HILIC Chromatography: Theory and Method Development Practices

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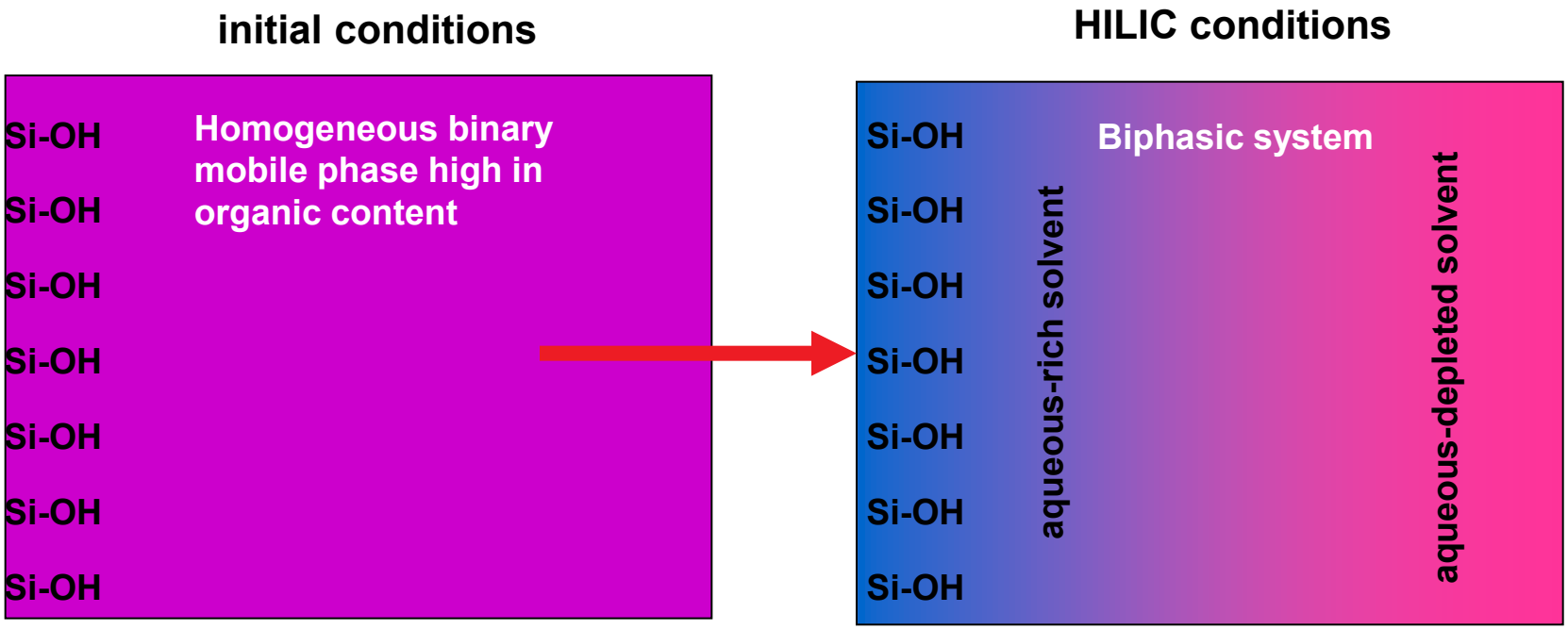
Introduction

1. Describe the HILIC system and prevailing theories on retention mechanisms
2. Discuss various HILIC stationary phases and the interactions they exhibit
3. Discuss method development practices/approaches
4. Provide some tips and cautions as they related to method development in HILIC mode

Aim

- Understand retention mechanisms involved in HILIC – the basis for method development practices
- Provide concepts and models for retention
- Provide some method development hints/pitfalls to watch for
- Illustrate with a few examples

Biphasic Solvent Distribution at Silica Surface

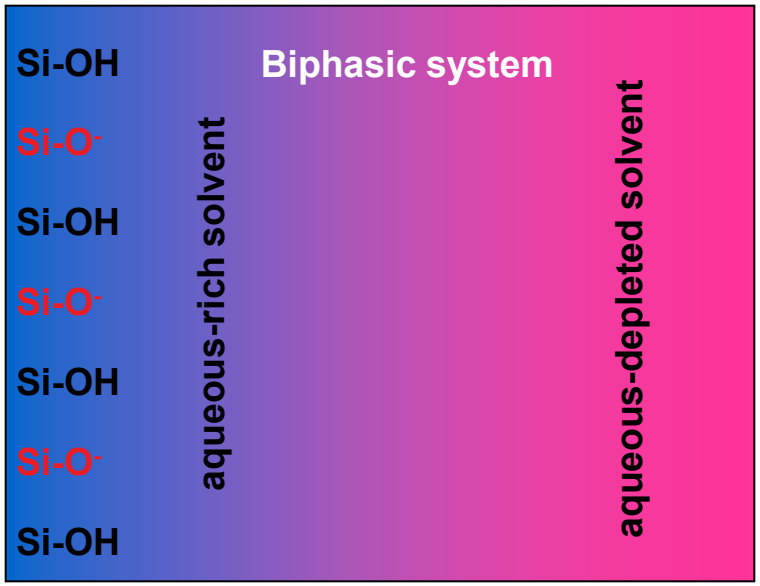


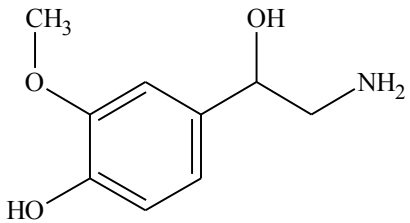
When a polar phase is utilized with a mobile phase high in organic concentration, the more polar water will preferentially adsorb on the surface creating a semi-stagnant, water-rich stationary phase and a water depleted mobile phase. Polar analytes can then partition in to aqueous-enriched phase

If it were only that simple….

- Polar analytes are just that because they are ionic or exhibit strong dipoles – naturally interactive
- We're bringing them close to a polar surface and therefore can expect (should expect) other strong interactions to take place
- Strong dipole interactions (H-bonding) and ion-exchange are an integral and important part of HILIC chromatography

HILIC conditions





Normetanephrine

Interactions in HILIC Chromatography

Partition – phase transfer of analytes to and from a polar stationary solvent (aqueous) and relatively non-polar organic mobile phase

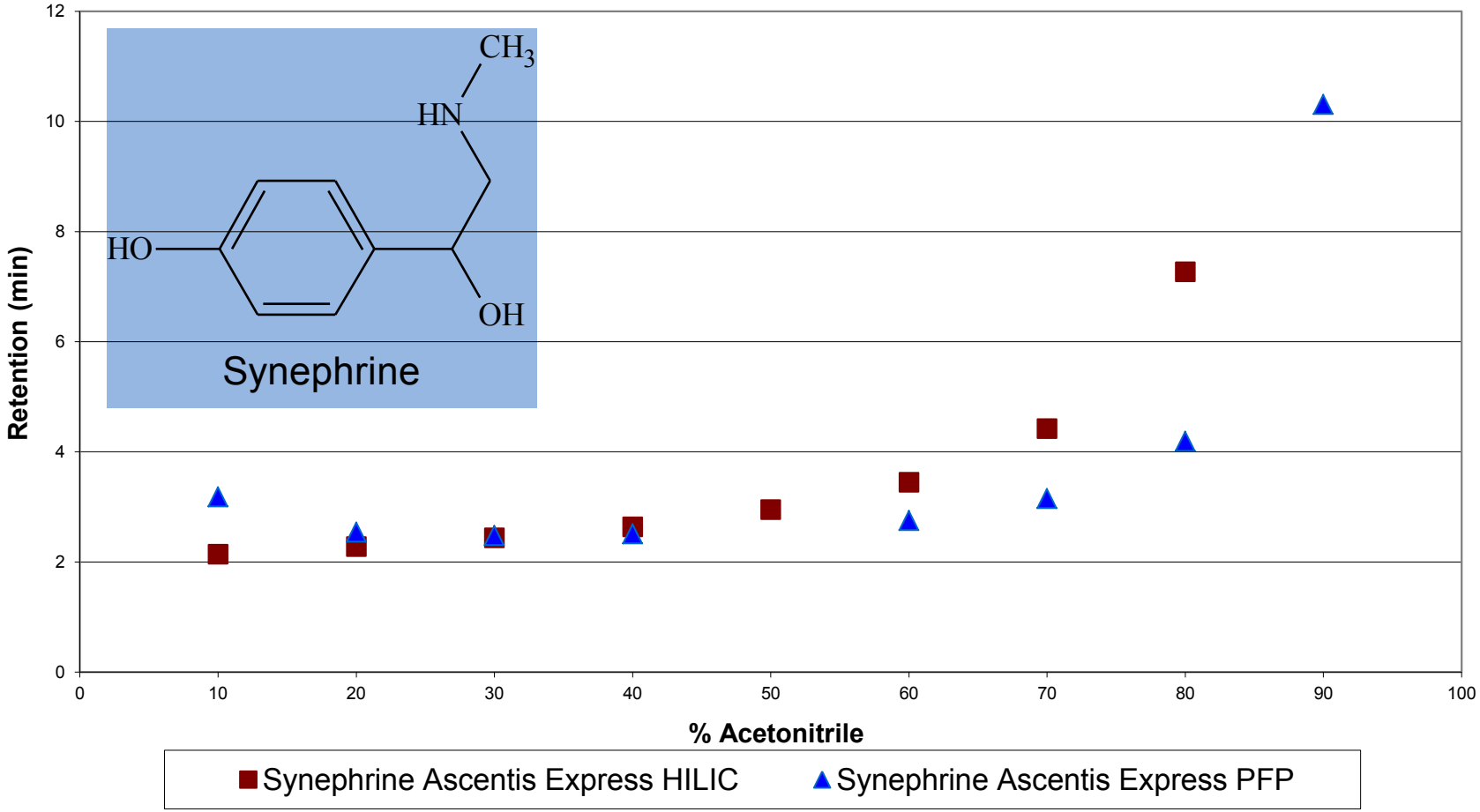
Polar interactions – Dipole (hydrogen bonding), induced dipole, etc

Ionic – Ion-exchange with bonded phase or ionized surface silanol groups

One major difference between reversed-phase and HILIC is the relative importance of these mechanisms. In RP partition dominates and selectivity/retention is generally 'tweaked' by supporting secondary polar or ionic interactions. In HILIC polar and ionic often dominate.

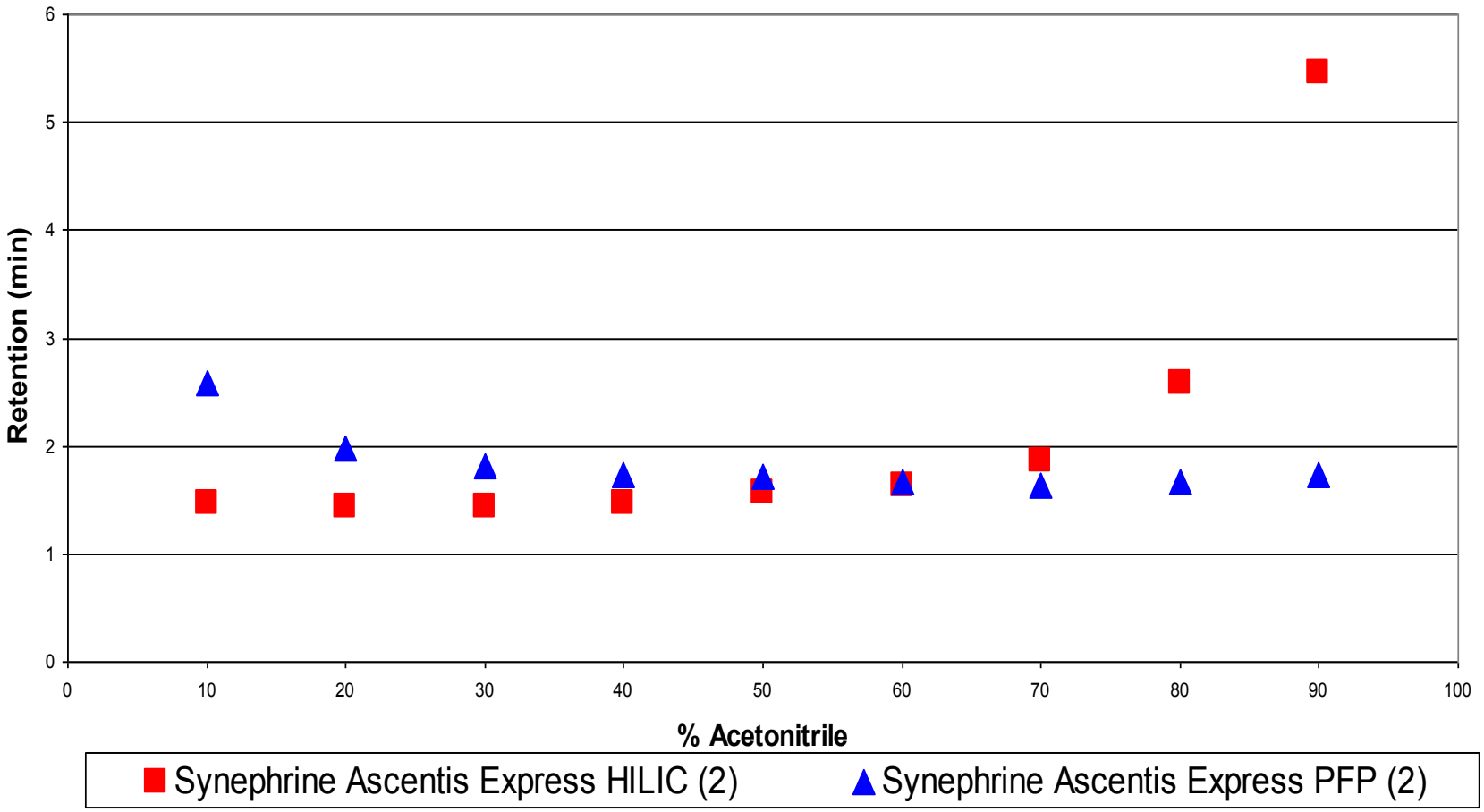
Retention Profile of Synephrine on Bare Silica and Pentafluorophenyl Phases

13 mM ammonium acetate, pH 6.7: acetonitrile – buffer diluted as organic is added



Retention Profile of Synephrine on Bare Silica and Pentafluorophenyl Phases –

13 mM ammonium acetate, pH 6.7: 13 mM AA in acetonitrile – buffer held constant



So What?

The previous example shows that there may be very different mechanisms causing retention and selectivity in HILIC systems:

Bare silica phase exhibits partition (not impacted by buffer concentration)

PFP phase exhibits IEX (highly impacted by buffer concentration)

Differing retention mechanisms give rise to alternative selectivity and retention – same as in reversed-phase

So What, What?

A fundamental understanding of underlying retention mechanisms is vital for:

- Choosing the right set of initial conditions for a given separation problem (column chemistry, mobile phase additives, aqueous/organic ratios)
- Knowing the correct knobs to turn to facilitate method development and optimization
- Placing the needed controls on the system to establish a rugged and robust method

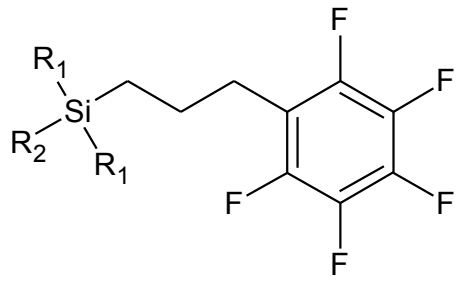
Are HILIC Phases the Same?

Much like in reversed phase, changing stationary phases in HILIC provides retention and selectivity differences

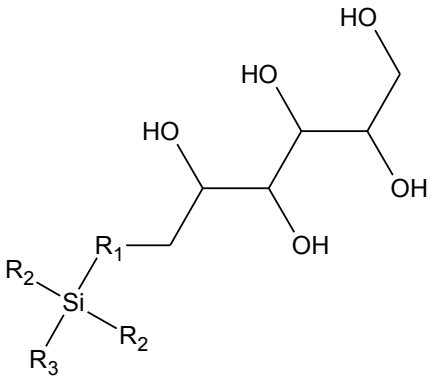
This study focuses on the interaction differences that might be expected for three different HILIC stationary phases: Ascentis Express F5 (pentafluorophenyl), Ascentis Express HILIC (bare silica) and Ascentis Express OH5 (pentahydroxy phase)

Using ephedrine as a probe molecule, retention as a function of buffer concentration was collected and interpreted.

Several related compounds were also run simultaneously and retention and selectivity noted. Interpretation of the observed results in terms of the dominant interactions prevalent using each phase is provided.



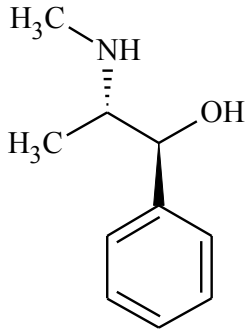
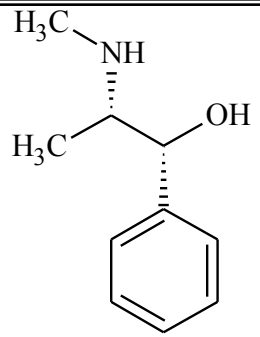
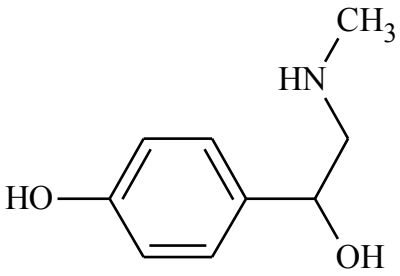
F5



OH5

Selected Probes

ACD/Labs, PhysChemProp, v. 12

Structure	pKa(MB)	LogD(8.0)	LogP	MW	name
	9.38	-0.37	1.08	165.23	pseudoephedrine
	9.38	-0.37	1.08	165.23	ephedrine
	9.37	-1.35	0.13	167.21	synephrine

Focus on Understanding the IEX Component

For any analysis where the potential for IEX exists, a good general practice is to evaluate the IEX contribution to the retention (and selectivity)

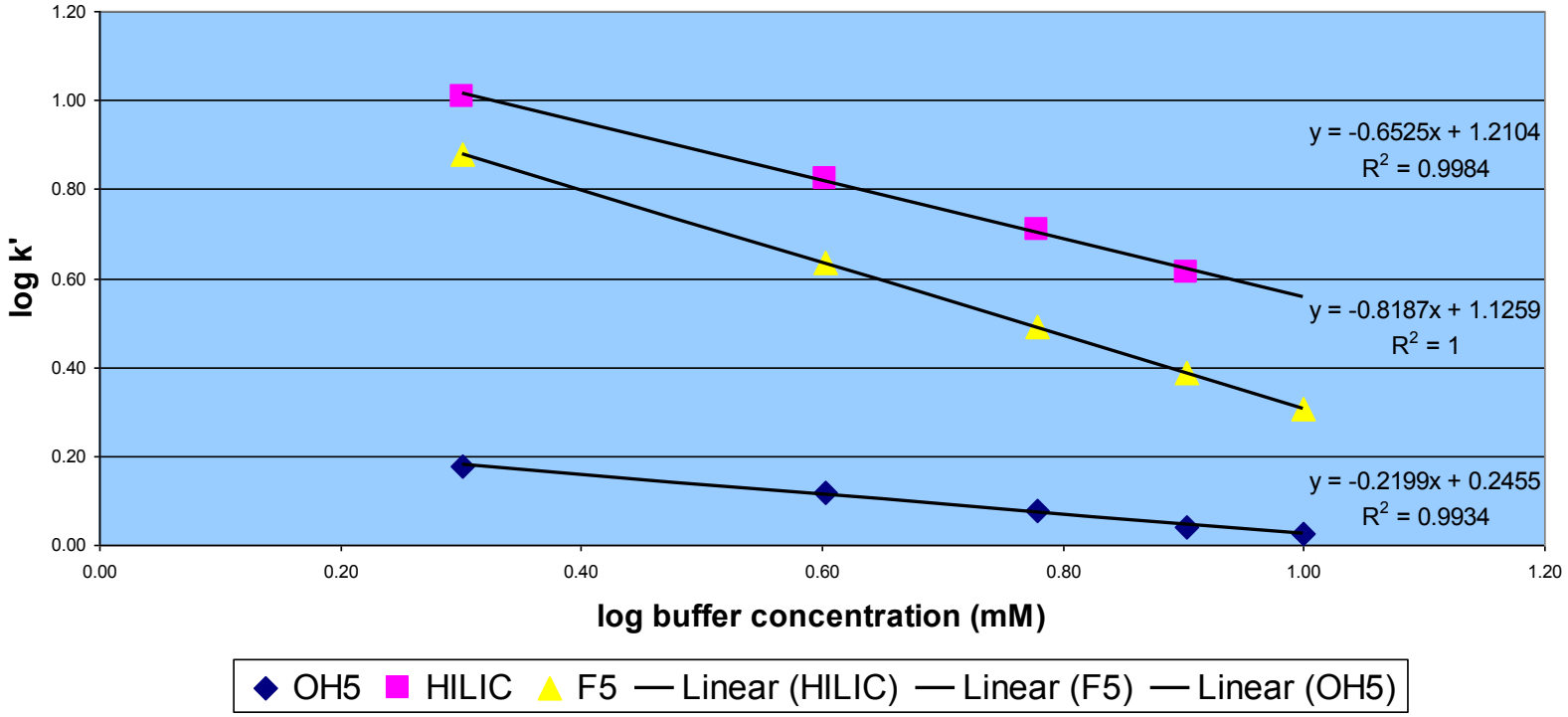
For an ion-exchange process involving singly charged analytes, the dependence of retention on mobile phase counter ion may be expressed as

$$\text{Log } k = -\log[C^+]_m + \log\beta_{IEX}$$

Where $[C^+]_m$ represents the concentration of the competing ion in the mobile phase and β_{IEX} is a constant for a given system which includes the phase ratio, ion-exchange capacity of the stationary phase and the ion-exchange equilibrium constant.

A plot of log k vs log $[C^+]_m$ will thus yield a slope of -1 when ion-exchange is solely responsible for retention, whereas the plot would yield a slope of 0 where ion-exchange is not present. The closeness of the resultant slopes to -1 is an indication of the relative dominance of IEX in the overall retention process.

Response of Ephedrine Retention on Buffer Concentration on Three HILIC Phases



Discussion

Examining observed retention in the present study:

- Keys
  - All three analytes have very similar pK_a values and thus should, excluding any outside interference, IEX the same
  - Synephrine is more polar than the ephedrine pair
  - The ephedrine pair are identical in physical properties except for their orientation in space

OH5 –

- Little IEX behavior (lack of surface interactions)
- Synephrine elutes after the ephedrine pair (partitioning)
  - Conclusion – close to pure HILIC partitioning

Discussion (continued)

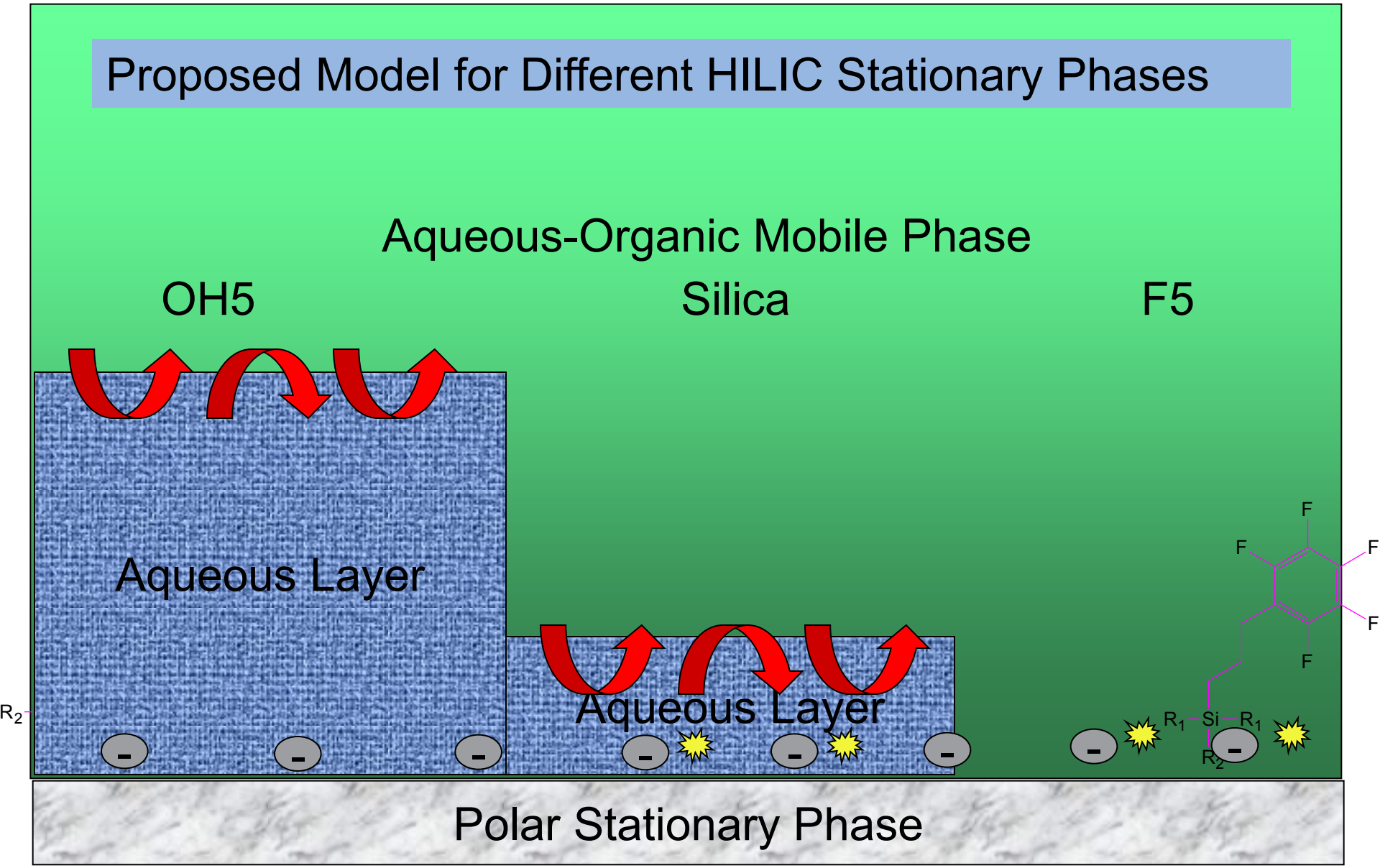
HILIC (bare silica phase)

- Mid range IEX behavior (surface interactions)
- Synephrine elutes after the ephedrine pair (partitioning)
- Greater overall retention (relative to the two other phases) – mechanistic synergy
  - Conclusion: synergistic combination of partitioning and ion-exchange

PFP (F5)

- Near total IEX behavior (surface interactions – at least long range)
- Synephrine elutes prior to ephedrine pair (lack of HILIC partitioning)
  - Conclusion: Mainly Ion-exchange

Proposed Model for Different HILIC Stationary Phases



Attributes of Common HILIC Phases

Stationary Phase	Interactions		
	Partitioning	Polar	Ionic
Bare Silica	moderate		moderate
Zwitterionic	strong		weak
Amide	strong		weak
Diol/Polyol	strong		weak
PFP	weak		strong
Cyano	weak		strong

Matching the Sample Analytes to the Right Retention Mechanisms

There are many different sets of analytes we may wish to apply HILIC to

A good start is to identify the stationary phase with the appropriate mechanisms to retain and separate them or at least eliminate those that do not:

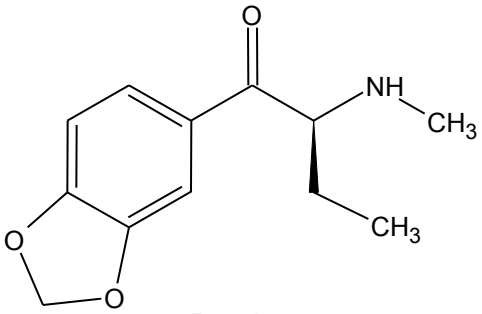
- For example – if we have a sample with polar neutral molecules, we know we will need partition as they will not interact ionically – F5 would be a bad choice, OH5 and bare silica would provide more promise

A key to utilizing any chromatographic mode is to have some idea of where the greatest differences lie with respect to the analyte physiochemical attributes and invoke the appropriate retention mechanism(s)

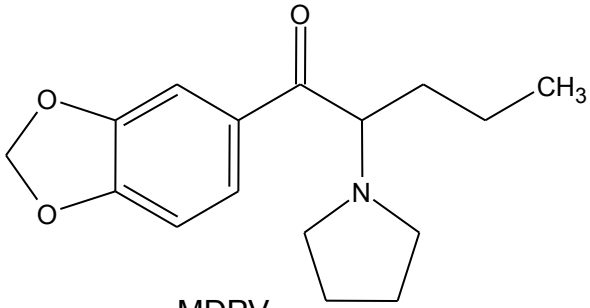
Method Development in HILIC

- Understand the analyte properties
  - Will they partition (polar?), will they ion-exchange (charged?), how do they differ most?
- Choose appropriate stationary phases based on potential interactions (conversely, eliminate those that do not make sense)
- Screen chosen stationary phases for retention and selectivity – isocratic
- Select phase that best fits the needs of the method and optimize

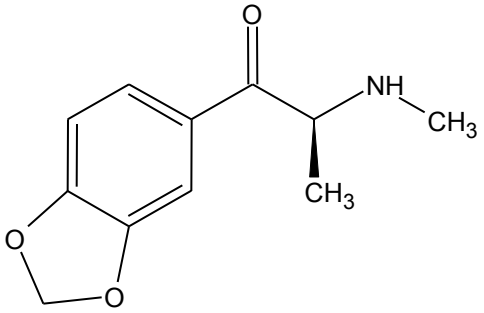
Bath Salt Analytes – Hydrophilic, Weak Bases



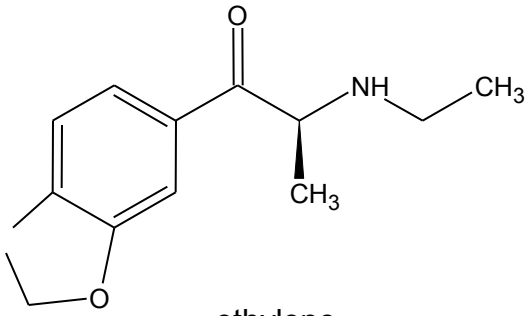
Butylone
Monoisotopic Mass = 221.105193 Da



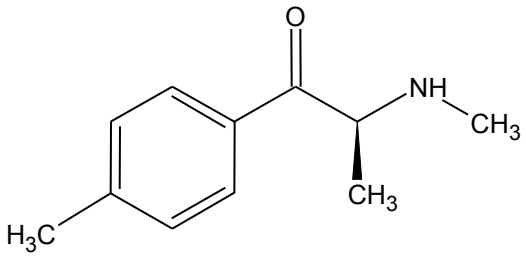
MDPV
Monoisotopic Mass = 275.152144 Da



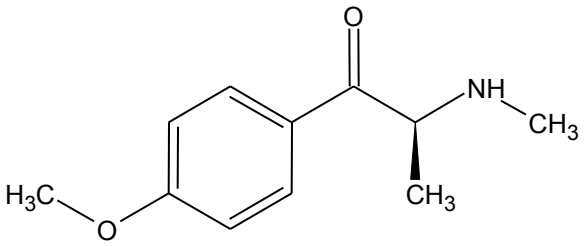
methylone
Monoisotopic Mass = 207.089543 Da



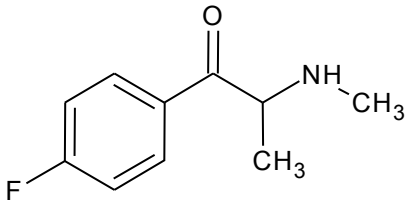
ethylone
Monoisotopic Mass = 221.105193 Da



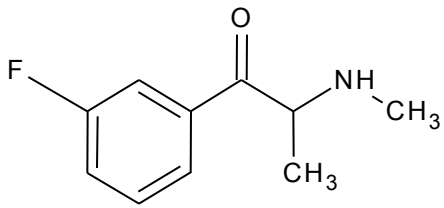
mephedrone
Monoisotopic Mass = 177.115364 Da



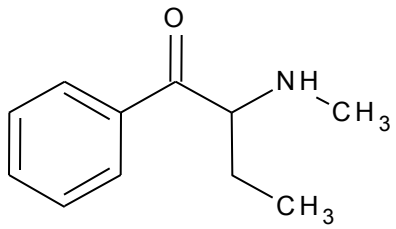
methedrone
Monoisotopic Mass = 193.110279 Da



4-fluoromethcathinone
Monoisotopic Mass = 181.090292 Da

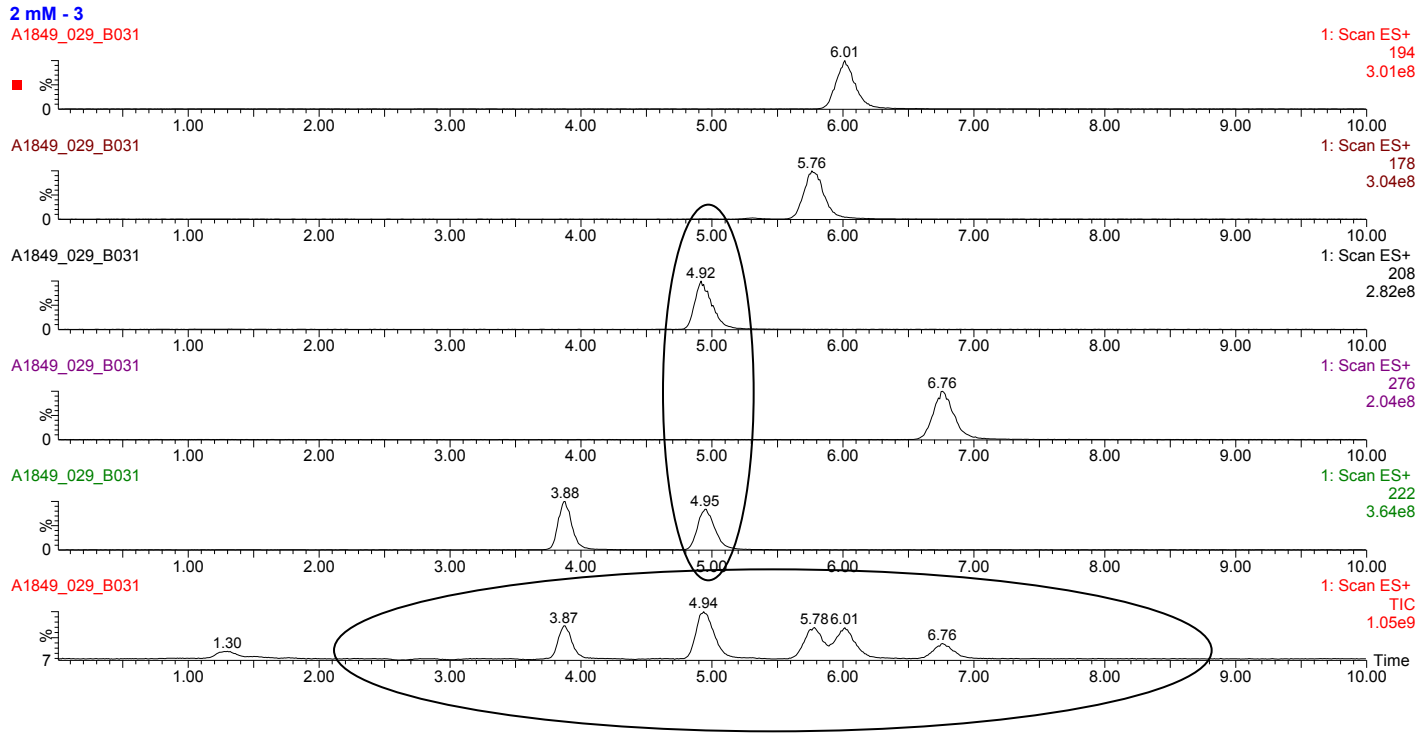


3-fluoromethcathinone
Monoisotopic Mass = 181.090292 Da



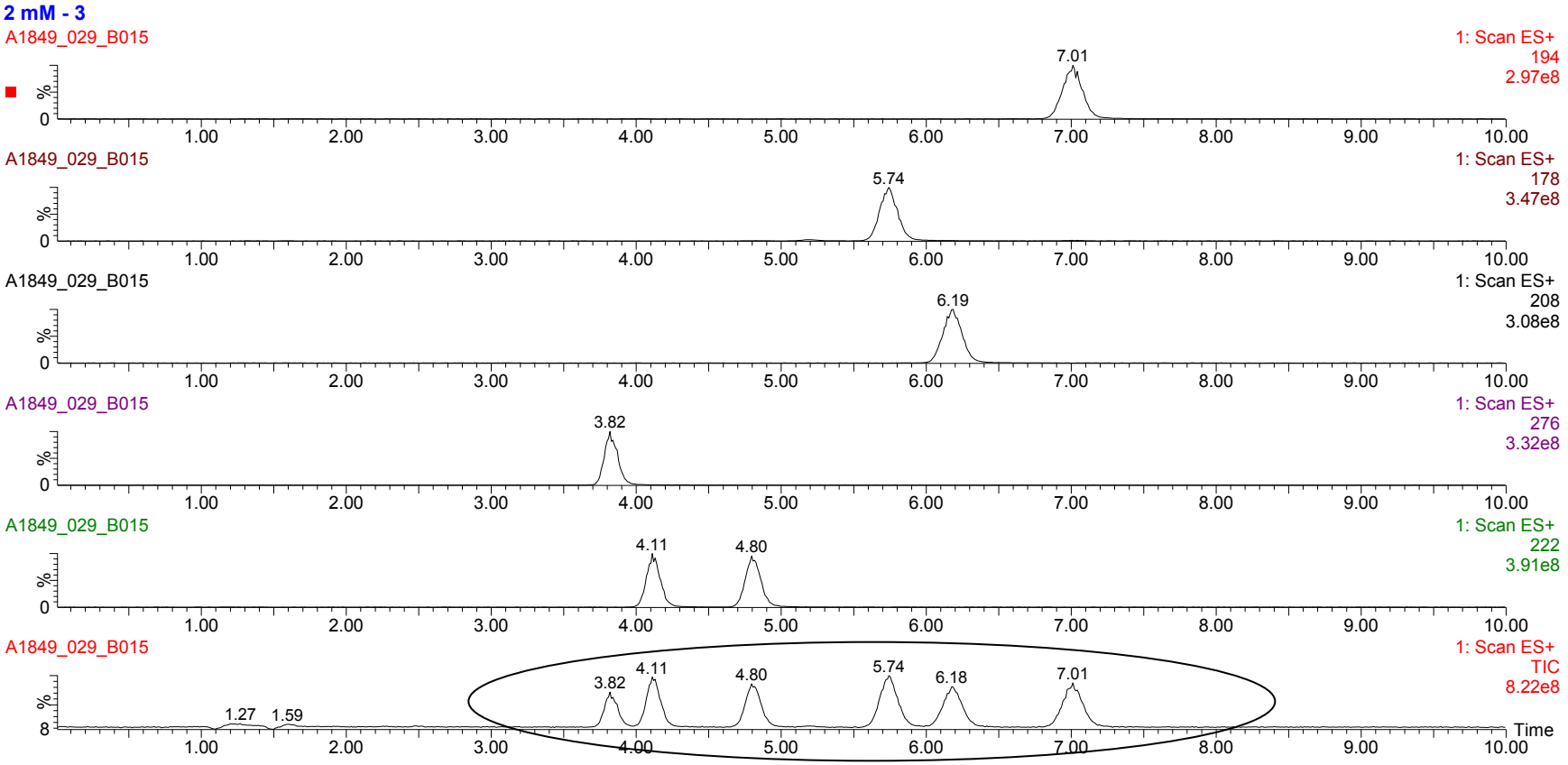
Buphedrone

Ascentis Express F5, 2 mM Ammonium Acetate in 90% Acetonitrile



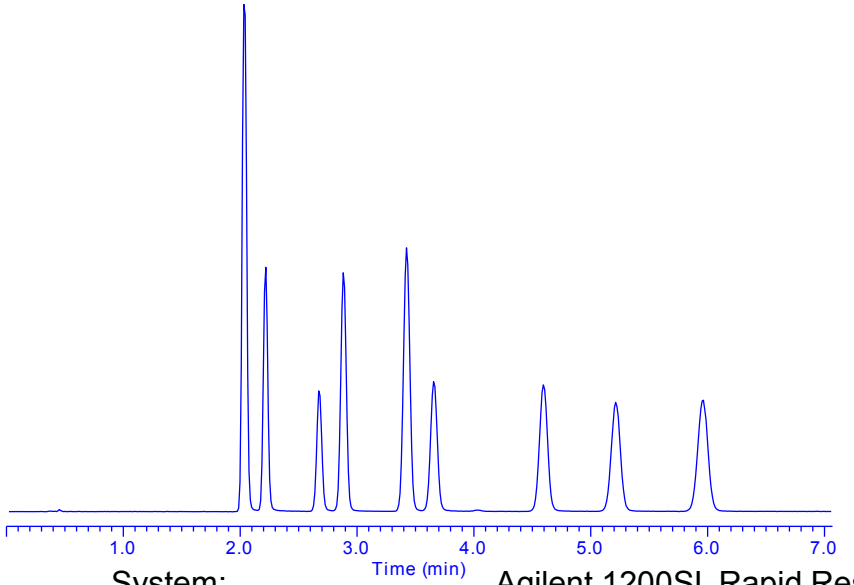
IEX results in some coelution in this case

Ascentis Express HILIC, 2 mM Ammonium Acetate in 90% Acetonitrile



IEX + partition provides the needed selectivity in this case

Optimized Separation of Bath Salts on Ascentis Express HILIC



Analyte	rt
MDPV	2.0
Buphedrone	2.2
3-fluoromethcathinone	2.7
butylone	2.9
ethylone	3.4
3-fluoromethcathinone	3.7
mephedrone	4.6
methylone	5.2
methedrone	6.0

System:	Agilent 1200SL Rapid Resolution, 6210 TOF
Column:	Ascentis Express HILIC 10cm X 2.1mm, 2.7um (53939-U)
Mobile Phase:	5mM ammonium formate (98:2 acetonitrile:water)
Flow:	0.6mL/min
Temperature:	35 ºC
System Pressure:	127bar
Injection Vol:	1uL
MS Detection:	ESI+, 100-1000m/z ,
Sample:	200ng/mL Bath Salts in acetonitrile

Craig R. Aurand, Robert Shirey, Leonard Sidisky Janusz Pawliszyn, and Yong Chen, Application of Bio-SPME for the Enrichment of Illicit Phenethylamine and Cathinone Compounds from Biological Samples, Poster, ASMS national Meeting, Vancouver, BC, 2012

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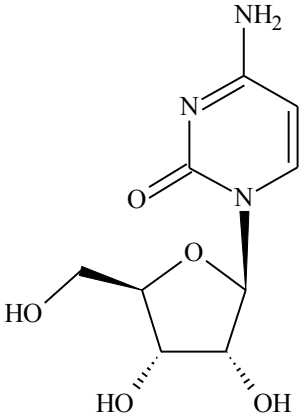
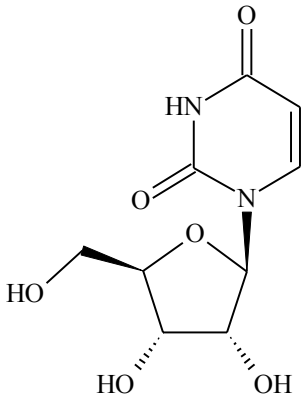
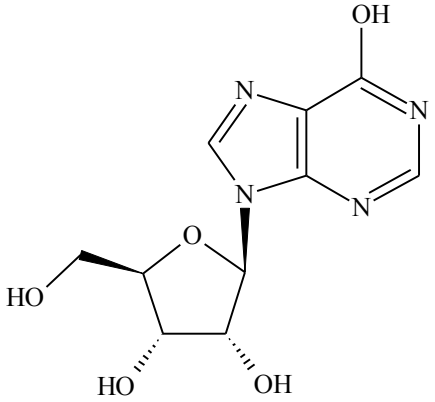
Nucleoside Structures – Polar Neutral (for the most part)



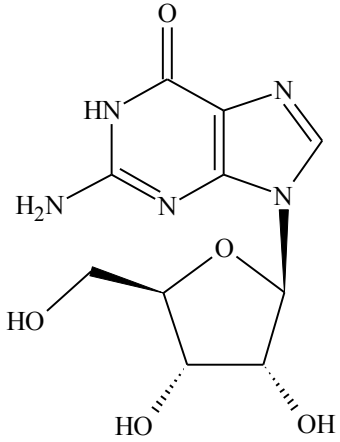
Cytidine



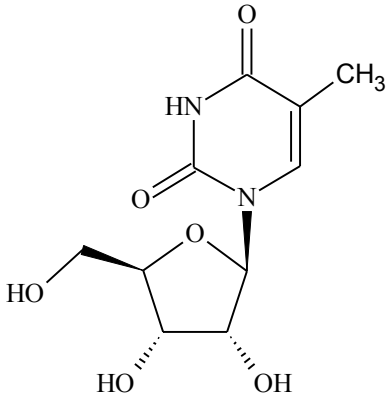
Uridine



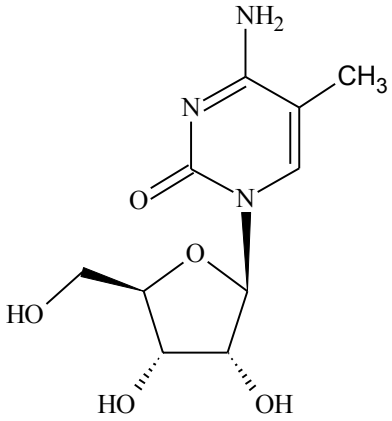
Inosine



Guanosine

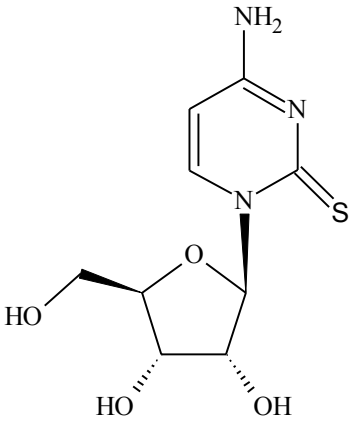


Ribothymidine
5-Methyluridine

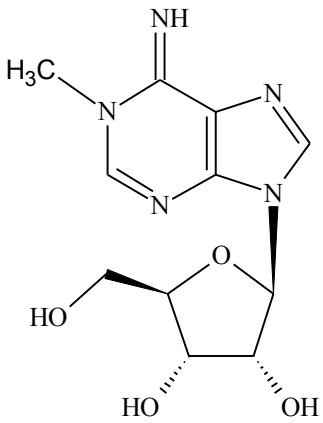


5-Methylcytidine

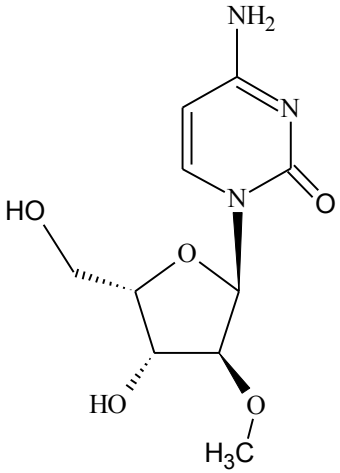
Structures



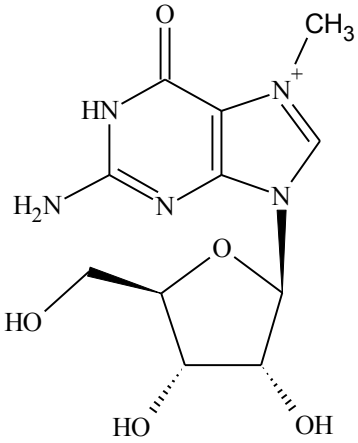
2-Thiocytidine



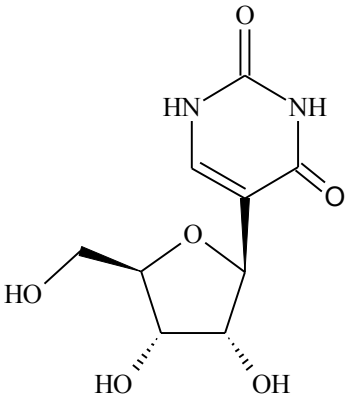
1-Methyladenosine



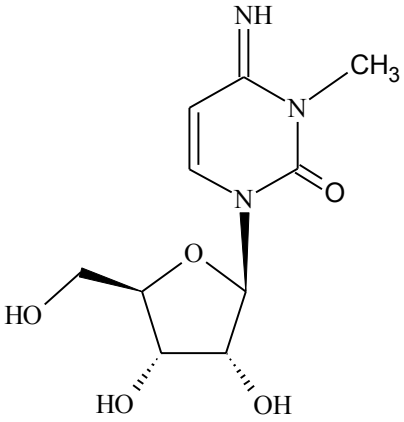
2'-O-Methylcytidine

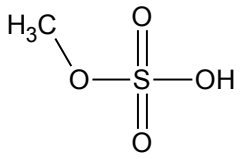


7-Methylguanosine



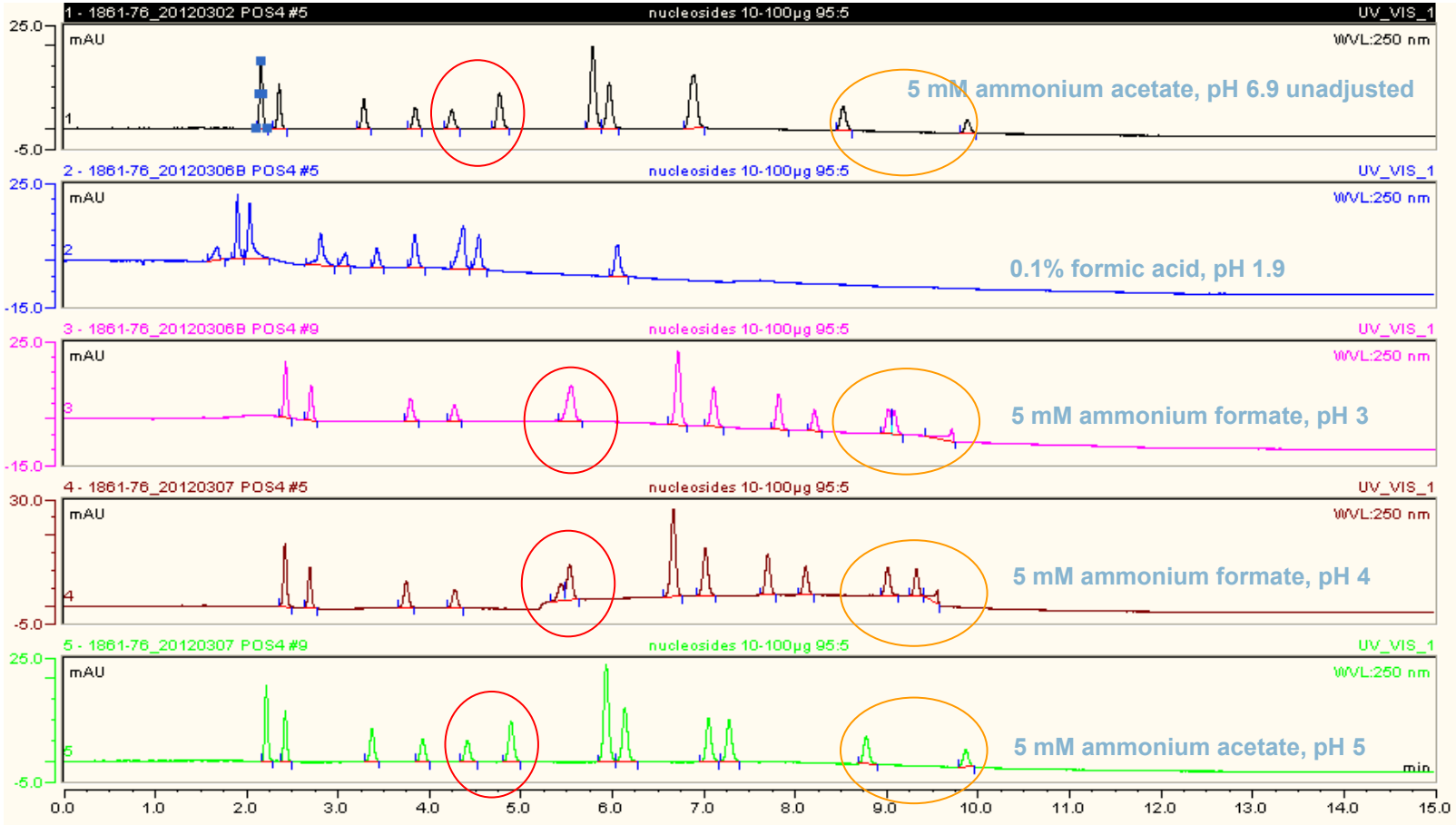
Pseudouridine





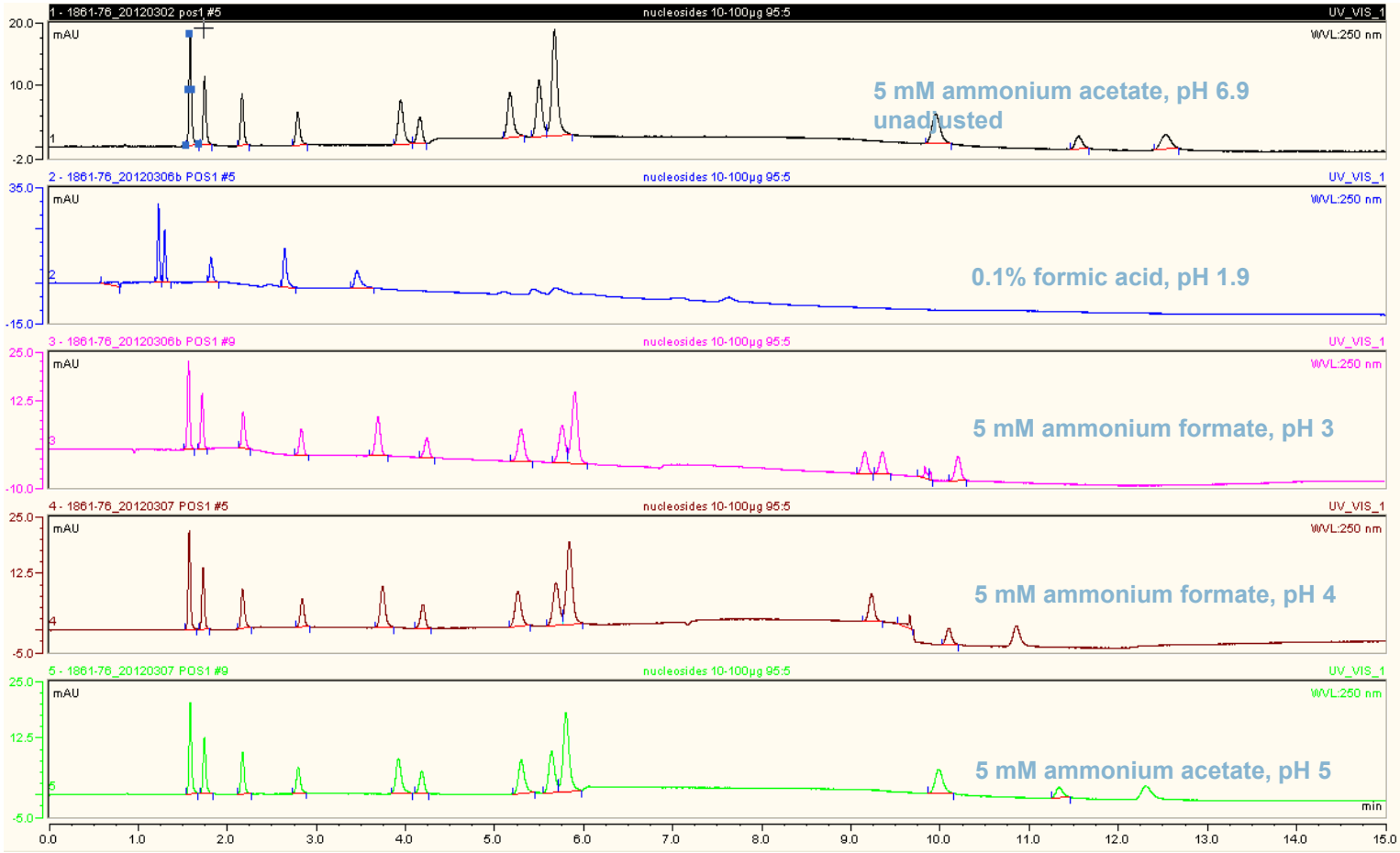
3-Methylcytidine metosulfate

Ascentis Express OH5



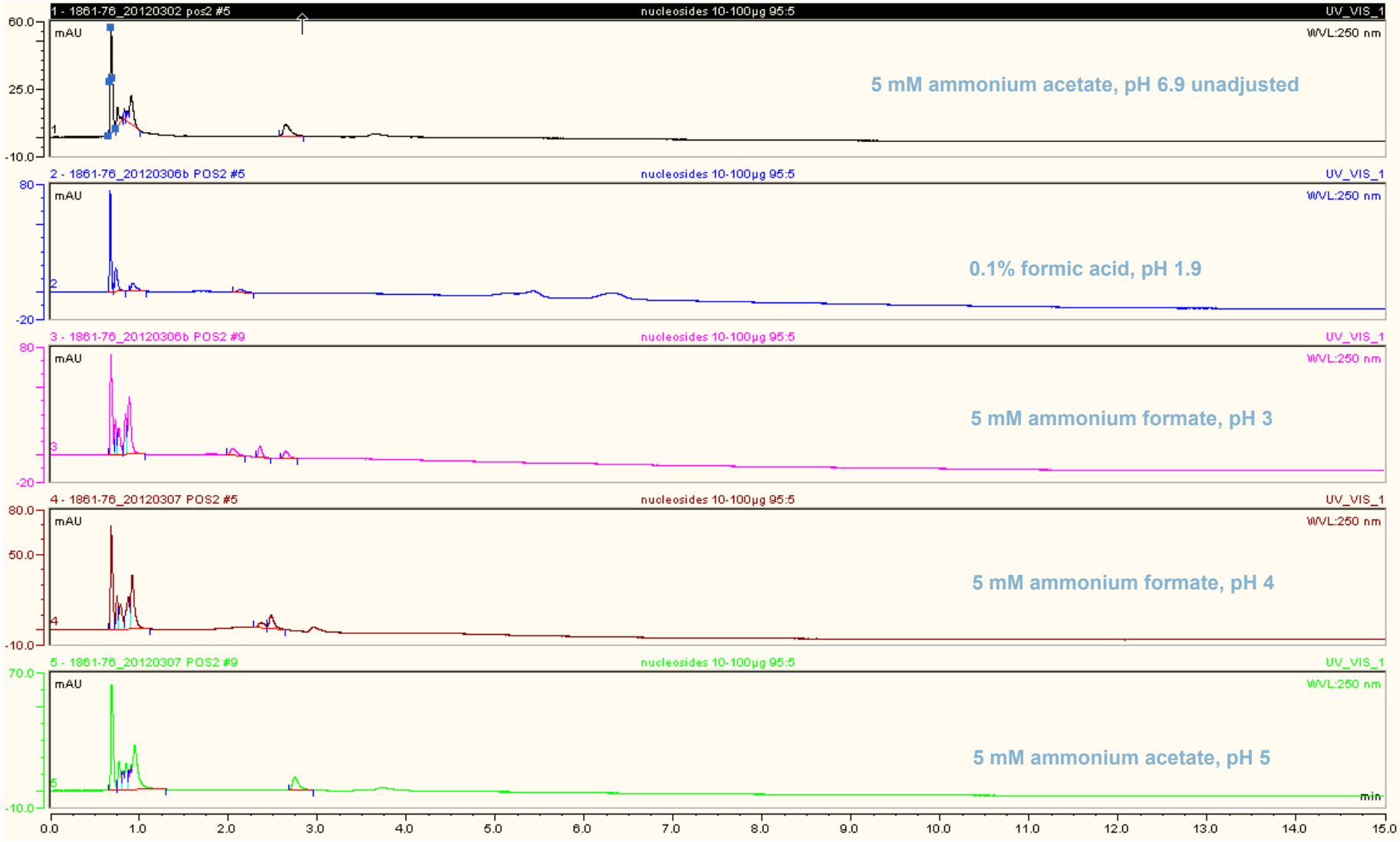
Good retention and selectivity – reasonably little change as a function of buffer pH

Ascentis Express HILIC

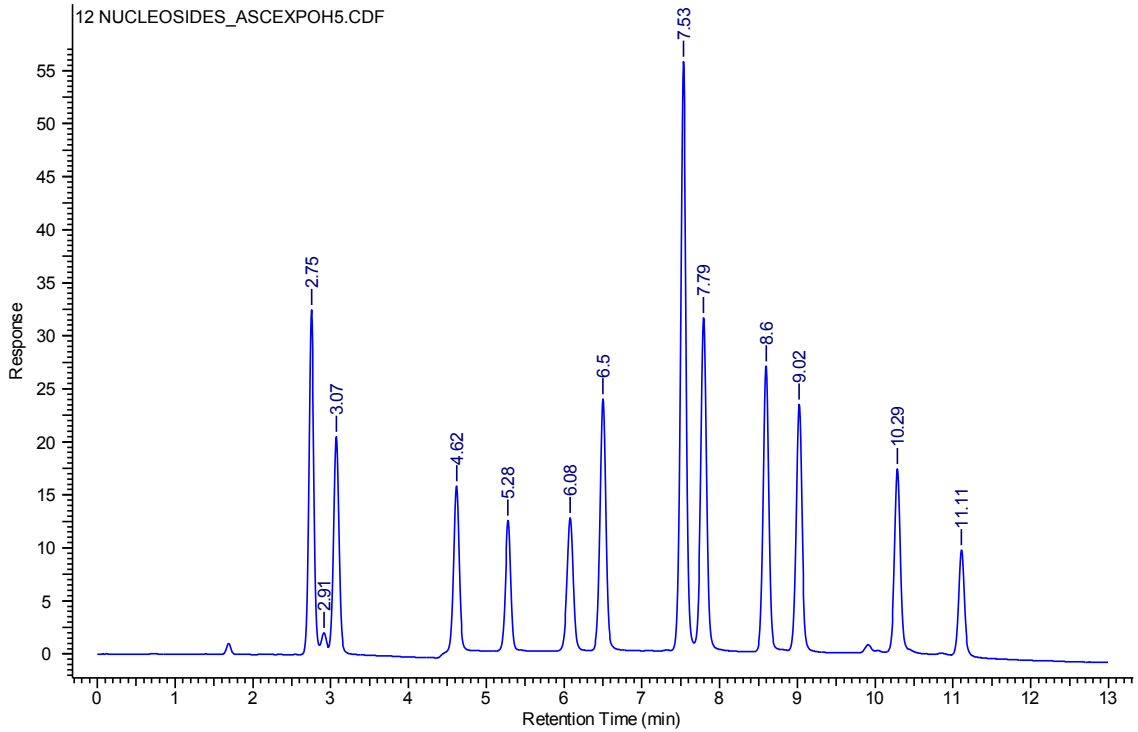


Good retention and selectivity – group of more basic analytes preferentially retained that are sensitive to changes in buffer pH

Ascentis Express F5

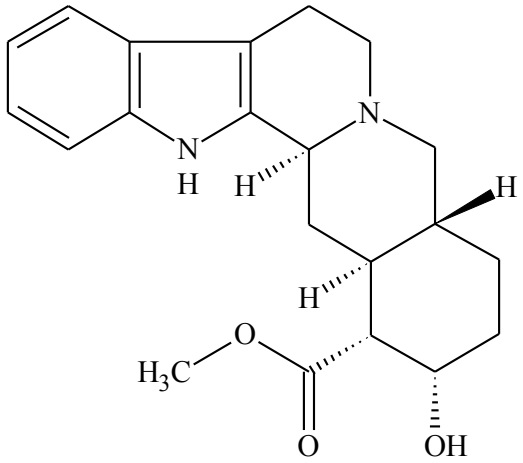


Optimized Separation of Nucleosides on Ascentis Express OH5

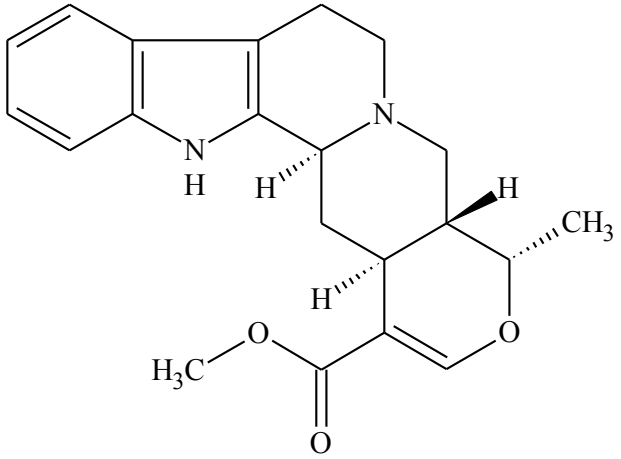


column: Ascentis Express OH5, 10 cm x 2.1 mm, 2.7 µm (53757-U)
mobile phase: (A) 5 mM ammonium acetate, pH 5.0 with acetic acid in 95:5, acetonitrile:water; (B) 5 mM ammonium acetate, pH 5.0 with acetic acid in 80:20, acetonitrile:water
gradient: 0% B held for 1 min; to 100% B in 10 min; held at 100% B for 1 min
flow rate: 0.3 mL/min
column temp.: 25 ° C
detector: UV at 250 nm
injection: 2 µL
sample: 10 - 100 µg/mL

Structures of Yohimbine and Ajmalicine Alkaloids



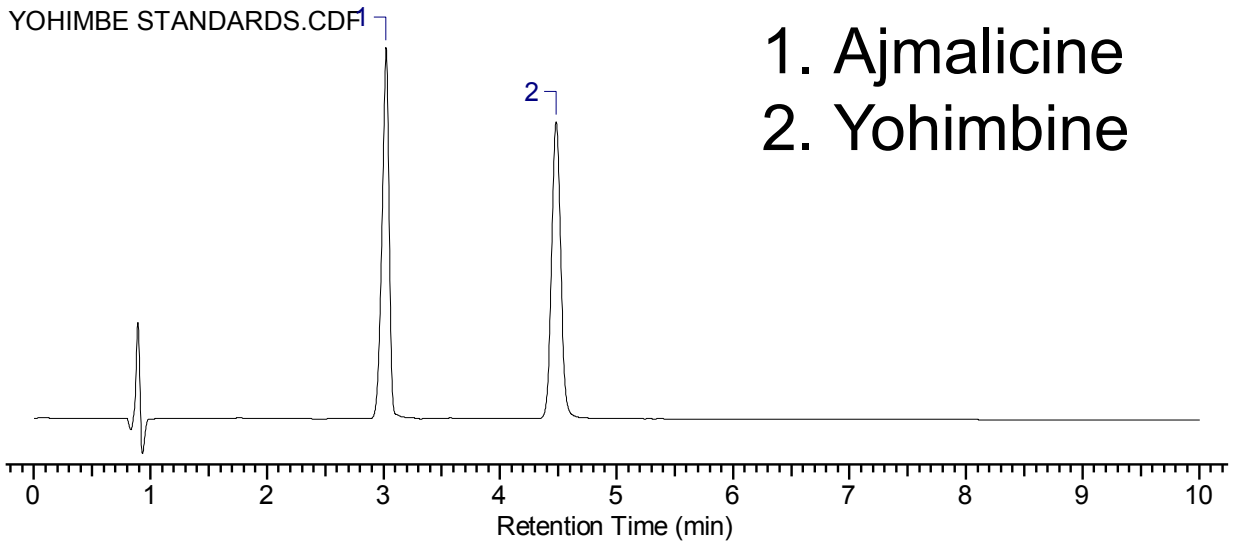
Yohimbine



Ajmalicine

Relatively nonpolar, basic analytes

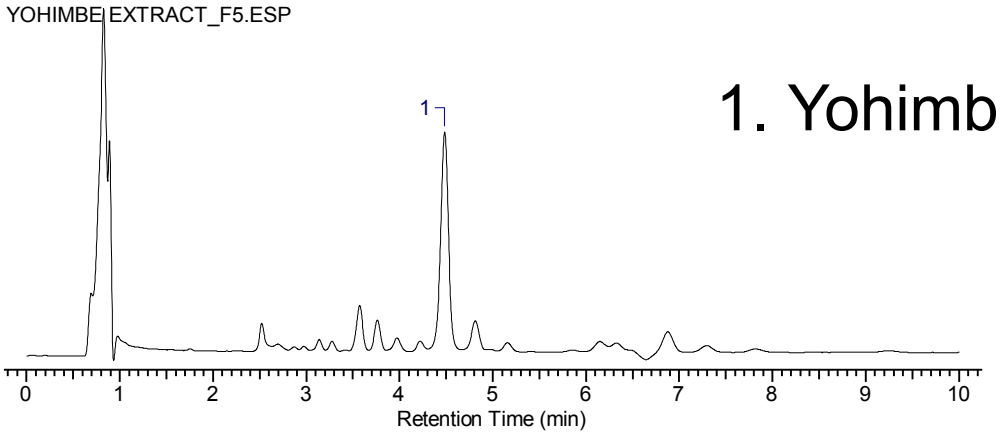
Separation of Yohimbe Alkaloid Standards on Ascentis Express F5



1. Ajmalicine
2. Yohimbine

Conditions:
Column: Ascentis Express F5, 10 cm x 3.0 mm, 2.7 µm (53766-U)
Mobile Phase: 2 mM ammonium acetate in 90% acetonitrile, pH adjusted to 6.0 with acetic acid (post addition of organic)
Flow Rate: 0.5 mL/min
Temperature : 35° C
Detection: UV. 275 nm
Injection: 2 µL

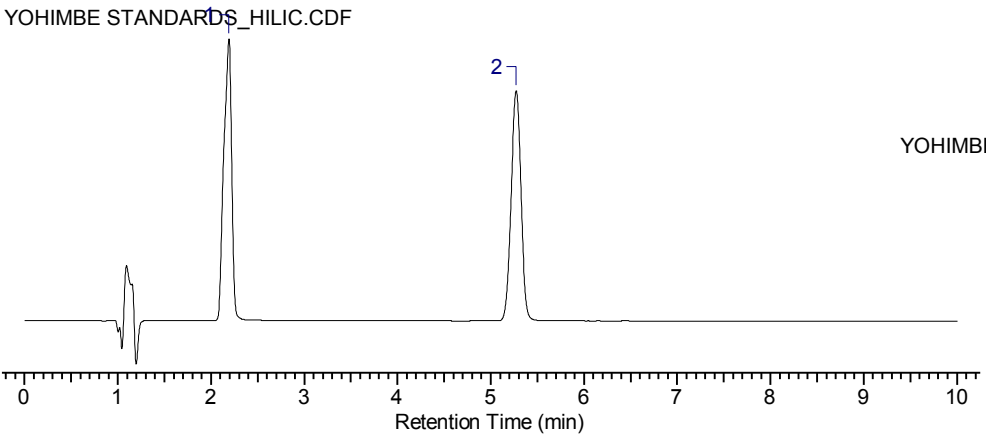
HILIC Analysis of Yohimbe Extract on Ascentis Express F5

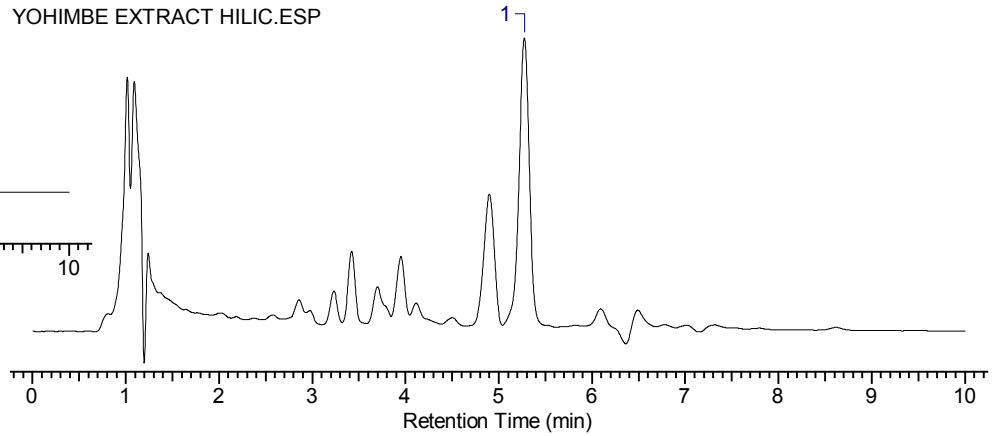


1. Yohimbine

Conditions:
Column: Ascentis Express F5, 10 cm x 3.0 mm, 2.7 µm (53766-U)
Mobile Phase: 2 mM ammonium acetate in 90% acetonitrile, pH adjusted to 6.0 with acetic acid (post addition of organic)
Flow Rate: 0.5 mL/min
Temperature : 35° C
Detection: UV. 275 nm
Injection: 2 µL

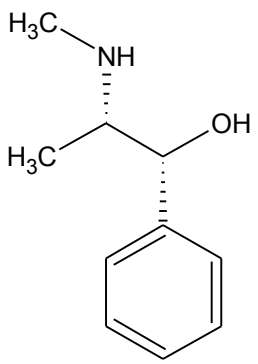
HILIC Analysis of Yohimbe Alkaloids on Ascentis Express HILIC



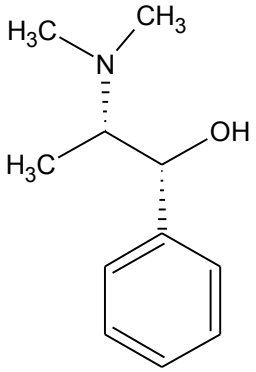


Conditions:
Column: Ascentis Express HILIC, 10 cm x 3.0 mm, 2.7 µm (53970-U)
Mobile Phase: 2 mM ammonium acetate in 90% acetonitrile, pH adjusted to 6.0 with acetic acid (post addition of organic)
Flow Rate: 0.5 mL/min
Temperature : 35° C
Detection: UV. 275 nm
Injection: 2 µL

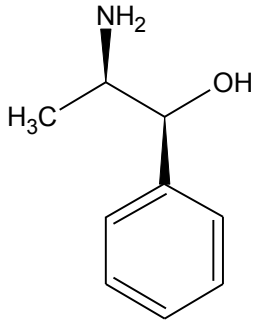
Ephedrine Alkaloids – Polar, Basic Compounds



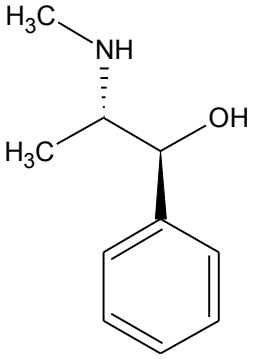
ephedrine



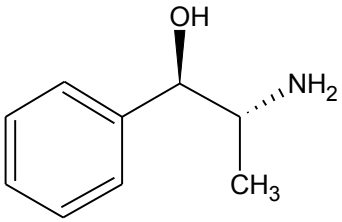
methylephedrine



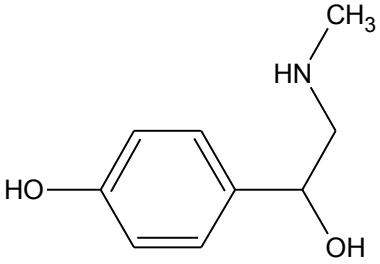
norephedrine



pseudoephedrine



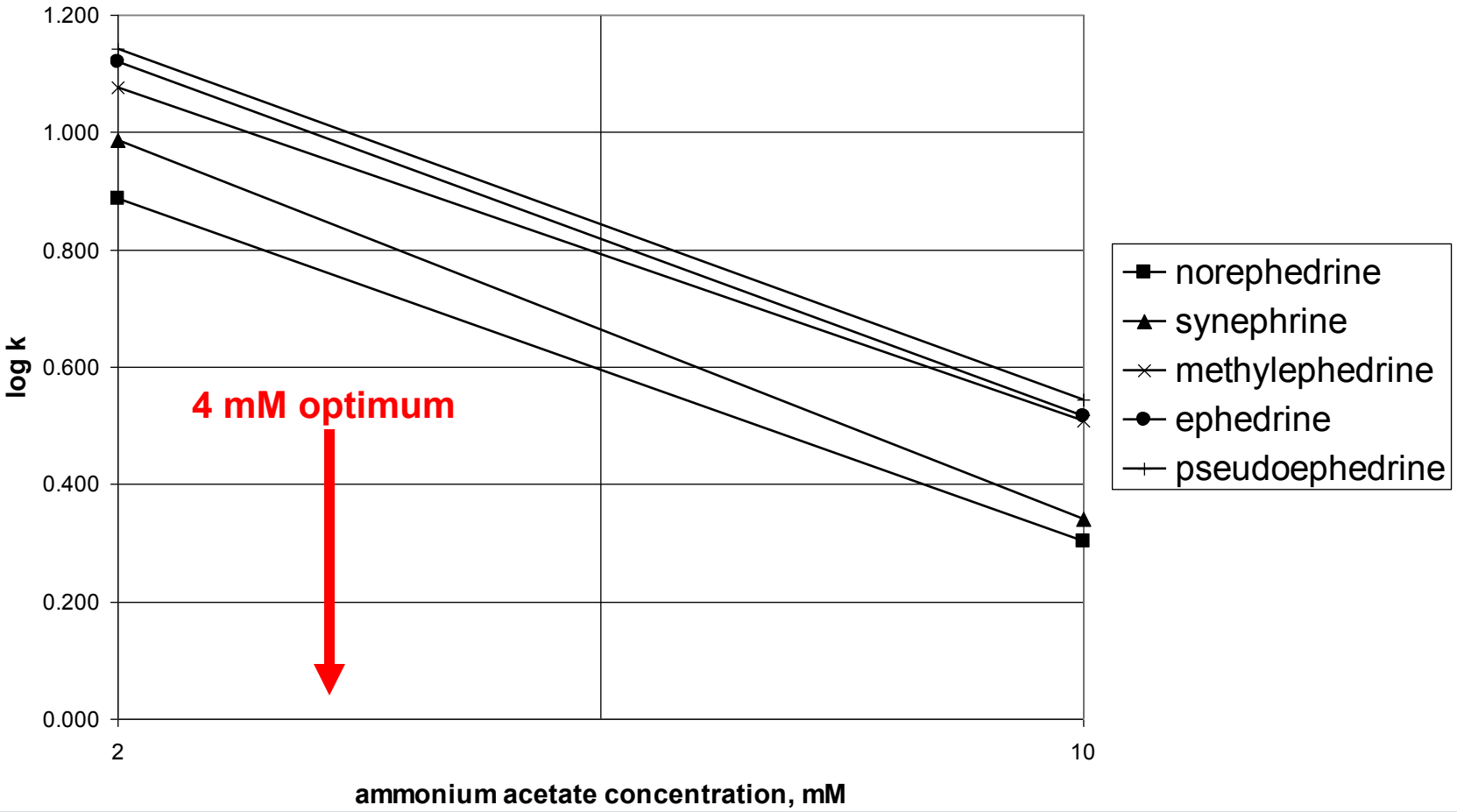
norpseudoephedrine



synephrine

Bell, D. S., H. M. Cramer, et al. (2005). "Rational method development strategies on a fluorinated liquid chromatography stationary phase: Mobile phase ion concentration and temperature effects on the separation of ephedrine alkaloids." Journal of Chromatography A **1095**(1-2): 113-118.

Optimization of Mobile Phase Ion Concentration – Linear Dependence



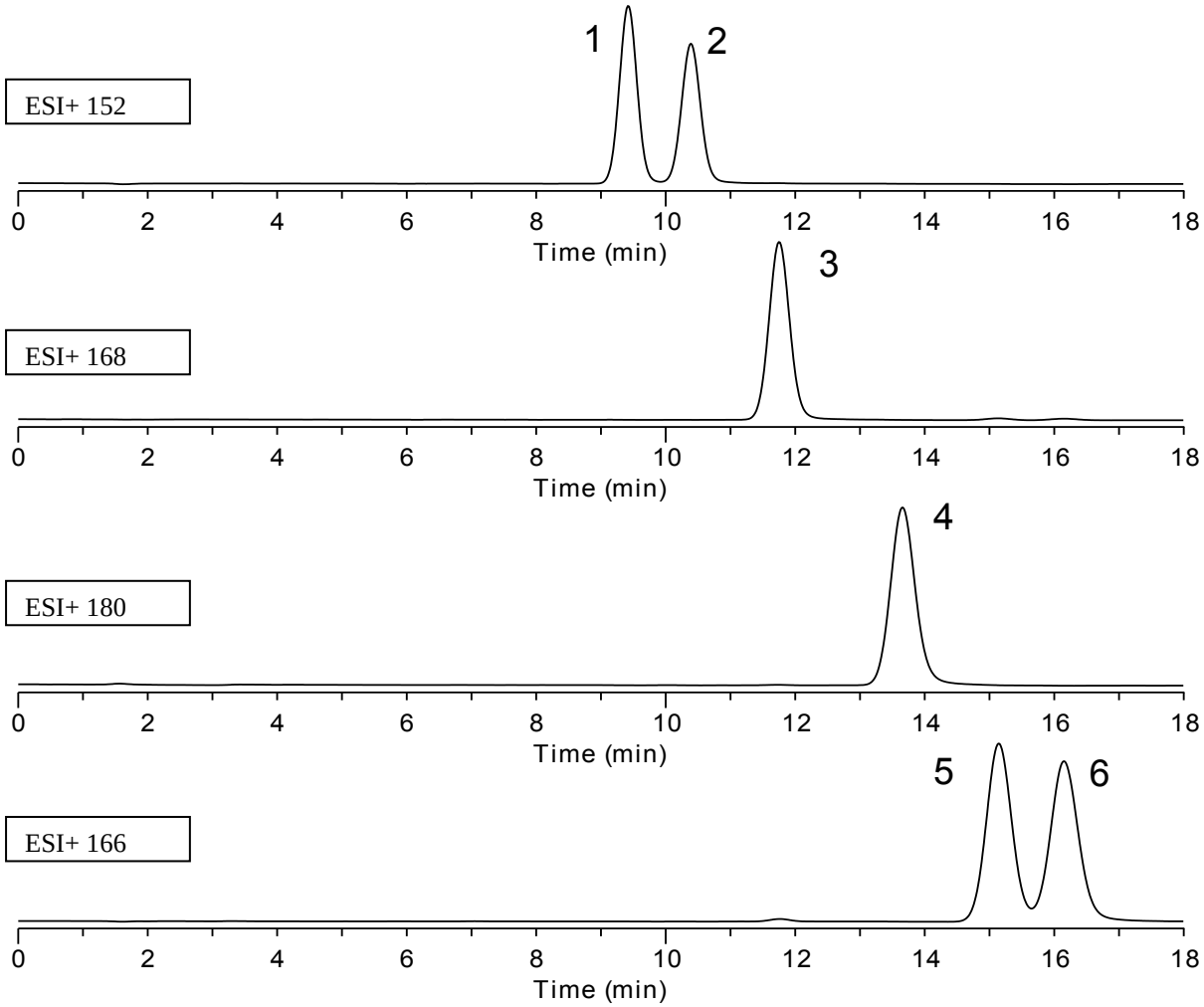
Experimental

Retention data was first acquired at 2 mM and 10 mM ammonium acetate in 90% acetonitrile

Acquisitions were performed on a Shimadzu 10A VP HPLC system

Column:	Discovery HS F5 (PFP), 15 cm x 4.6 mm, 5 µm
Mobile Phase:	2 or 10 mM ammonium acetate in 90:10, CH_3CN:water
Flow Rate:	1 mL/min
Temp.:	35° C
Det.:	UV at 215 nm
Inj.:	10 µL
Sample:	mix of ephedrine alkaloids + synephrine at 100 µg/mL each in 2 mM ammonium acetate mobile phase

LC-MS Analysis of Ephedrine Alkaloids



1. norephedrine
2. norpseudoephedrine
3. synephrine
4. methylephedrine
5. ephedrine
6. pseudoephedrine

Understanding dominant interactions greatly facilitates method development

Notes on Method Development

These few applications highlight the need to:

1) choose a column with the interactions needed for retention/selectivity based on analyte properties/method goals

2) manipulate retention and selectivity using the strongest variables first – then hone in with remaining variables

   if IEX dominant – buffer concentration/pH

   if partition – aqueous/organic composition

The result is facile method development of robust and rugged methods

Method Development in HILIC - Tips

Sample consideration

- HILIC is generally restricted to highly polar and/or ionic analytes. Low octanol-water coefficients (Log P generally less than 1) and ionizable bases are considered good candidates for this mode of chromatography.
- Make sure samples are prepared in weak HILIC solvents (high organic, low salt concentrations)

Mobile phase conditions

- A good general starting mobile phase for HILIC is 2-10 mM ammonium acetate or formate in 90% acetonitrile leaving pH unadjusted (~pH7 where both strong bases and surface silanols are ionized)
- If the analytes are nonionic or IEX is not desirable, a starting point could be 10 mM ammonium formate, pH 3.0, in 90-95% acetonitrile.
- Choice of formate or acetate buffer informed primarily by volatility and MS compatibility, and solubility at high levels of organic
- Run isocratically if possible, if gradients are necessary, change one variable only (ie organic or buffer, not both)

Method Development in HILIC - Tips

System Variables:

It may be difficult to predict the operative retention mechanisms in HILIC; however, it is imperative to gain some understanding of the mechanisms in order to facilitate the development and ensure rugged and robust methods result.

A quick buffer concentration study like the one discussed in this seminar will provide good insight into the relative dominance of IEX

If IEX is present, buffer concentration, pH and temperature ***must*** be controlled variables

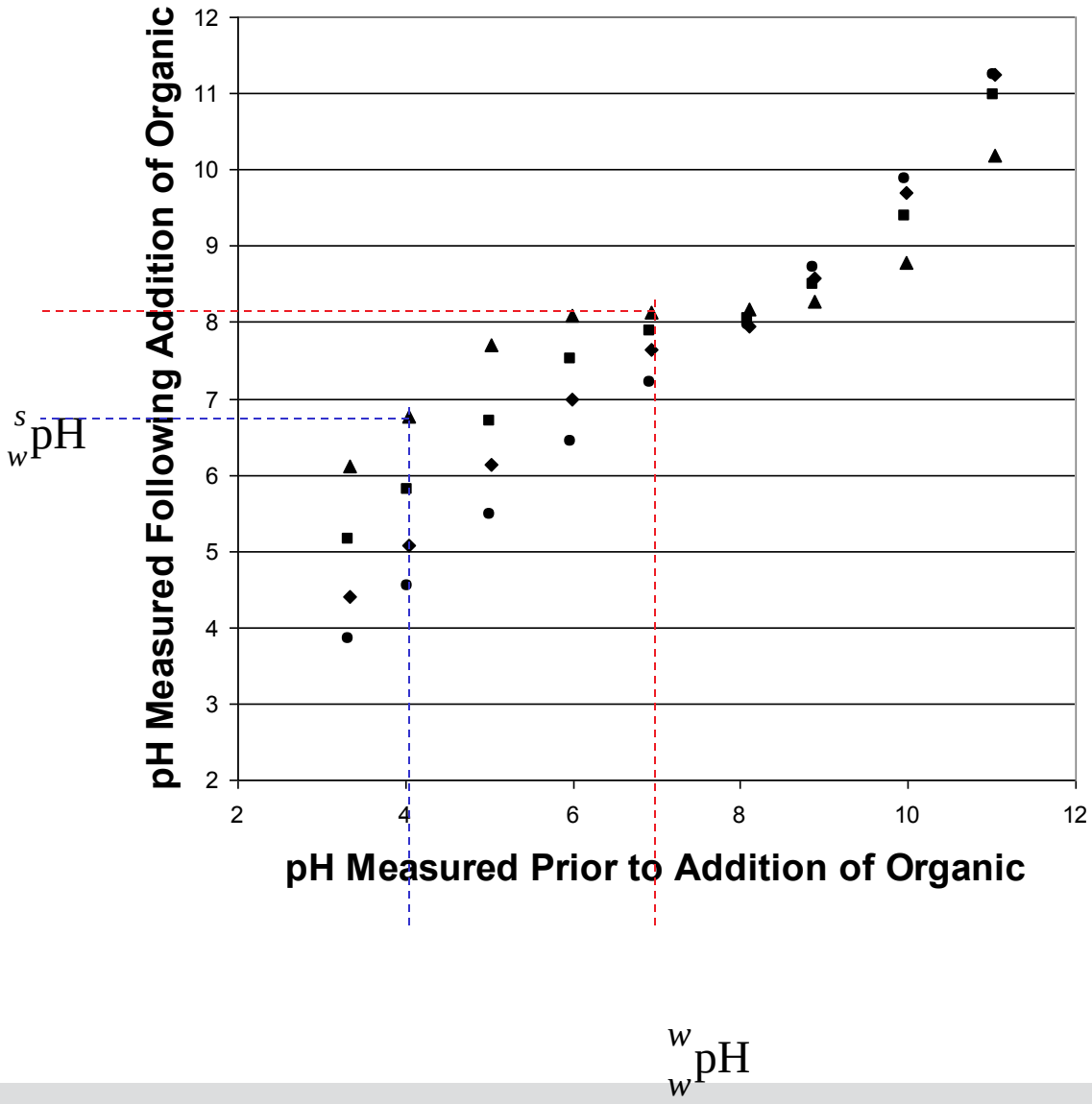
Make sure instrument wash solvents are compatible with HILIC mobile phases, especially if used in certain injection modes

Determination of Dominant Interactions

pH adjustment

- Be aware that pH can modify the ionization of both the analyte and the chromatographic surface
- Modern surface silanols exhibit a range of pK_a values – finding the right pH is often a balance between analyte and surface degrees of ionization
- Buffer concentrations need to be as exact as possible
- Best to measure and state the pH after the addition of organic

Effect of Acetonitrile on pH of Ammonium Acetate



The pH of the aqueous ammonium hydroxide solution was adjusted with acetic acid prior to the addition of acetonitrile ($^{w}_{w}\mathrm{pH}$). Subsequent pH measurement was taken following the addition of acetonitrile ($^{s}_{w}\mathrm{pH}$). Each measurement utilized a glass electrode filled with saturated KCl calibrated using pH 4, pH 7 and pH 10 NIST standardized aqueous reference. Measurements were taken at 25ºC.

Triangle: 90.0% ACN,
Square: 75% ACN,
Diamond: 50% ACN,
Circle: 32.5% ACN

Determination of pK_a Values using NMR

Amitriptyline

% Acetonitrile	$^{s}_{w}pK_a$	Correlation (R^2)
25	9.32	0.9997
50	9.02	0.9996
75	8.88	0.9956
90	8.34	0.9923

- pK_a values for bases decrease with increasing acetonitrile
- At 90% each analyte exhibited a pK_a value about 1 full pH unit less than the literature pK_a value

Analyte	Literature pK_a	$^{s}_{w}pK_a$	Correlation (R^2)
Amitriptyline	9.4	8.34	0.9923
Nortriptyline	9.7	8.92	0.9920
Diphenhydramine	9.0	8.33	0.9978
Verapamil	8.9	7.98	0.9976
Alprenolol	9.7	8.73	0.9855

Impact of pH and pK_a Variation on Ion-Exchange

In order for an ion-exchange interaction to take place, both the analyte and the stationary phase must posses opposite charges

Taking only the aqueous-based values:

- Analyte pK_a = 8.0
- Mobile phase pH = 7

The degree of analyte ionization would be:

- $10^{(pKa - pH)} / (1 + 10^{(pKa-pH)}) = 0.90$ or 90% ionized

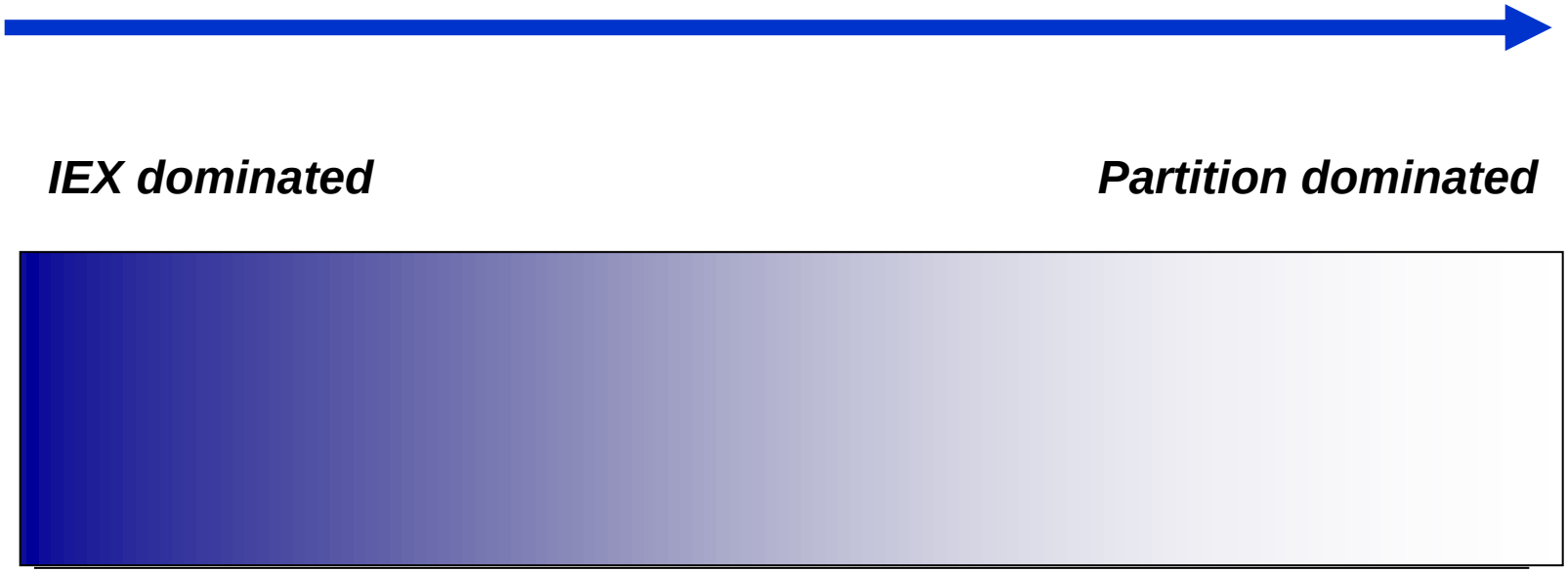
In 90% acetonitrile, however:

- Analyte pK_a ~ 7.0
- Mobile phase pH ~ 8

The degree of analyte ionization would be only about 10% and thus much less apt to interact via ion-exchange

It is thus extremely important to take the variation of both pH and pK_a into account when developing methods

Balancing HILIC and IEX



IEX dominated ***Partition dominated***

Low ionic concentration ***High ionic concentration***

Fluorinated/Cyano phases ***Bare silica*** ***OH5 phase/Amide Zwiterionic***

Summary

Developed some models to describe what is happening in HILIC

- Take home message: if you have basic analytes in your system, expect IEX mechanisms to be prevalent –
- Employing and controlling IEX is different than partitioning – need to be prepared to do both

Showed that different phases offer different degrees of partition and IEX

- OH5/Amide/Zwitterionic – mainly partition
- Bare Silica – partition and IEX
- F5/Cyano – IEX

Demonstrated through application how one can choose and omit column chemistries based on analyte knowledge

- If analytes are neutral (or acidic), partition will need to be invoked
- If all analytes are bases, IEX and/or partition may be most suitable

Finally provided a few tips on method development and system parameters to consider

Acknowledgements

Hillel Brandes
Xiaoning Lu
Hugh Cramer
Craig Aurand
Errol Fernandes

Carmen Santasania
Gaurang Parmar
Wayne Way

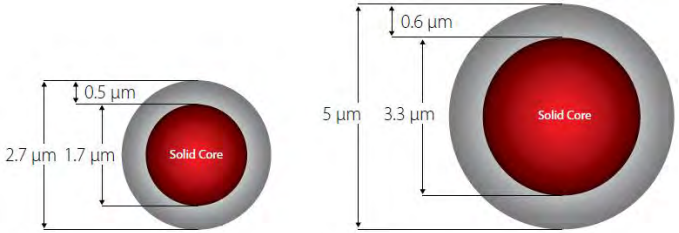
Separation Science

Q&A

Follow up questions on HILIC? Contact: Dave.Bell@sial.com

Ascentis Express HPLC Columns are available in 2 particle sizes and multiple phases. For product information, visit: sigmaaldrich.com/express

2.7 µm
9 Phases



5 µm
7 Phases