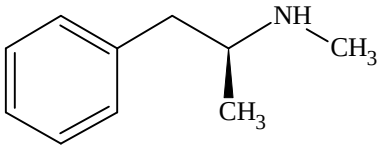
CDF = 1.294

	Parameters					
Weightings	kPB (1.00)	aCH2 (1.00)	aTO (1.00)	aCP (1.00)	aBP76 (1.00)	aBP27 (1.00)
Column 1: Discovery C18 HS	6.68	1.49	1.55	0.4	0.38	0.1
Column 2: Discovery F5 HS	1.7	1.26	2.55	0.68	0.85	0.34

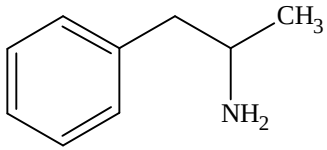
Selegiline and Amphetamines Retained and Separated using PFP



Selegiline

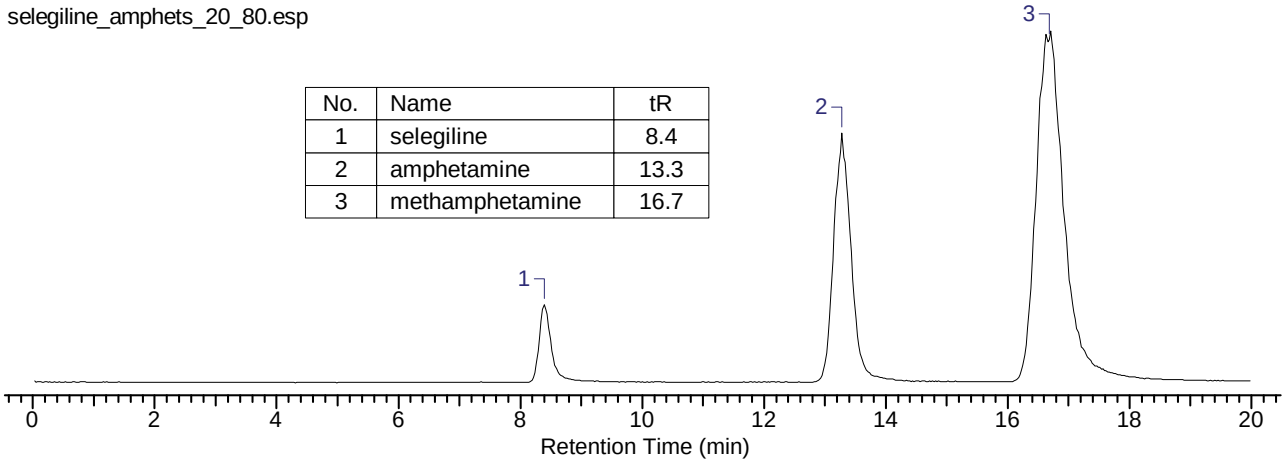


Methamphetamine



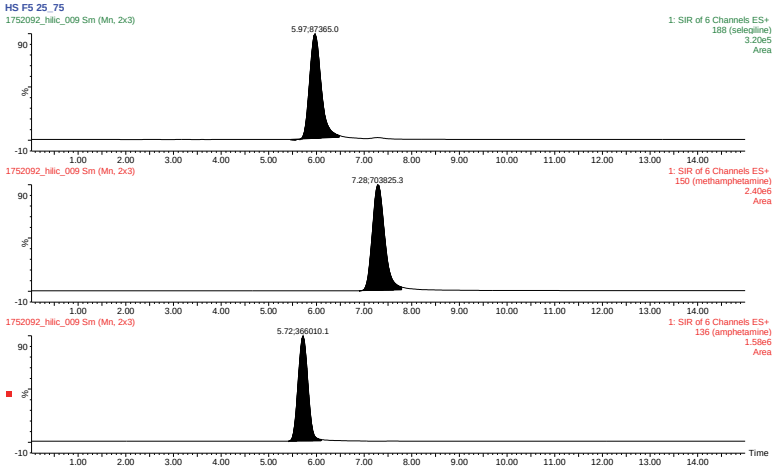
Amphetamine

column:	Ascentis Express F5, 10 cm x 4.6 mm, 2.7 µm particles,
mobile phase:	(A) 10 mM ammonium acetate, pH 4.0 with acetic acid
	(B) acetonitrile; A:B = 20:80
flow rate:	1.0 mL/min.
pressure:	160 bar (2300 psi)
temp.:	35 °C
det.:	MS ESI (+), SIR m/z 136, 150, 188
injection:	2 µL
sample:	10 µg/mL in methanol

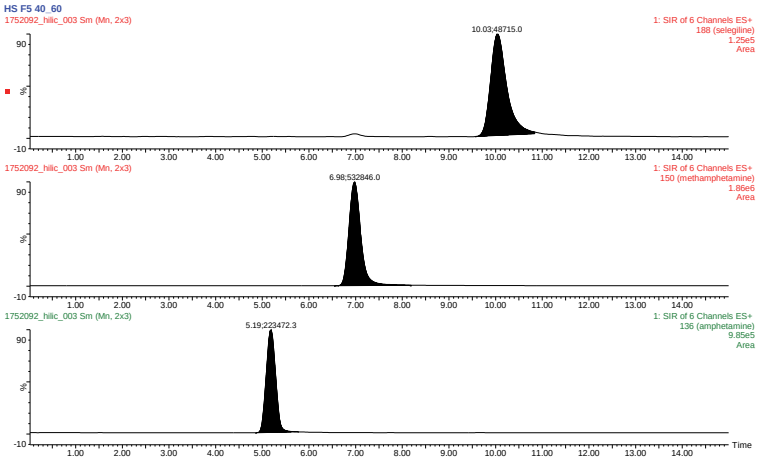


Altering Retention and Selectivity through Manipulation of Ion-Exchange and Percent Organic Components

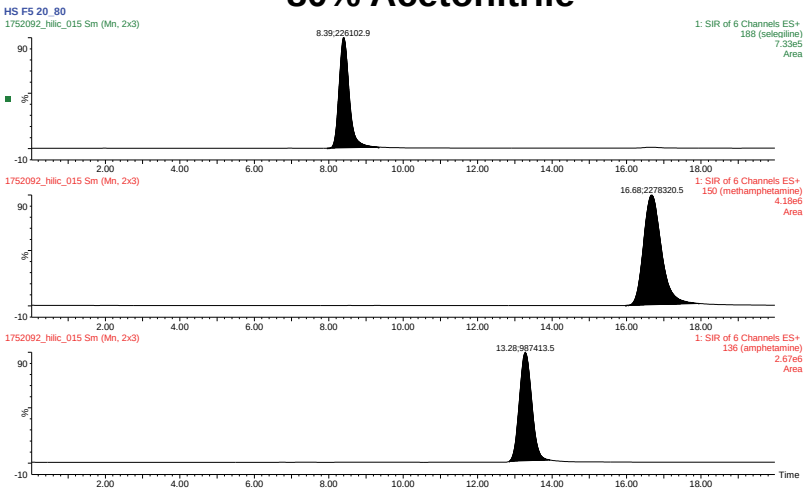
75% Acetonitrile



60% Acetonitrile



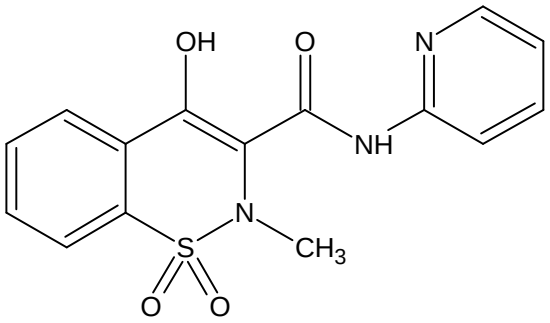
80% Acetonitrile

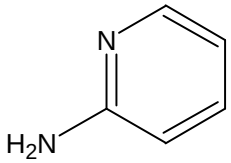


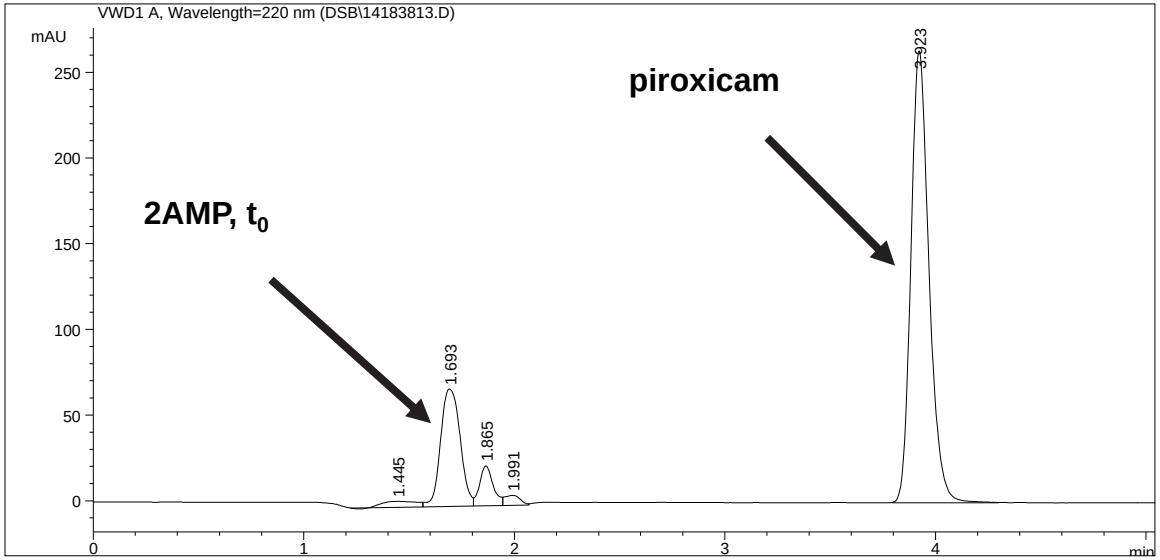
Solving Real Issues with Alternative Selectivity

Conditions:

column:	Discovery C18, 15 cm x 4.6 mm, 5 µm
mobile phase:	10 mM KH_2PO_4, pH 2.5, acetonitrile (55:45, v/v)
det.:	UV @220 nm



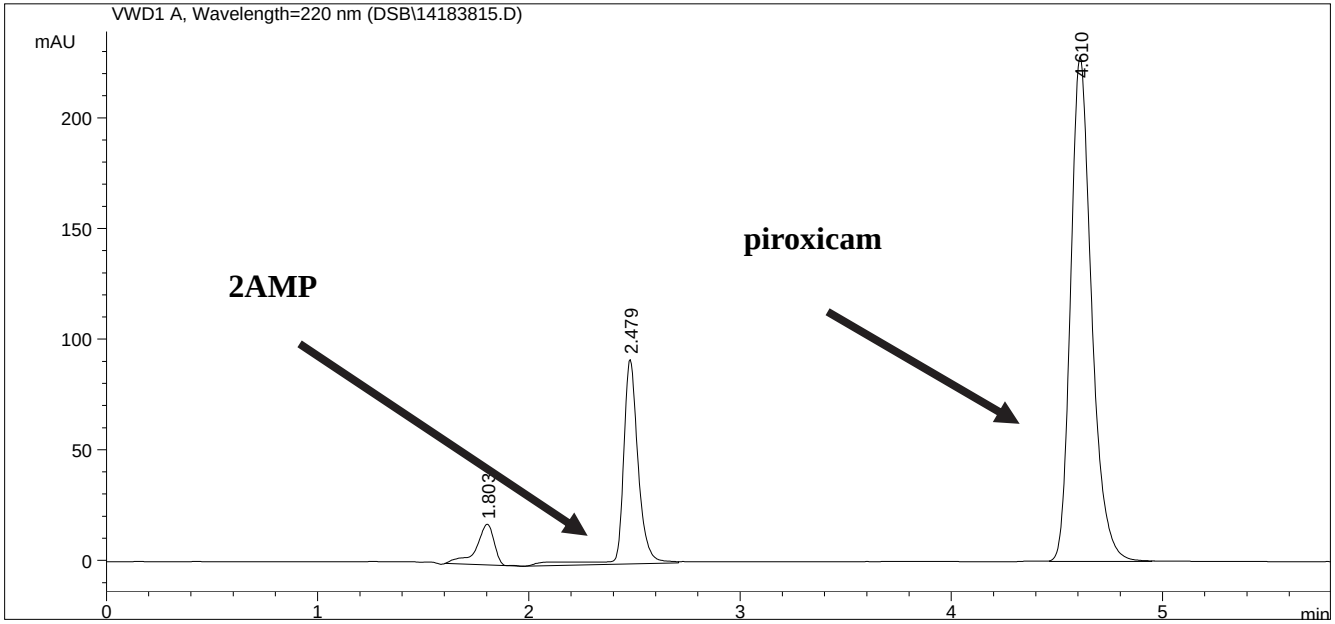




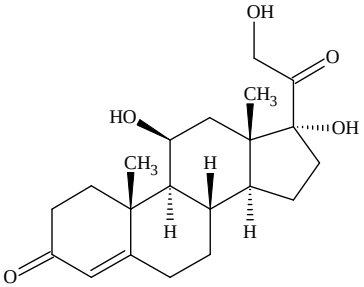
Solving Real Issues with Alternative Selectivity (contd.)

Conditions:

column:	Discovery HS F5, 15 cm x 4.6 mm, 5 µm
mobile phase:	10 mM KH_2PO_4, pH 2.5, acetonitrile (55:45, v/v)
det.:	UV @220 nm

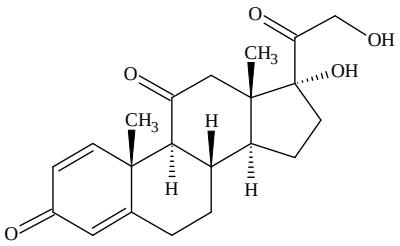


Apparent Shape Selectivity using PFP



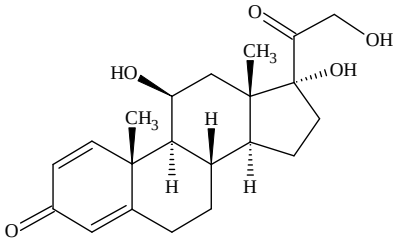
Hydrocortisone [*{BAN}; *{INN}; *{JAN}]

Monoisotopic Mass = 362.209324 Da



Prednisone [*{BAN}; *{INN}]

Monoisotopic Mass = 358.178024 Da

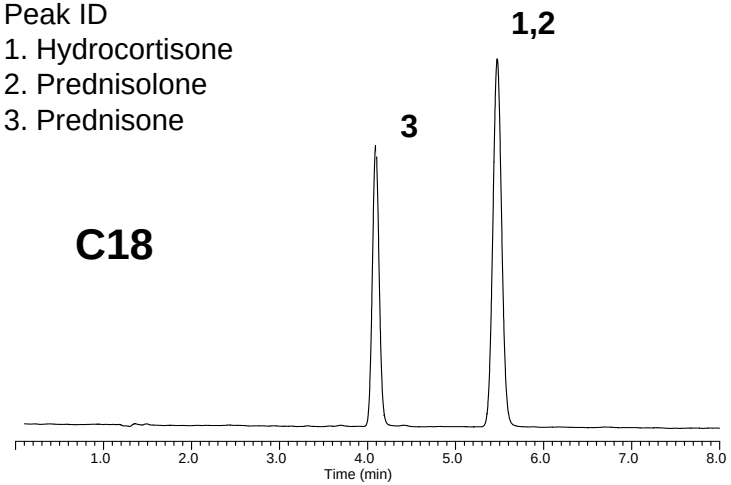


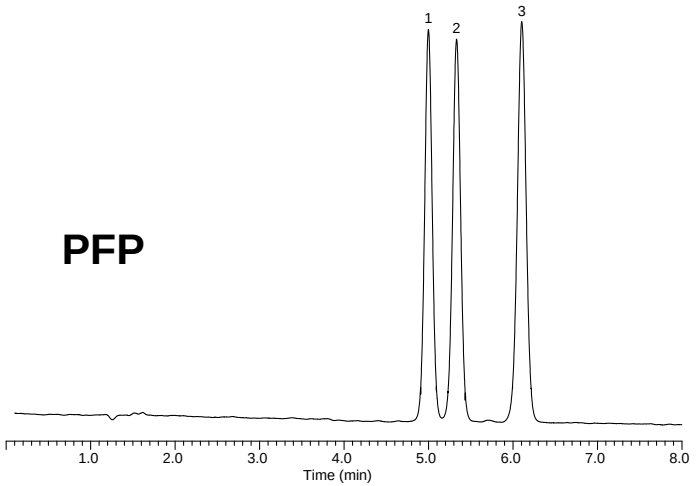
Prednisolone [*{BAN}; *{INN}; *{JAN}]

Monoisotopic Mass = 360.193674 Da

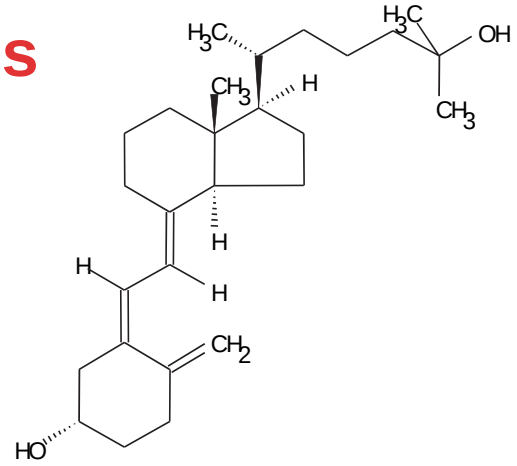
mobile phase: water:methanol (50:50, v/v)

Peak ID
1. Hydrocortisone
2. Prednisolone
3. Prednisone

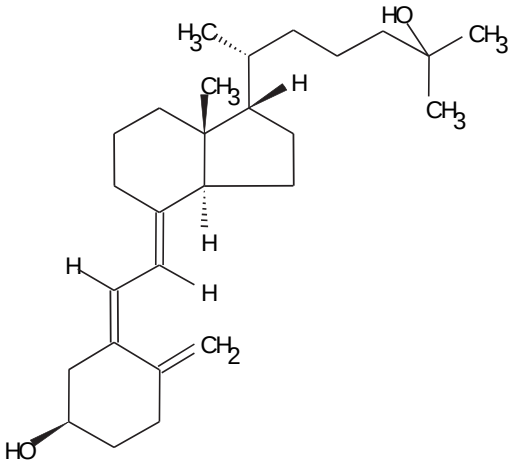




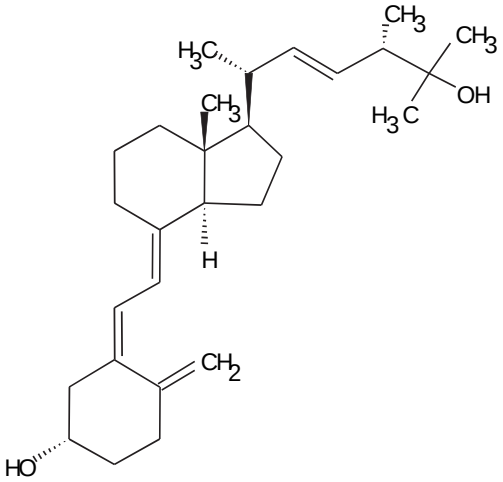
Structures



25-Hydroxyvitamin D



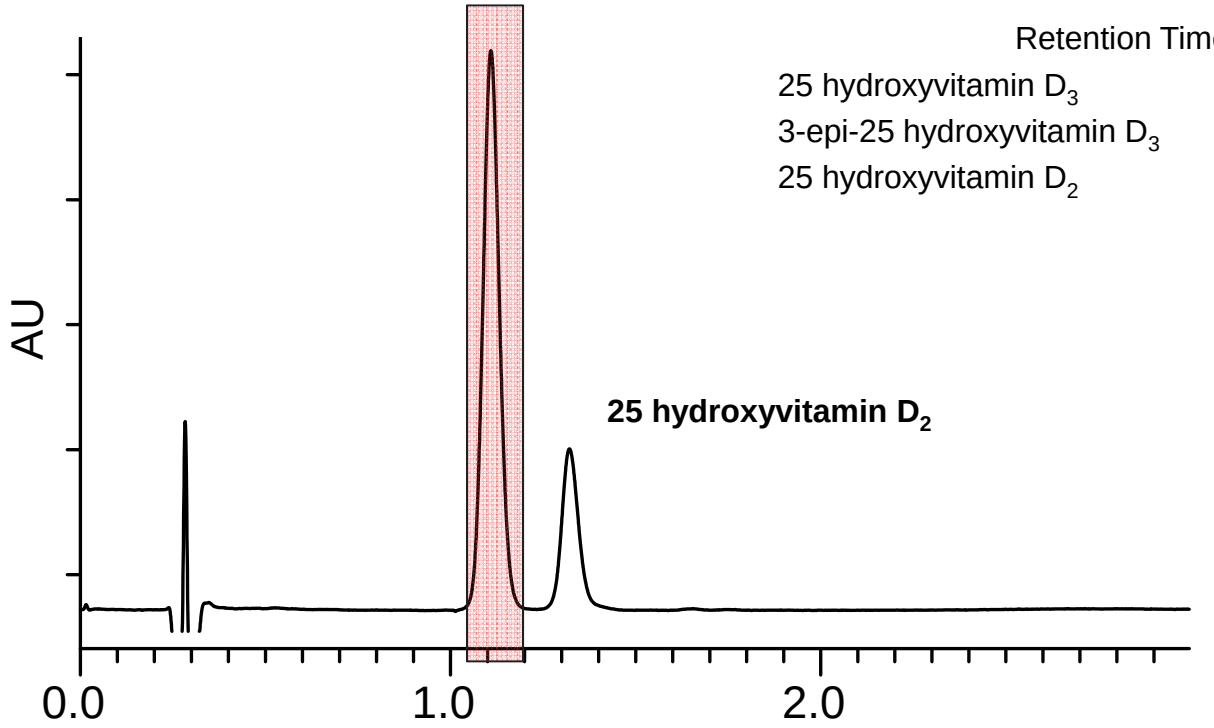
3-epi-25-Hydroxyvitamin D



25-Hydroxyvitamin D2

Vitamin D Metabolites on C18

column:	Ascentis Express C18, 5 cm x 2.1 mm, 2.7 µm (53822-U)
mobile phase:	(A) 15%, 5 mM ammonium formate water
	(B) 85%, 5 mM ammonium formate (95:5 acetonitrile:water)
flow rate:	0.4 mL/min
temp.:	35 °C
det.:	UV 265 nm, MS ESI+ 100-1000m/z
injection:	1 µL

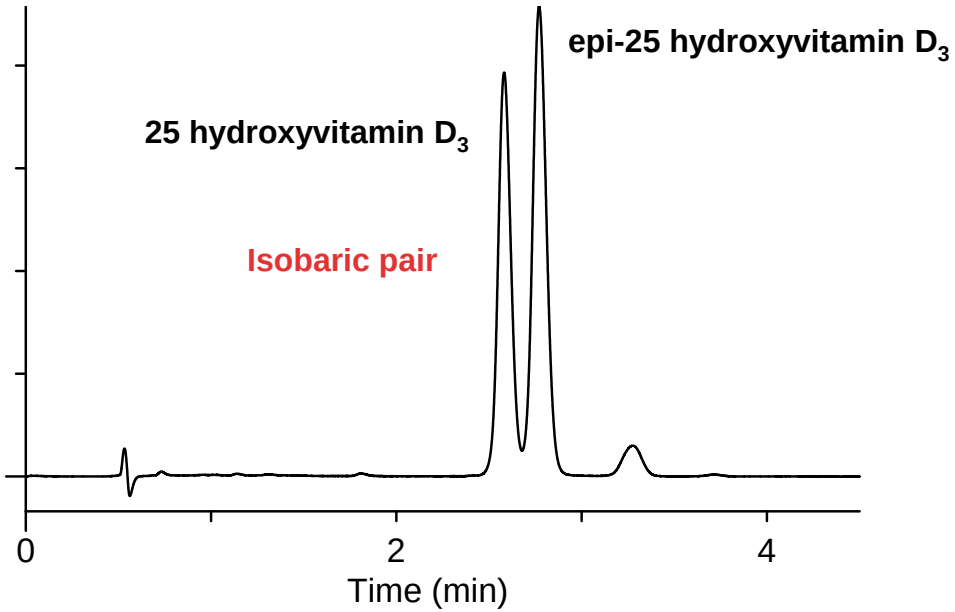


Retention Time:	
25 hydroxyvitamin D_3	1.09 min
3-epi-25 hydroxyvitamin D_3	1.09 min
25 hydroxyvitamin D_2	1.29 min

Vitamin D Metabolites on PFP

column:	Ascentis Express F5, 10 cm x 2.1 mm, 2.7 µm (53569-U)
mobile phase:	(A) 25%, 5 mM ammonium formate water
	(B) 75%, 5 mM ammonium formate methanol
flow rate:	0.4 mL/min
temp.:	40 °C
det.:	UV 265 nm, MS ESI+ 100-1000m/z
injection:	1 µL

	Retention Time:
25 hydroxyvitamin D_3	2.57 min
epi-25 hydroxyvitamin D_3	2.76 min
25 hydroxyvitamin D_2	2.77 min



Aurand, C. R., Bell, D. S, Wright, M., (2012). *Bioanalysis* 4(22): 2681-2691.

Fluorophenyl Phases

Increased IEX behavior over alkyl and phenyl phases (let it happen)

Increased shape selectivity.

Notable for separation improvements for basic (polar and nonpolar) as well as rigid structures.

Classification by Chemical Interaction Type[a]

Bonded Phase	Hydrophobic	H-Bonding	Dipolar	π-π	Steric	Ion-Exchange[b]
C18	V. Strong	Weak	No	No	No	Moderate
C8	Strong	Weak	No	No	No	Moderate
Amide	Strong	Strong Acceptor	Moderate	No	Weak	Very weak
Phenyl	Moderate	Weak Acceptor	Weak	Strong Donor	Strong (Rigid)	Moderate
Cyano	Weak	Moderate	Strong	Weak	No	Strong
PFP	Moderate	Moderate Acceptor	Strong	Strong Acceptor	Strong (Rigid)	Strong

a. Using Euerby adaptation of Snyder-Dolan-Carr Hydrophobic Subtraction Model.

b. May include interactions with acidic silica substrate

I. Choice of Column and the 12 Problems

Resolution
- Retention, efficiency and selectivity

Peak shape
- Silanol activity

Retention of polar compounds
- Availability of polar and ionic interactions

Time to develop a method
- The right tool speeds the job

Separation of critical pairs
- Availability of needed interactions

High throughput
- Smaller dimension = faster analysis
- Superior selectivity = faster analysis
- UPLC/SP particles = flatter van D

Wide range of sample polarities
- Correct blend of interactions

Ruggedness/robustness
- Column stability

Mass spec compatibility
- Low column bleed

Low detection limits
- Efficiency

Scale-up to prep
- Availability of preparative dimensions and particle sizes

Dealing with some of the other problems

Retention of polar compounds: HILIC

- *Same reasoning in terms of stationary phases*
- *Must keep mechanisms in mind*
- *Retention Mechanisms in HILIC Chromatography - http://www.sigmaaldrich.com/analytical-chromatography/video/brighttalk.html*

High throughput, Low detection limits: Sub 2 µm, Superficially Porous

- *Both approaches provide increased efficiency*
- *Both approaches provide flatter van Deemter profiles*

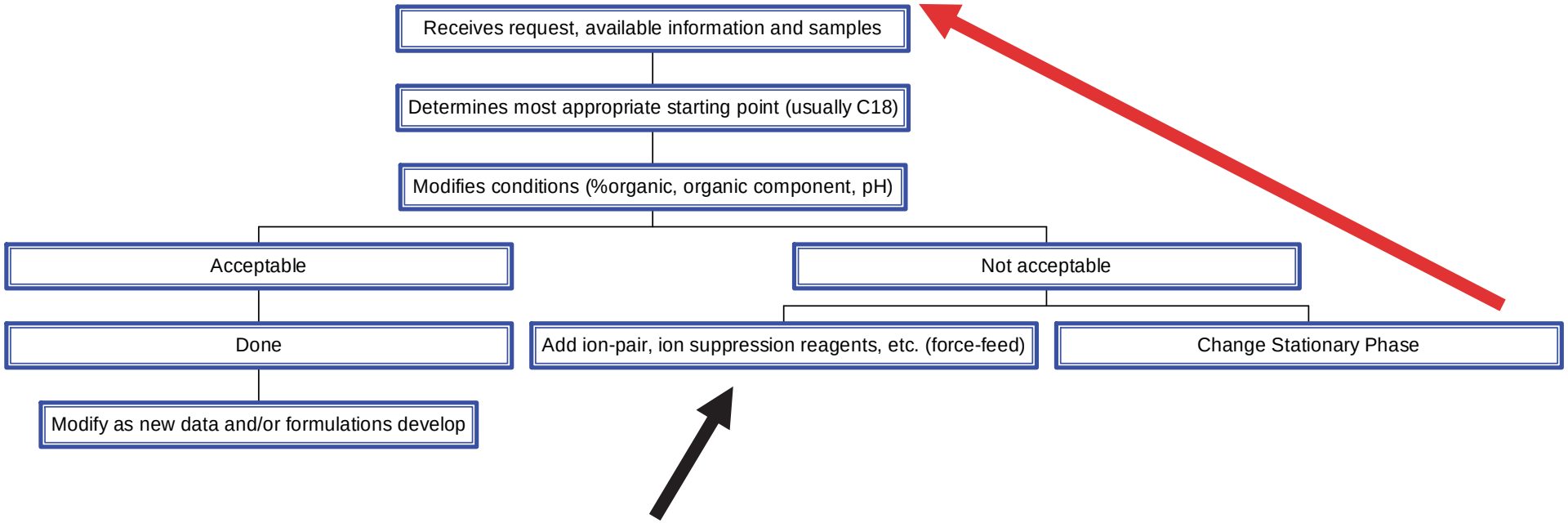
Ruggedness/robustness

- *Most modern phases provide intrinsic robustness*
- *As long as methods are developed with appropriate controls and away from edges…..*

Mass spec compatibility – use modern phases

Scale-up to prep

Speeding Up the Method Development Process



Often a poor choice

Mobile Phase Modifiers: Buffers and the 12 Problems

- Resolution
  - Retention, efficiency and selectivity
- Peak shape
  - Silanol activity
- Retention of polar compounds
  - Availability of polar interactions
- Time to develop a method
  - The right tool speeds the job
- Separation of critical pairs
  - Availability of needed interactions
- High throughput
- Wide range of sample polarities
  - Correct blend of interactions
- Ruggedness/robustness
- Column stability
- Mass spec compatibility
  - Mobile phase considerations
- Low detection limits
  - Mobile phase considerations
  - Efficiency
- Scale-up to prep

Mobile Phase: Buffer

Buffers

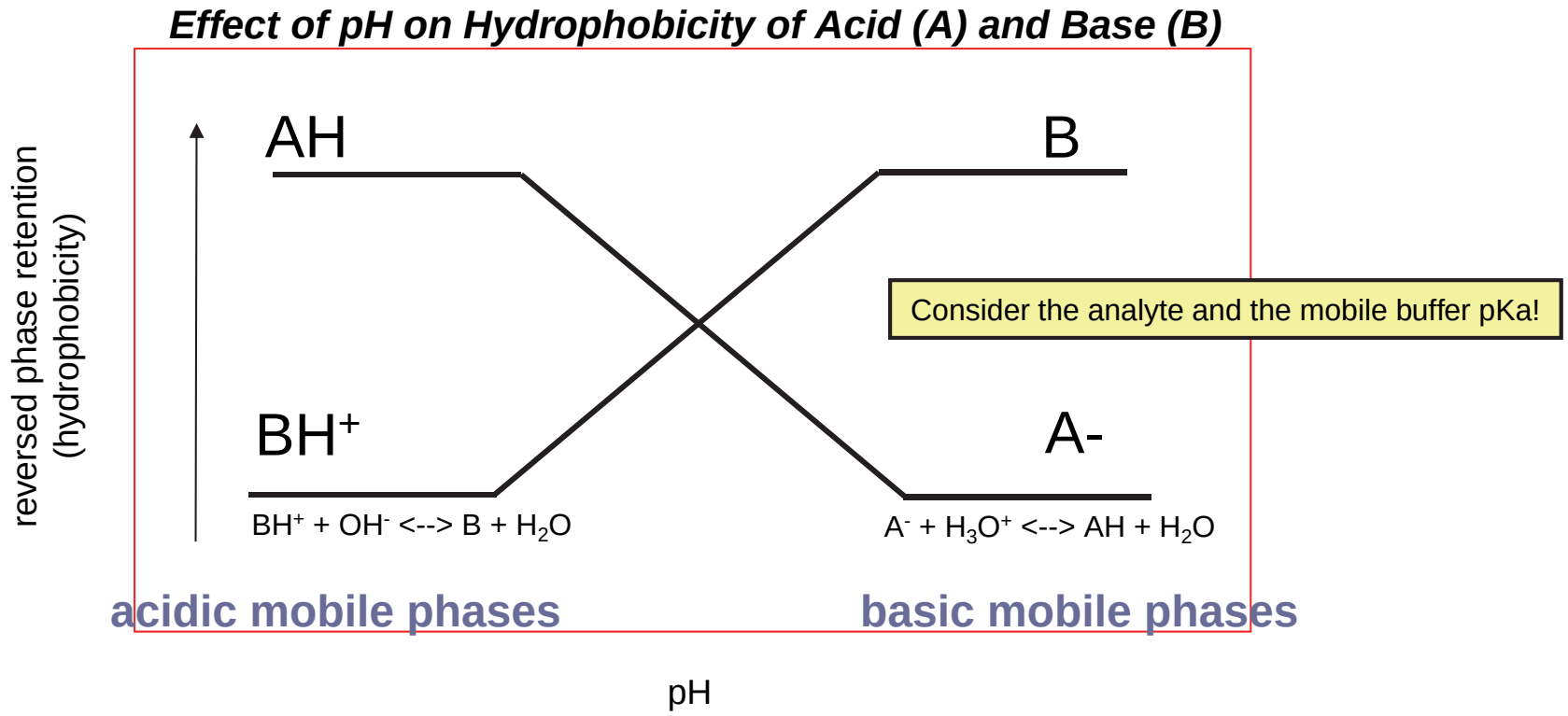
- Ionizable analytes make pH control a must
- Buffers, by definition, help to control pH but only near their pK_a
- A common problem is that developers use the wrong buffer for a given pH

Rule: adjust pH +/- 1 pH unit about a buffer pK_a

- Note that some situations require the use of a given buffer outside this range

Controlling Retention in RP-HPLC: *pH*

Acids are more non-polar at low pH where they are protonated, and bases are more non-polar at high pH were they are not protonated.



Buffers and Salts for HPLC

Buffers	pk_a	Salts
Acetate	4.75	NaCl
Ammonium	9.24	KCl
Borate 1	9.24	NH_4OAc
Borate 2	12.7	
Borate 3	13.8	**Acids**
Citrate 1	3.13	H_3PO_4
Citrate 2	4.76	HCl
Citrate 3	7.56	TFA
Formate	3.75	Acetic
Oxalate 1	1.27	Formic
Oxalate 2	4.27	Carbonic
Phosphate 1	2.15	
Phosphate 2	7.20	**Bases**
Phosphate 3	12.3	TEA
Triethylamine	10.72	KOH
Trifluoroacetic acid	0.5	NaOH
Tris*	8.08	

*Tris(hydroxymethyl)aminomethane

Qualities

- UV-transparent
- Clean
- Particle-free
- Non-reactive
- Miscible with other solvents and samples
- LC/MS-compatible (volatile)

Mobile Phase (contd.)

Buffers

- Control the ionization state of the analyte AND the surface silanols
- Consider both
- Generally, 10 mM is enough – if greater shows better peak shape – likely dealing with secondary interactions
- Watch high organic percentage and buffer solubility
- Use volatile organic buffers for MS (ammonium acetate, ammonium formate)
- Counterion often very important – mediates ion-exchange interactions
- Consider pH AFTER mixing with organic

Mobile Phase: Organic Modifier and the 12 Problems

- Resolution
  - Retention, efficiency and selectivity
- Peak shape
  - Silanol activity
- Retention of polar compounds
  - Availability of polar interactions
- Time to develop a method
  - The right tool speeds the job
- Separation of critical pairs
  - Availability of needed interactions
- High throughput
- Wide range of sample polarities
  - Correct blend of interactions
- Ruggedness/robustness
- Column stability
- Mass spec compatibility
  - Mobile phase considerations
- Low detection limits
  - Mobile phase considerations
  - Efficiency
- Scale-up to prep

Mobile Phase: Organic Modifier

Organic modifier: Why acetonitrile, for example?

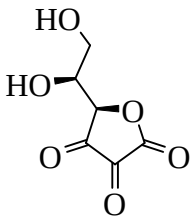
- Used to control the retention based on dispersive interactions
- Can effect polar interactions as well
- Choices: Acetonitrile, Methanol, THF
- Why these?
  - Low viscosity – backpressure issues
  - Low UV absorbance – detection issues
  - Water miscibility
- Can other be used?
  - Sure, but often with penalties

Mobile Phase: Organic Modifier

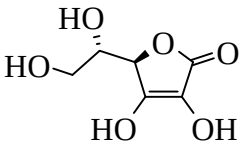
- Avoid very low organic on standard C18 phases (so-called phase collapse)
  - If very low organic is required use a stationary phase that is 100% aqueous compatable
- Avoid very high organic when using inorganic buffers (solubility)
- Avoid >20% THF with PEEK

100% Aqueous Stability

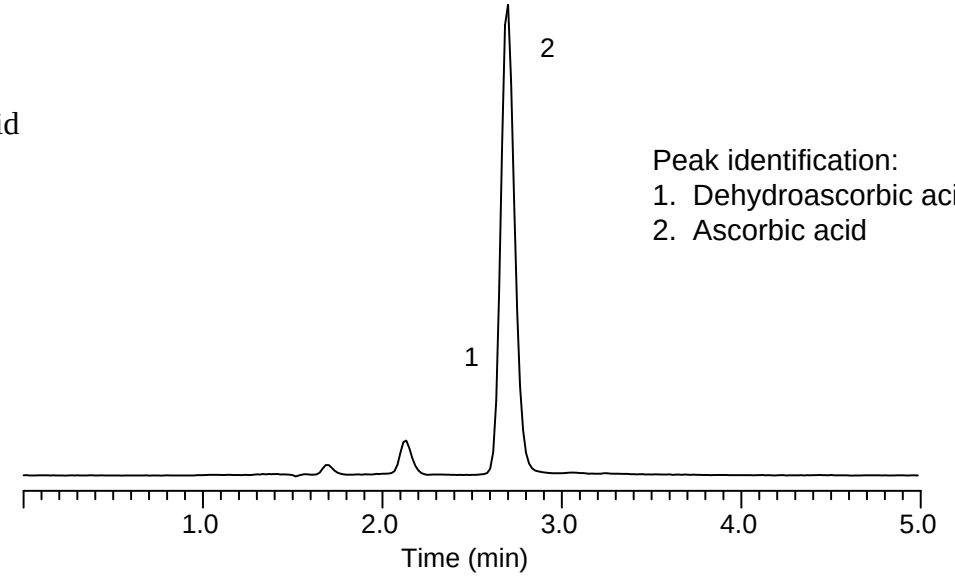
column:	Ascentis RP-Amide, 15 cm x 4.6 mm, 5 µm (53569-U)
mobile phase:	25mM monobasic potassium phosphate (pH 3.0 with H_3PO_4)
flow rate:	1 mL/min
temp.:	35 °C
det.:	UV 230
injection:	10 µL



dehydroascorbic acid



ascorbic acid



Peak identification:
1. Dehydroascorbic acid
2. Ascorbic acid

Peak Tailing

Peak tailing due to secondary interactions with the silanol surface of C18 columns.

C18 phases are not designed to take advantage of these interactions, therefore we do not have control.

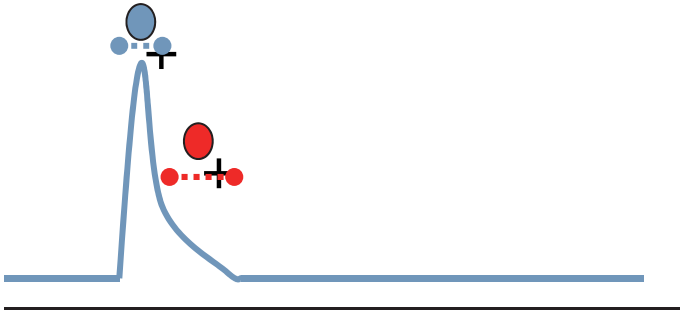
Only part of the analyte population sees this interaction, thus tailing occurs.

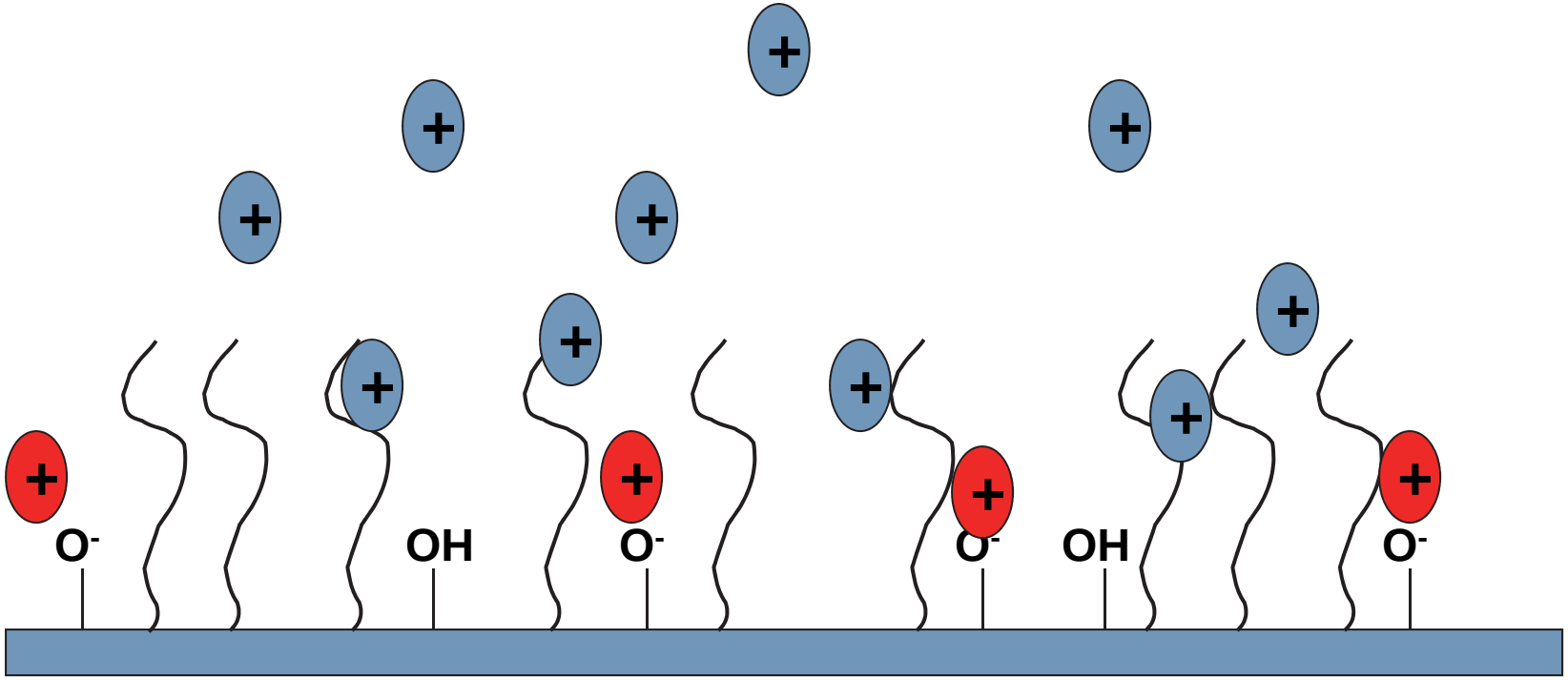
A more base-deactivated C18 means that there are less available ionized silanols for bases to interact with.

Polar phases take more advantage of silanol interactions.

Origins of Peak Tailing

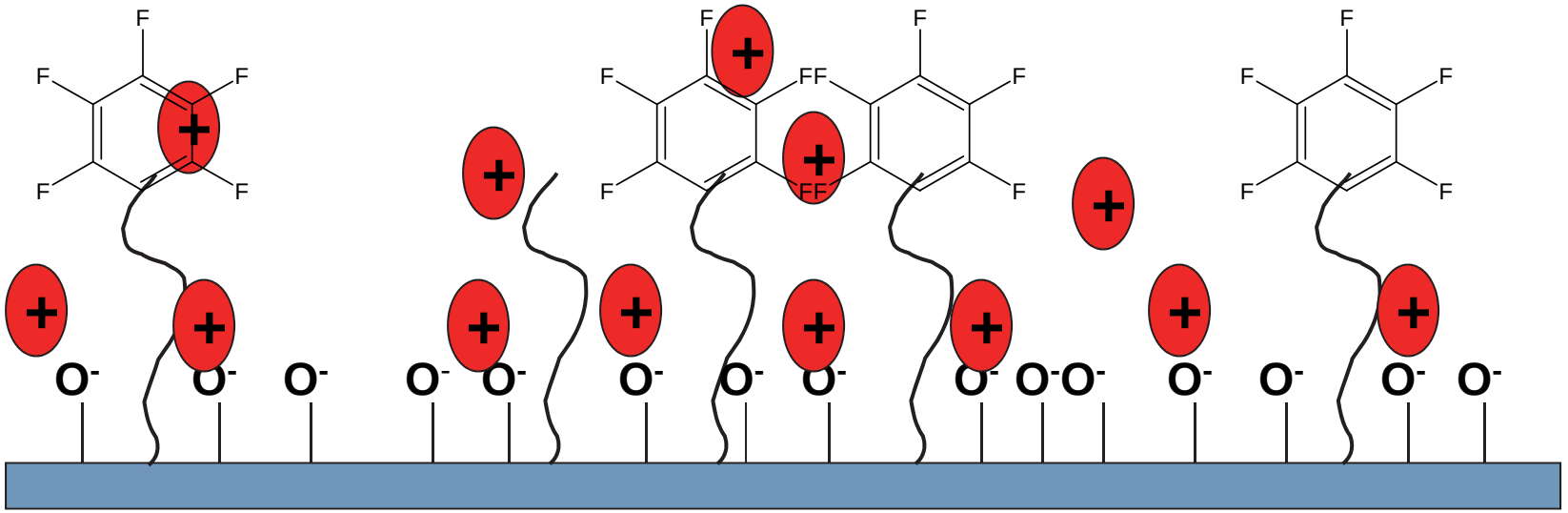
Only some of the analyte population has access to surface ionized silanols.



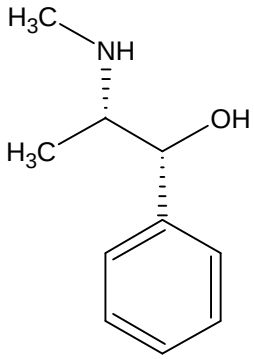


Origins of Peak Tailing (contd.)

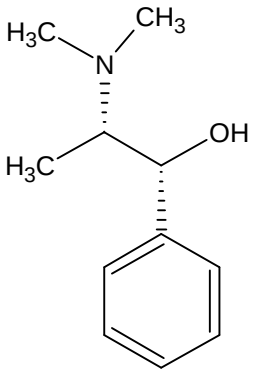
All analyte molecules have equal access to ionized surface silanols.



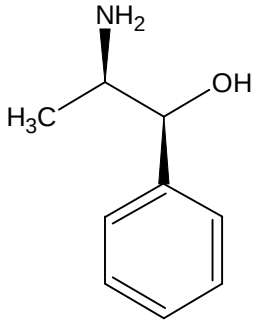
Ephedra Alkaloids



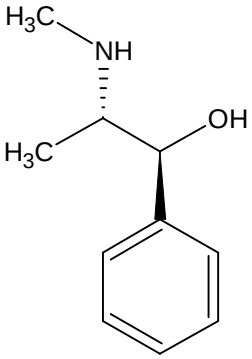
ephedrine



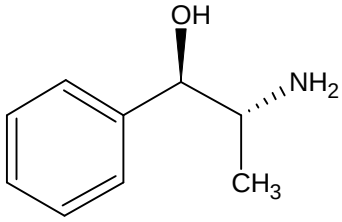
methylephedrine



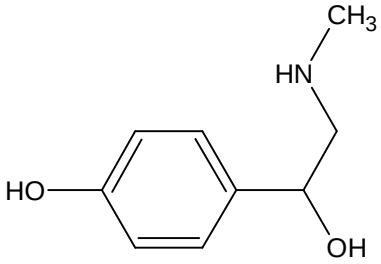
norephedrine



pseudoephedrine



norpseudoephedrine

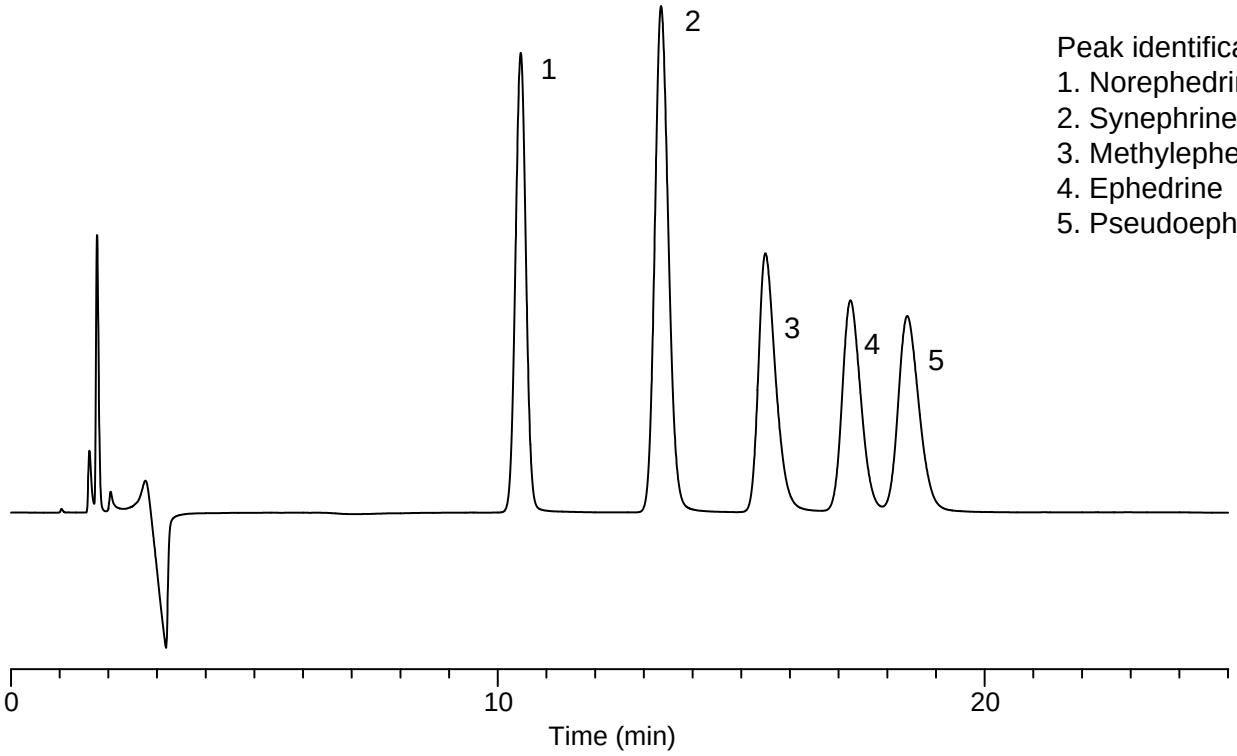


synephrine

D.S. Bell and A. D. Jones, *J. Chromatogr. A.,* 2003

Ephedrine Alkaloids

column: Discovery HS F5, 15 cm x 24.6 mm, 5 µm particles (567416-U)
mobile phase: 4 mM ammonium acetate in 90:10, CH_3CN:water
flow rate: 1 mL/min
temp.: 45 °C
det.: UV at 215 nm
injection: 10 µL
sample: 10 µg/mL in 40:60, mobile phase:CH_3OH



Peak identification:
1. Norephedrine
2. Synephrine
3. Methylephedrine
4. Ephedrine
5. Pseudoephedrine

Peak Tailing

Remedies for peak tailing issues

- Lower pH minimizes population of ionized silanols (pK_a 4-6)
  - There are always some ionized silanols
  - Reported pK_a values are averages
  - Solvent system has impact on silanol pK_a
    - Greater organic proportions = higher silanol pK_a values
  - Stationary phase chemistry also has an effect on silanol pK_a values
    - Solvation at surface is altered
- Sometimes better to allow ion-exchange interactions rather than try to hide them.
- Some stationary phases, such as polar embedded phases, show improved peak shape for basic analytes due to masking of surface silanols.

V. Injection Volume and Sample Composition

Generally effect peak shape/ruggedness of method

- Separation is based on setting up an equilibrium
- When samples are injected – equilibrium is disrupted
- Need to balance injection volume with required detection limits
- Typically greater concentration (if possible) and lower volume is better than the opposite
- Most injectors are capable of 1-100 μL
  - Stay away from the edges
  - Stay within calibrated (qualified) volumes

V. Injection Volume and Sample Composition (contd.)

Best to keep sample solvent as close to mobile phase as possible

Very common problem

- Sample solvent should be “weaker” elution solvent than MP
- Strong solvent results in fronting peaks
- Can limit the effect by using small volumes

Dirty samples!

- Use guard columns and filters
- Know when to change them

Summary

Although many HPLC issues are “instrument” related (leaks, faulty detectors, extra column volume…) – a large percentage are related to poor method development.

Understanding why certain components are in there and some knowledge of usable ranges is critical for avoiding issues.

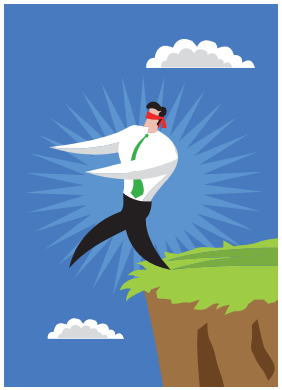
Trouble is usually avoidable through the development of sound methods.

Proper column, mobile phase and external parameters (ie temperature) are essential.

Column screening early in MD is highly recommended.

Use simplest system - KISS

Stay away from the edges



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