Predicting HPLC Selectivity in Ternary Reversed-Phase Solvent Systems

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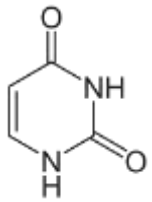
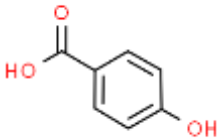
Analytical Solutions to Analytical Problems

Case Study: We Have a Mixture To Separate!

	Code	Name	Log P
1	Ur	**Uracil (Void Time Marker)**	-1.1
2	HBA	**4-Hydroxybenzoic Acid**	1.6
3	HIPhA	**4-Hydroxyisophthalic Acid**	1.5
4	PH	**Phenol**	1.5
5	SA	**Salicylic Acid**	2.3
6	DMP	**Dimethyl phthalate**	1.6
7	NB	**Nitrobenzene**	2.0
8	Ani	**Anisole**	2.1



1 (t_M)



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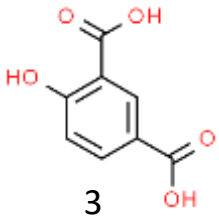

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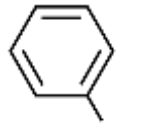

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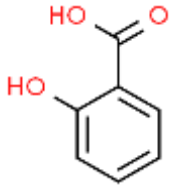

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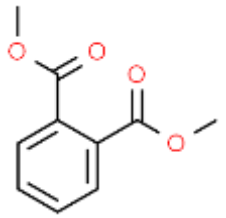

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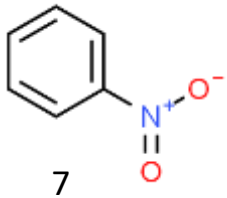

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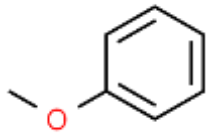

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Adjust mobile phase to acidic pH to suppress ionization of acids.

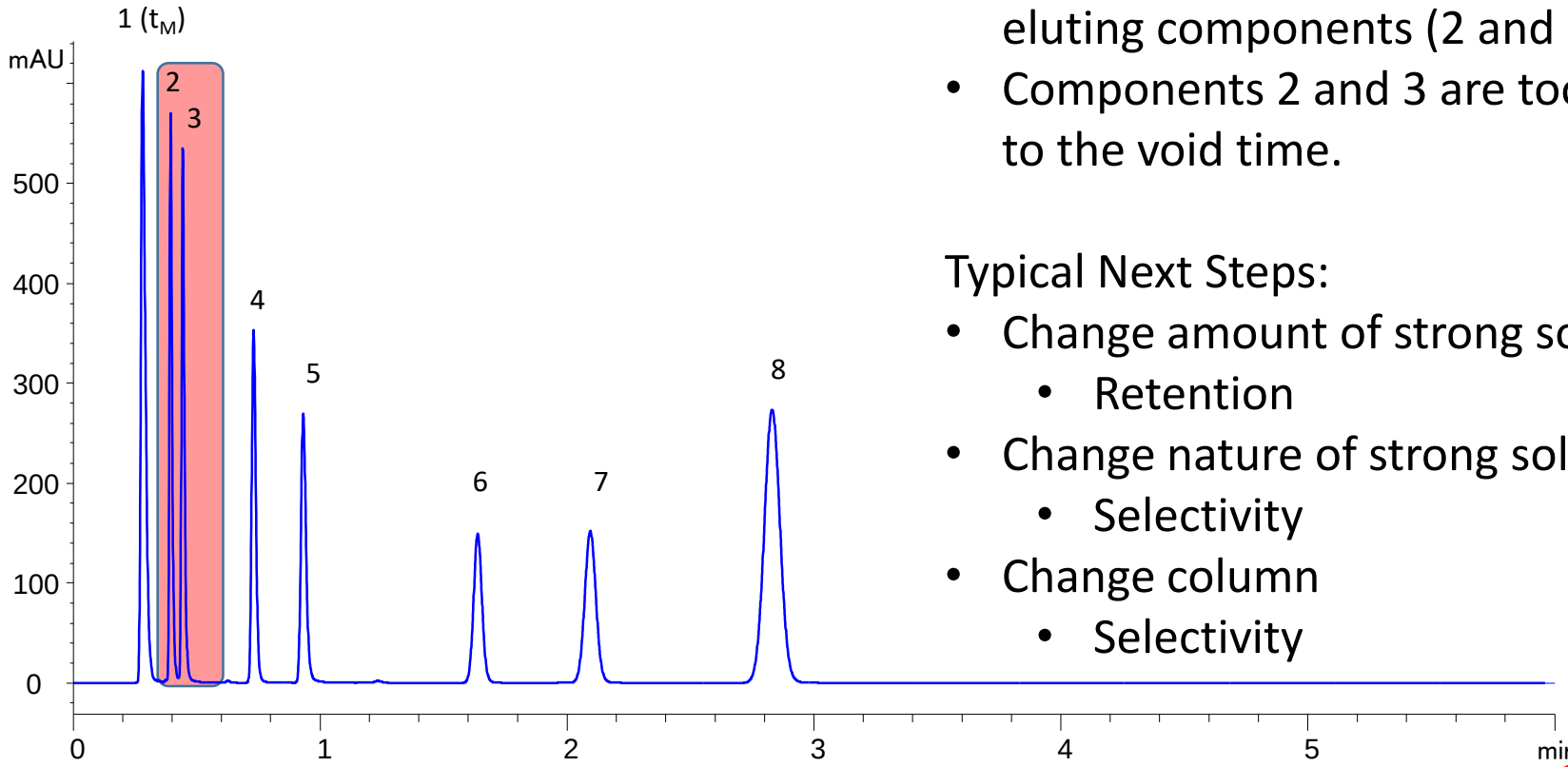
Separation Goals

- Isocratic separation
  - Typical for product/formulation analysis
- Minimize run time
  - Allow analysis of large batches each day
- Chromatography considerations
  - Retention: $k = 2 – 10$
  - Resolution: $Rs > 2$
  - Peak Shape: $T_f < 2$
  - Simple mobile phase
  - Readily available column

Initial Screening

Conditions:
Column: SPP C18, 4.6X50 mm, 2.7 um
Mobile Phase: 0.1 % H_3PO_4/**Acetonitrile** (70/30)
Flow: 1.5 mL/min.
Injection: 2 µL
Column Temperature: 35 °C
Detection: 250 nm (±30 nm)

- 30 % Acetonitrile is a good start, based on the log P values of these compounds.



Comments:

- Good separation except for early eluting components (2 and 3).
- Components 2 and 3 are too close to the void time.

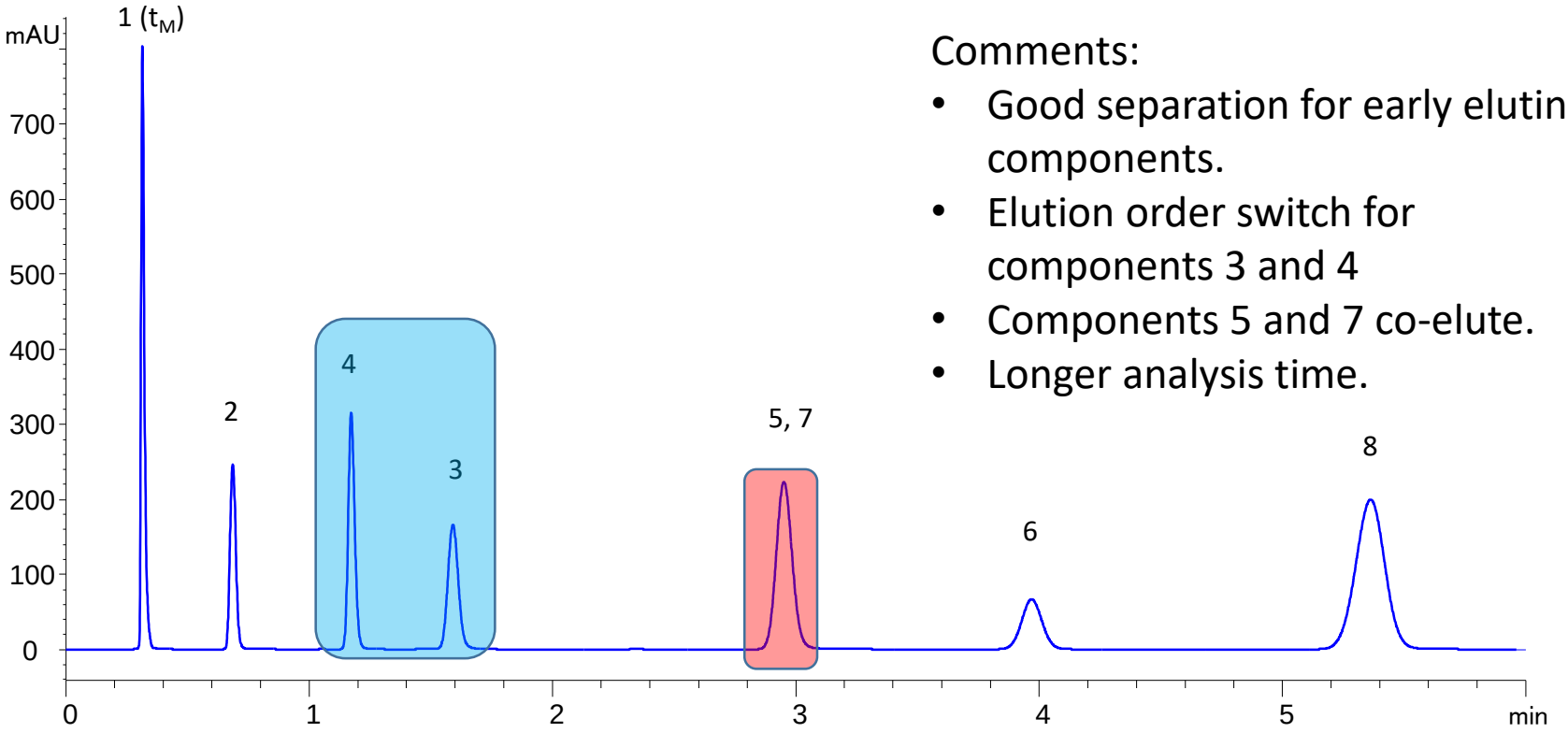
Typical Next Steps:

- Change amount of strong solvent
  - Retention
- Change nature of strong solvent
  - Selectivity
- Change column
  - Selectivity

Next Experiment

Conditions:
Column: C18, 4.6X50 mm, 2.7 um
Mobile Phase: 0.1 % H_3PO_4/**Methanol** (70/30)
Flow: 1.5 mL/min.
Injection: 2 µL
Column Temperature: 35 °C
Detection: 250 nm (±30 nm)

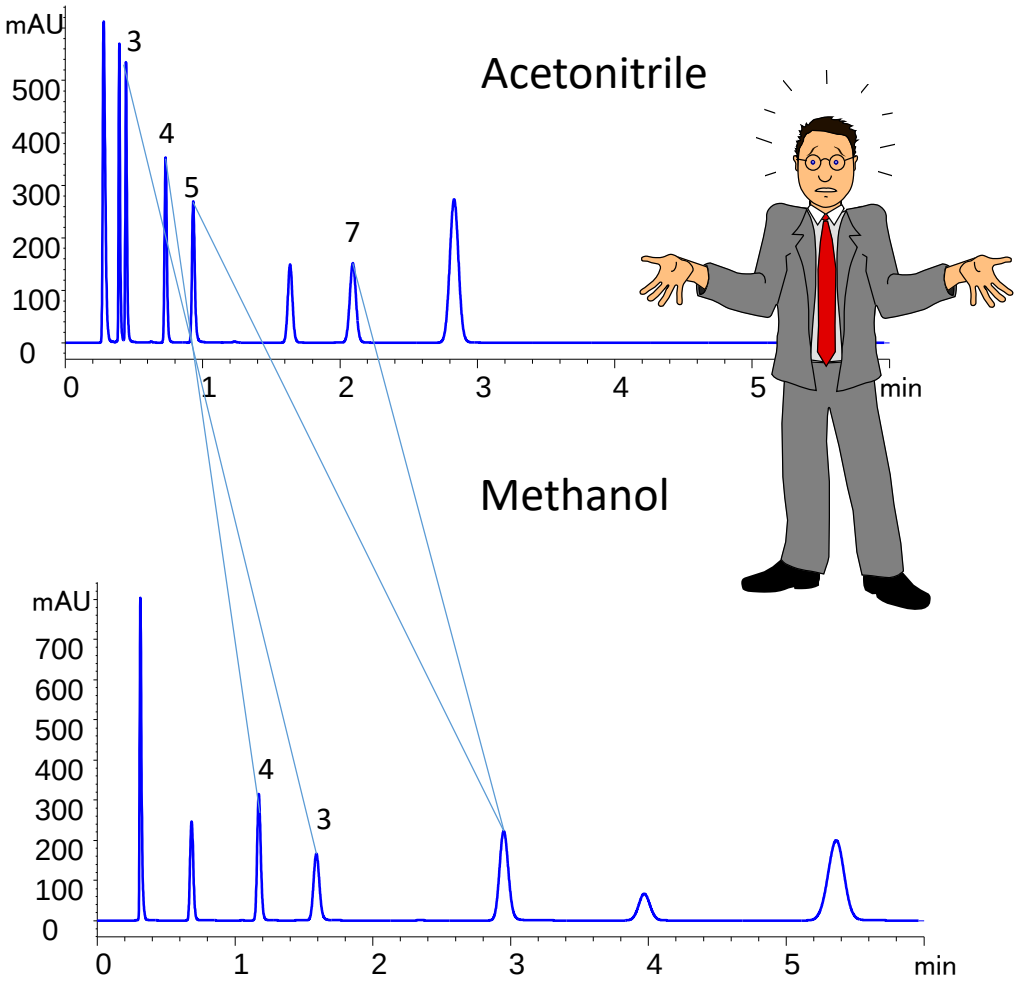
- Change Strong Solvent to Methanol



Comments:

- Good separation for early eluting components.
- Elution order switch for components 3 and 4
- Components 5 and 7 co-elute.
- Longer analysis time.

So Now What?



Common Next Steps:

- Temperature
  - Generally only produces smaller changes.
- pH
  - Possible solution since some of these components are acids. Requires additional solutions and may be time-consuming.
- Different strong solvent
  - THF is another common option but has safety and compatibility issues.
- Are there any other options?

What About a Ternary Mobile Phase?

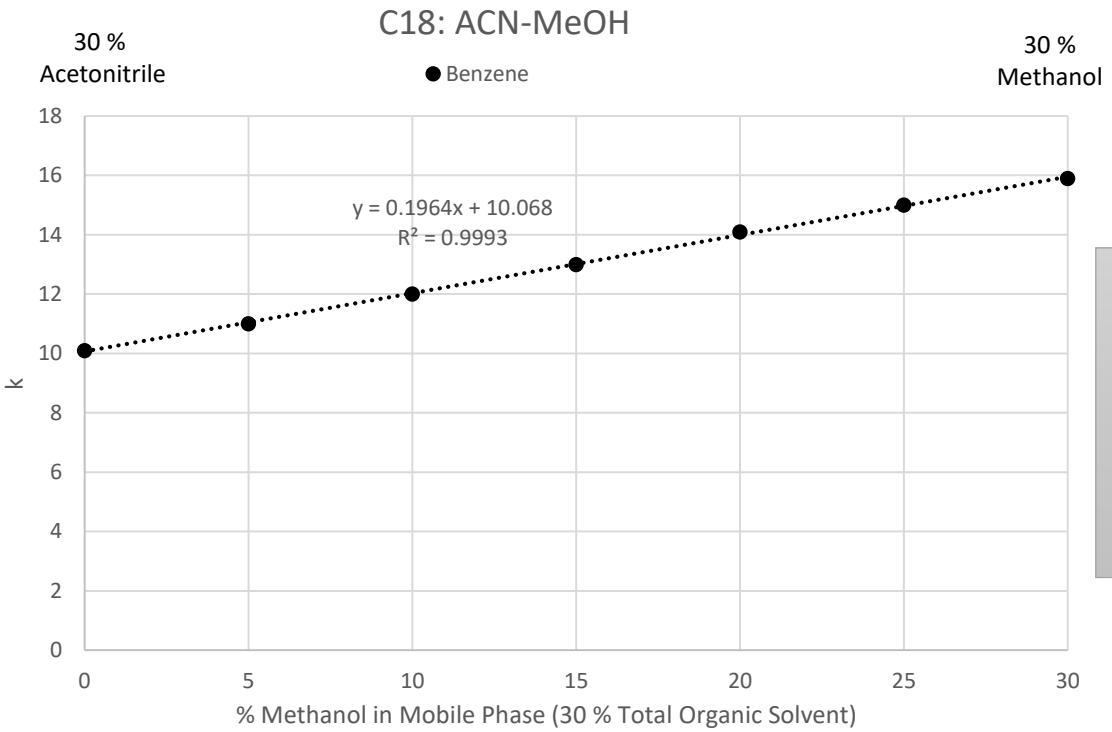
- Water/Strong Solvent 1/Strong Solvent 2
  - The two binary combinations have different problems
    - Acetonitrile: retention and separation (2 and 3)
    - Methanol: co-elution (5 and 7)
  - Would a mixture of all three solvents produce a better separation or make it worse?
  - Key Questions:
    - How does the separation of analytes change as we blend different amounts of each solvent type?
      - Is the change different for different compound classes?
      - Does adding a stronger Hydrogen-bonding component cause a sudden or gradual change in retention?
    - Is the change regular (linear, non-linear)?
    - Can we predict the results with a minimum number of experiments?
      - Allows faster identification of optimum conditions

Designing A Rapid Ternary System Study Keeping Strong Solvent Amount Constant

Activity	Selection		
Choose column	Superficially-porous Halo C18, 4.6X50 mm, 2.7 µm		
Establish desired retention window for an isocratic separation (k < 10)	30 % Strong Solvent (Acetonitrile)		
Choose strong blending solvents	Methanol, Acetonitrile, THF		
Blend two solvents in different ratios, **keeping total strong solvent amount constant at 30 %**: Aqueous/Solvent 1/Solvent 2	Aqueous	Solvent 1	Solvent 2
	70	30	0
	70	25	5
	70	20	10
	70	15	15
	70	10	20
	70	5	25
	70	0	30
Measure Retention (t_R and k)	Uracil used to measure t_M		

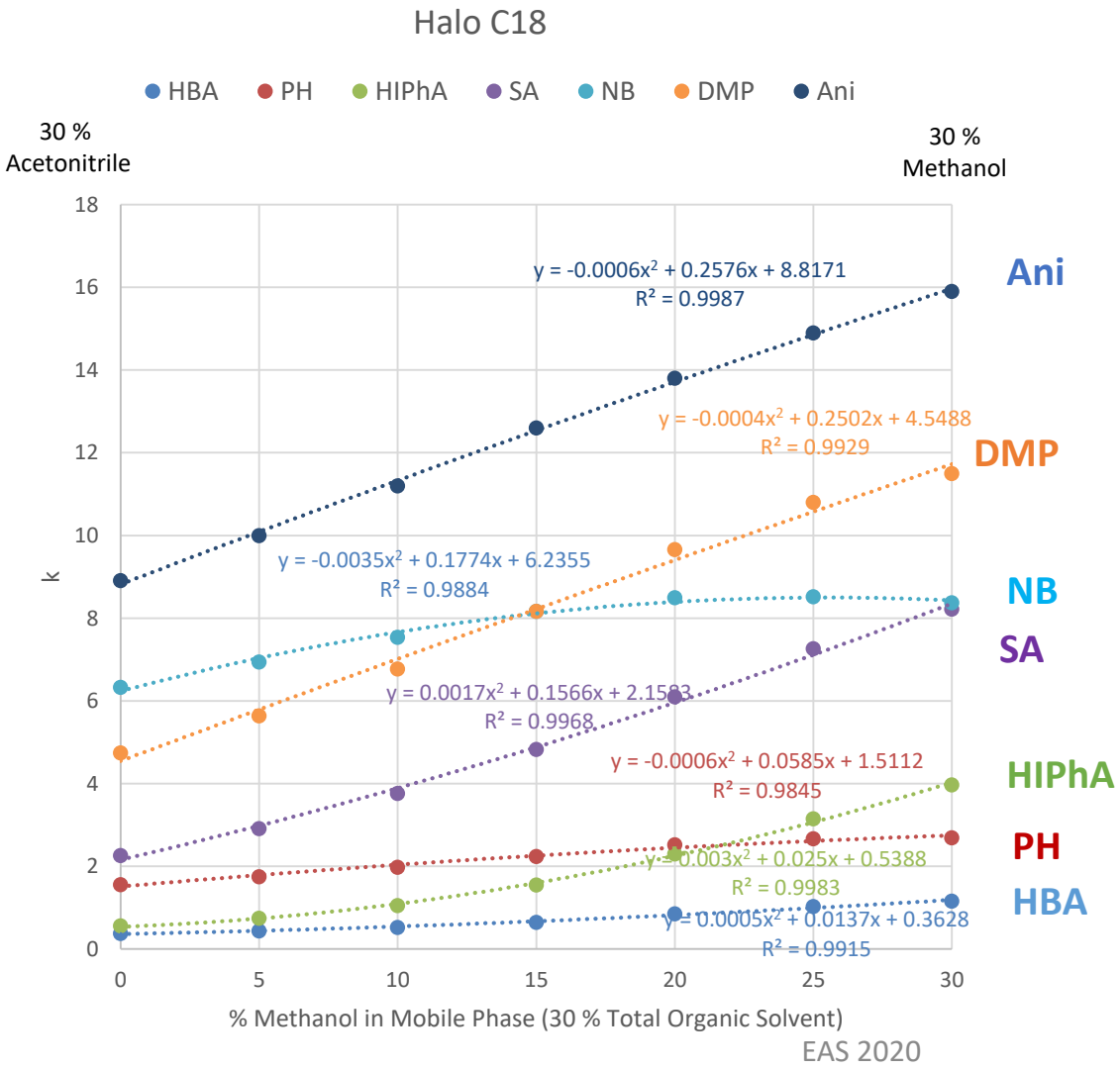
Retention for Benzene in the Acetonitrile-Methanol System

- Regular change in k from 30 % acetonitrile to 30 % methanol for this column
  - Highly linear
  - Suggests simple (hydrophobic) interactions



Question:
Will solute dipole interactions and Hydrogen-bonding produce similar trends?

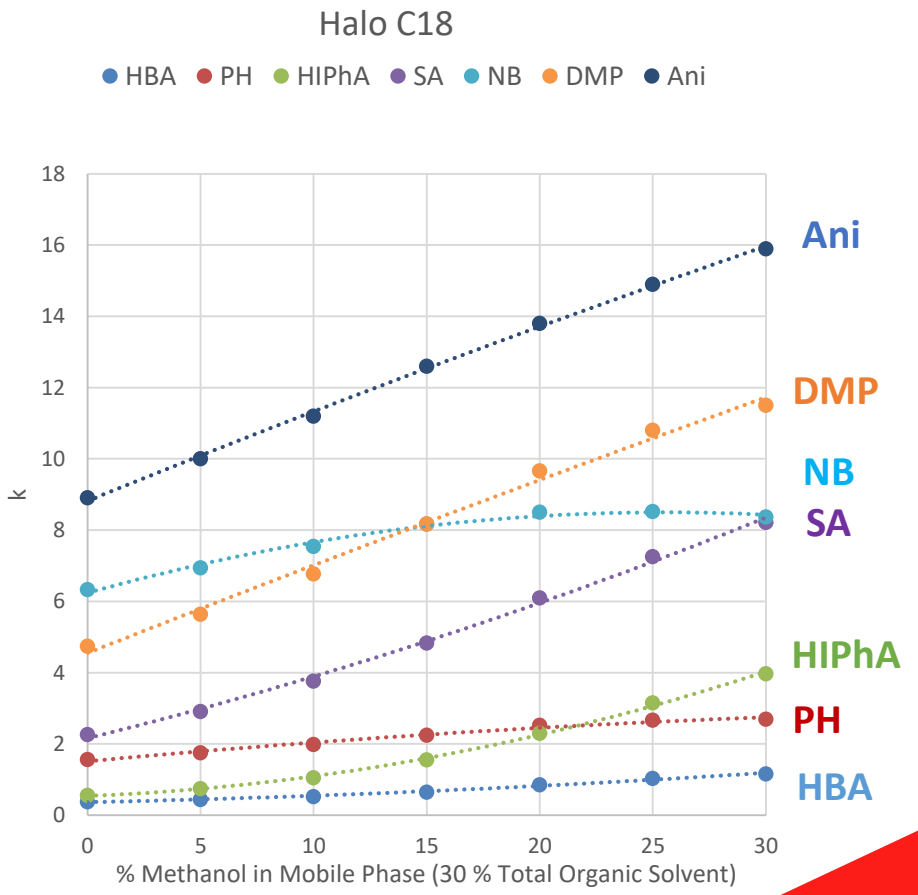
Retention for All Probes in the Acetonitrile-Methanol System



- Regular change in retention.
- Adequate fit with 2nd order polynomial.
- Phenol (PH) / HIPhA and Nitrobenzene (NB) / Dimethylphthalate (DMP) show selectivity changes.

Understanding the C18: Acetonitrile-Methanol System

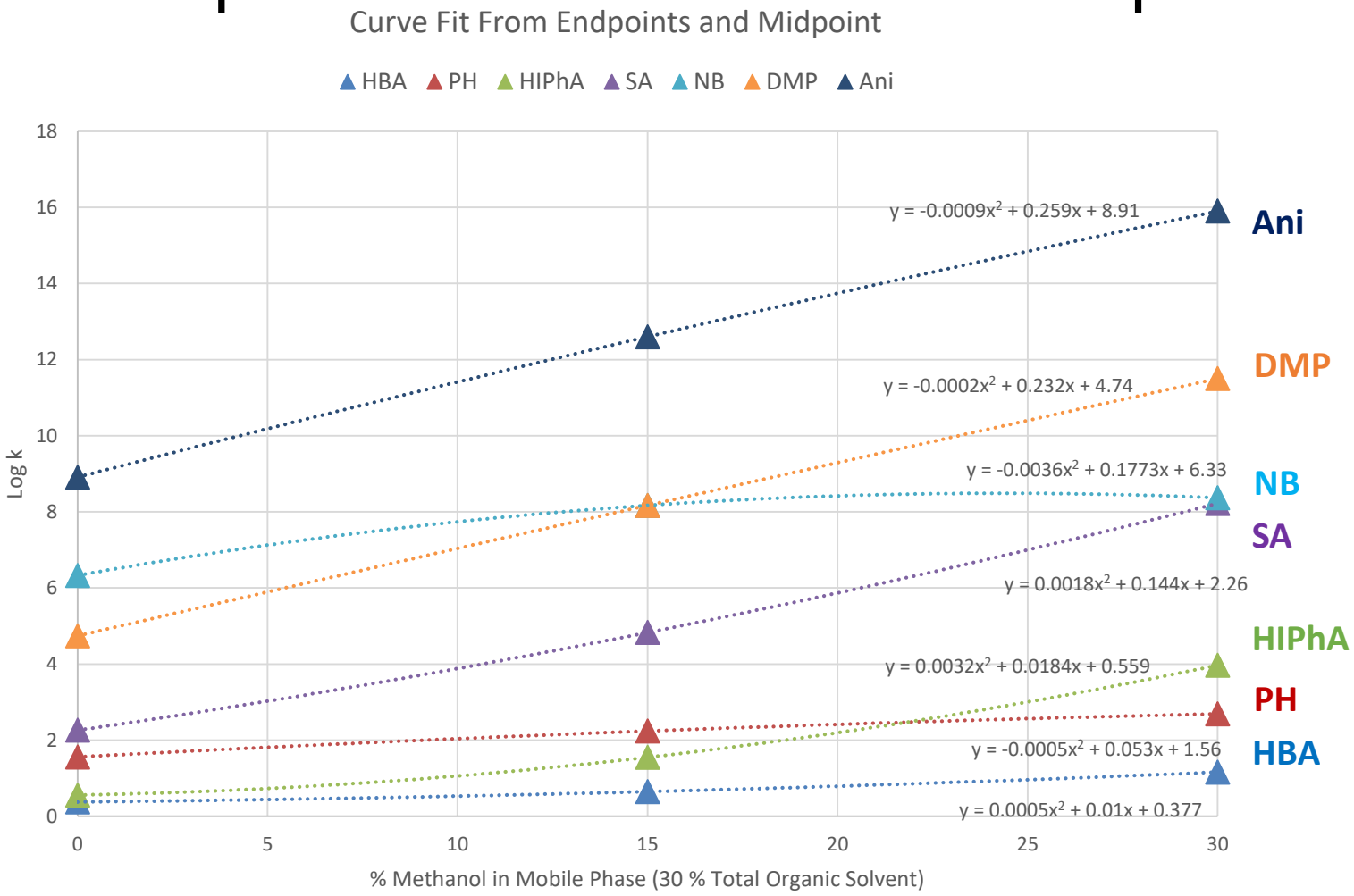
- The change is regular but not linear.
- Across several different functional groups, the change can be predicted by a 2nd order polynomial.
- The organic acids show enhanced convex non-linear retention with increasing amounts of methanol.
- Nitrobenzene exhibits a concave linear trend.



Predicting Retention in the C18: Acetonitrile-Methanol System

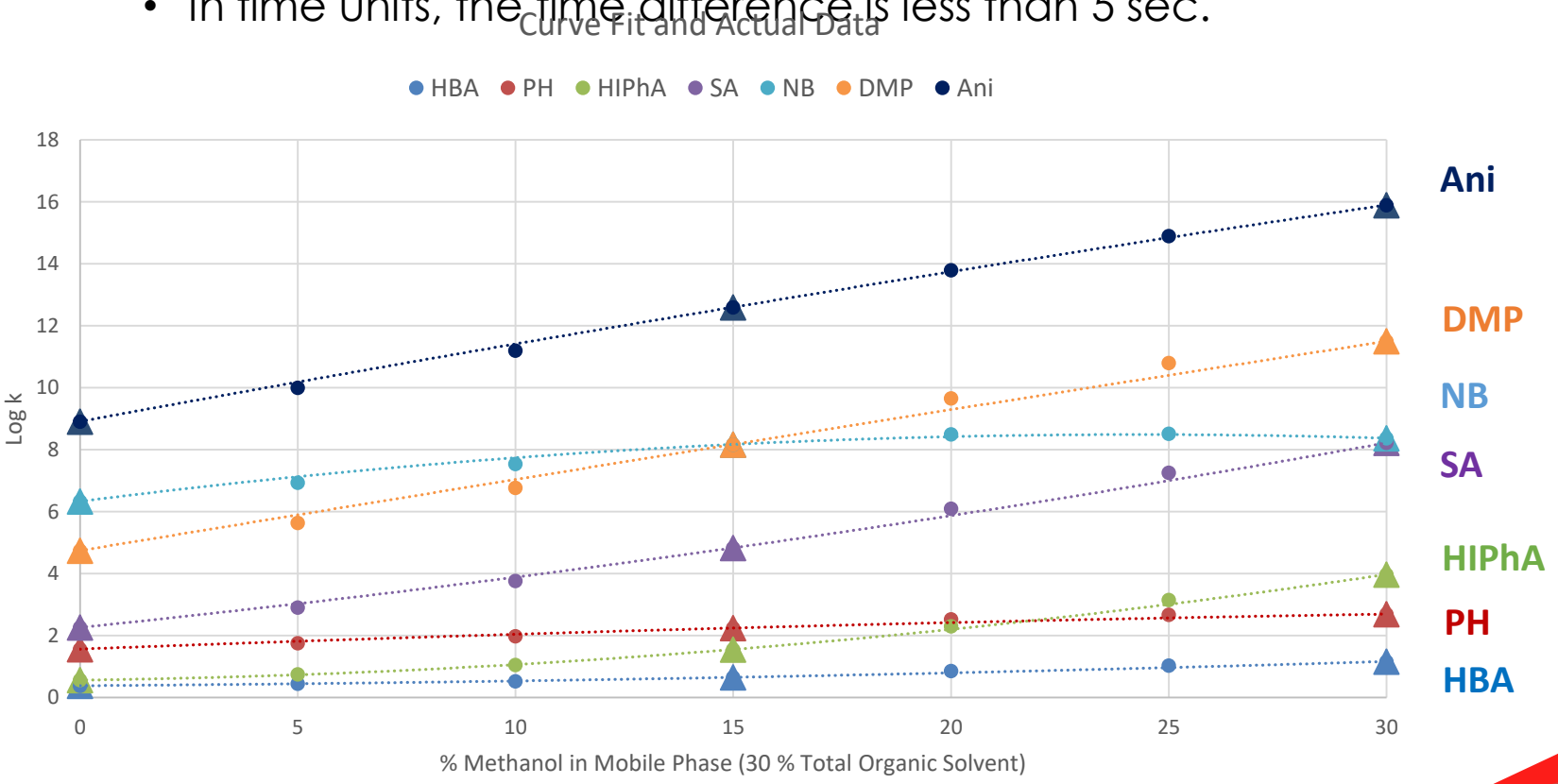
- A 2nd order polynomial produces an adequate fit to the data
- Using this information, retention can be modelled using only three injections: the endpoints and one midpoint
  - Aq/ACN/MeOH (70-30-0)
  - Aq/ACN/MeOH (70-15-15)
  - Aq/ACN/MeOH (70/0/30)
- Use fitted equations to predict retention across the series
  - Only relatively simple spreadsheet features required

Calculated Values From the Endpoints and One Midpoint



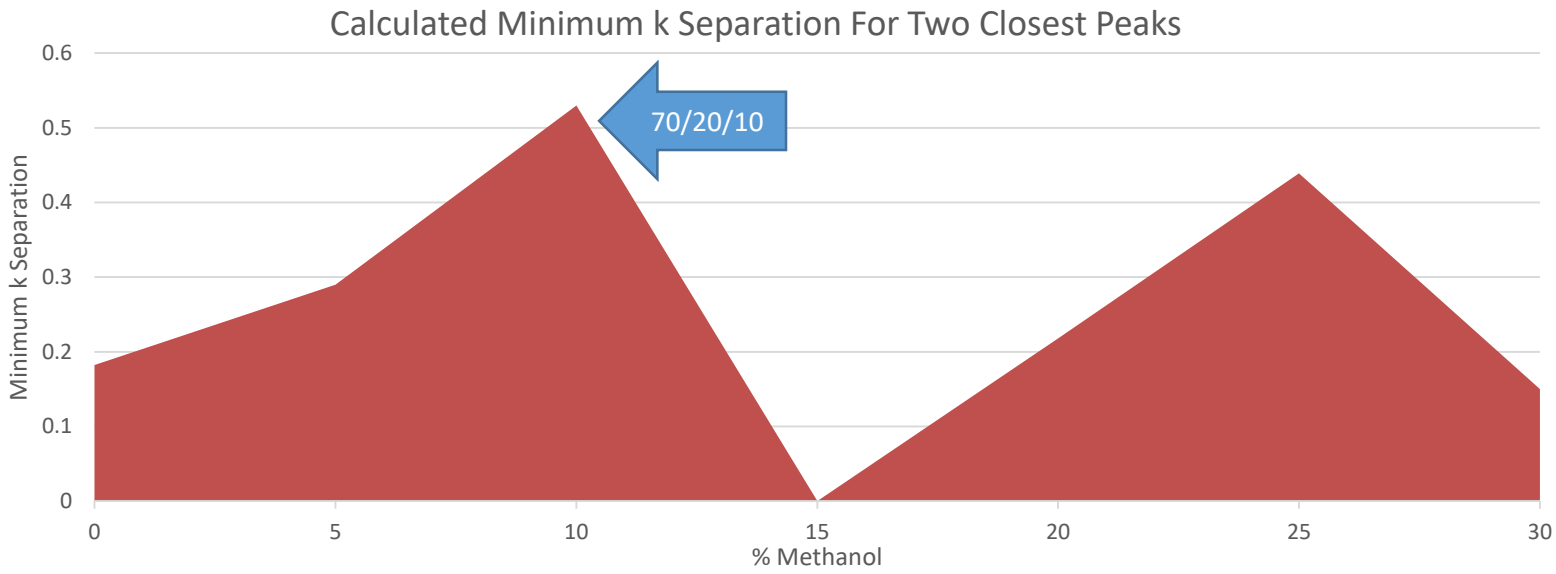
Overlay Curve Fits With Actual Data

- Triangles are points used for modeling the retention.
  - Trend lines are from three points only.
- Circles are other data points (not used in modeling).
- The maximum deviation from the real value (in k units) is less than 5 %
  - In time units, the time difference is less than 5 sec.



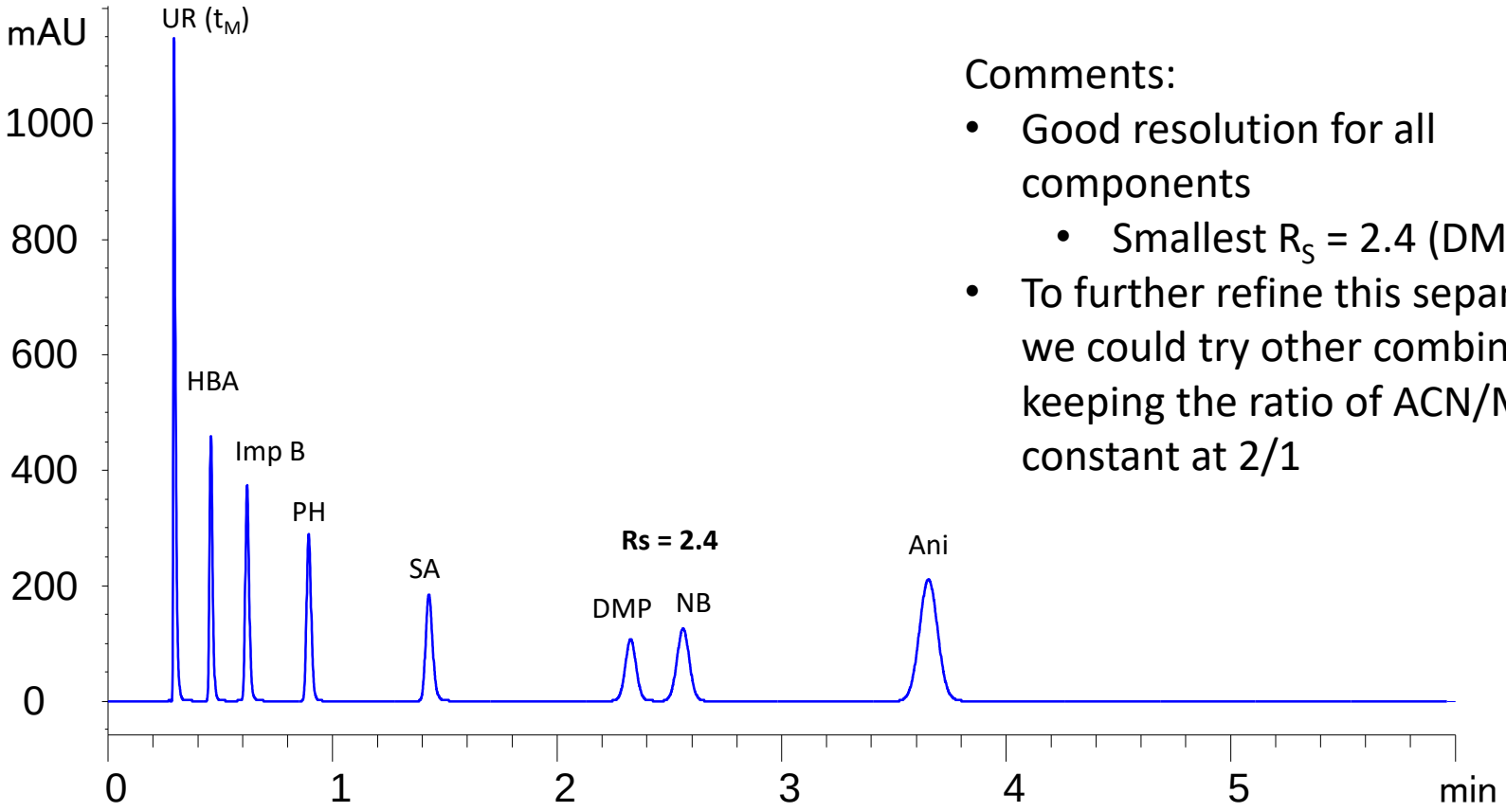
Finding the Best Separation

- Using the calculated k values, calculate the smallest predicted separation, in k units, for the two closest peaks across the series.
- Find conditions that maximize that separation.
- The best value is near Aq/ACN/MeOH (70/20/10)



Predicted Optimum Conditions

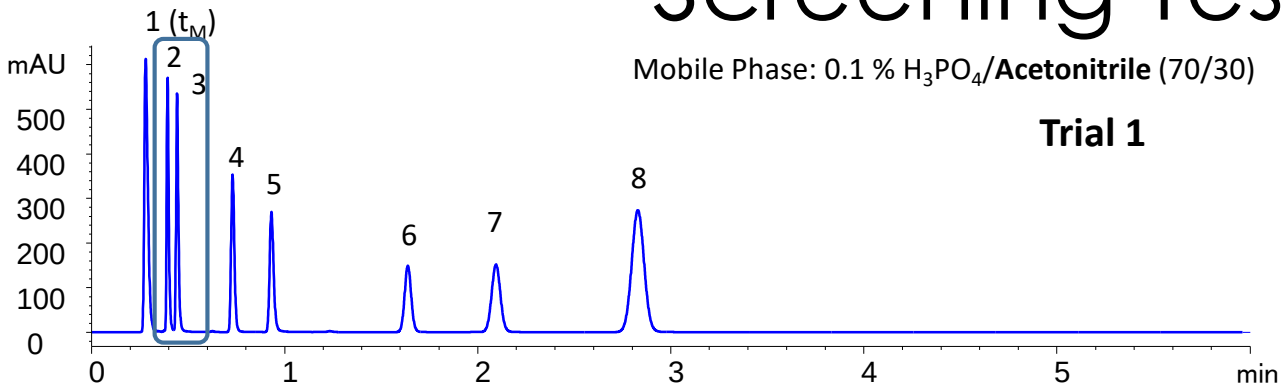
- Results at Aq/ACN/MeOH (70/20/10)



Comments:

- Good resolution for all components
  - Smallest R_S = 2.4 (DMP/NB)
- To further refine this separation, we could try other combinations, keeping the ratio of ACN/MeOH constant at 2/1

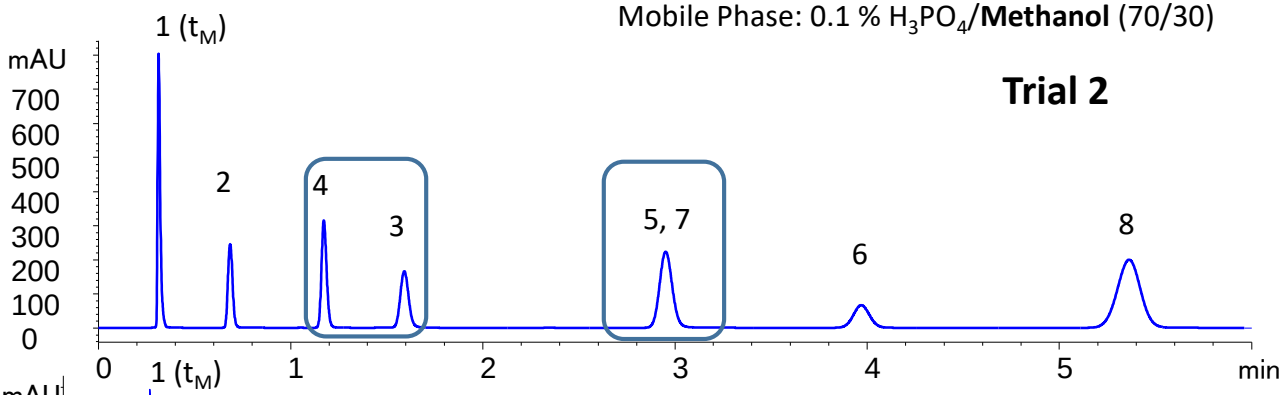
Summary of a Successful Screening Test



Mobile Phase: 0.1 % H_3PO_4/**Acetonitrile** (70/30)

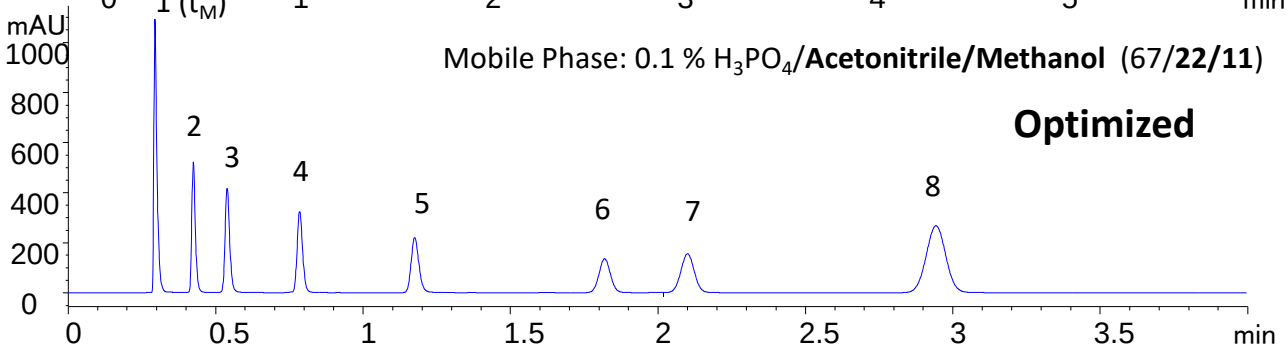
Trial 1

Conditions:
Column: SPP C18, 4.6X50 mm, 2.7 um
Flow: 1.5 mL/min.
Injection: 2 µL
Column Temperature: 35 °C
Detection: 250 nm (±30 nm)



Mobile Phase: 0.1 % H_3PO_4/**Methanol** (70/30)

Trial 2

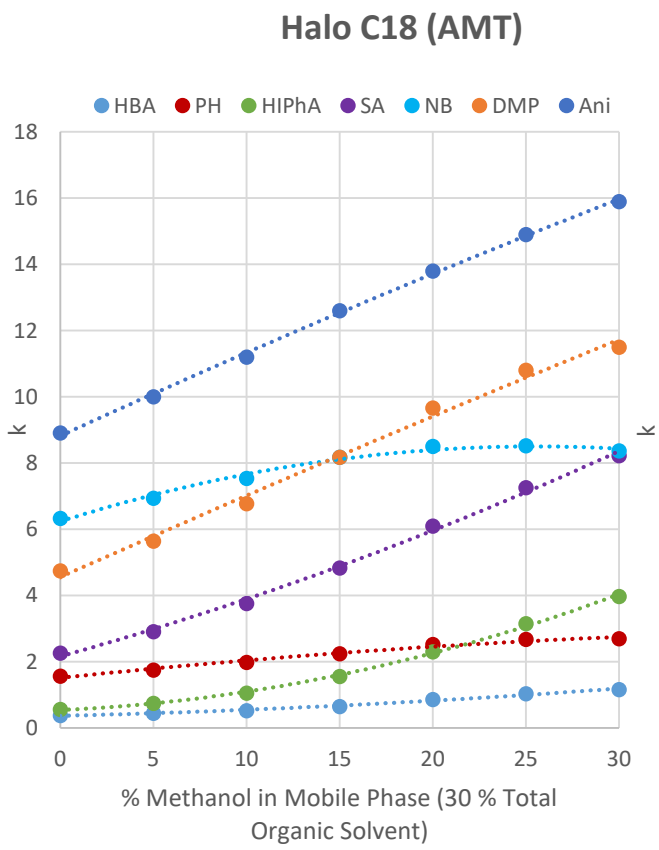


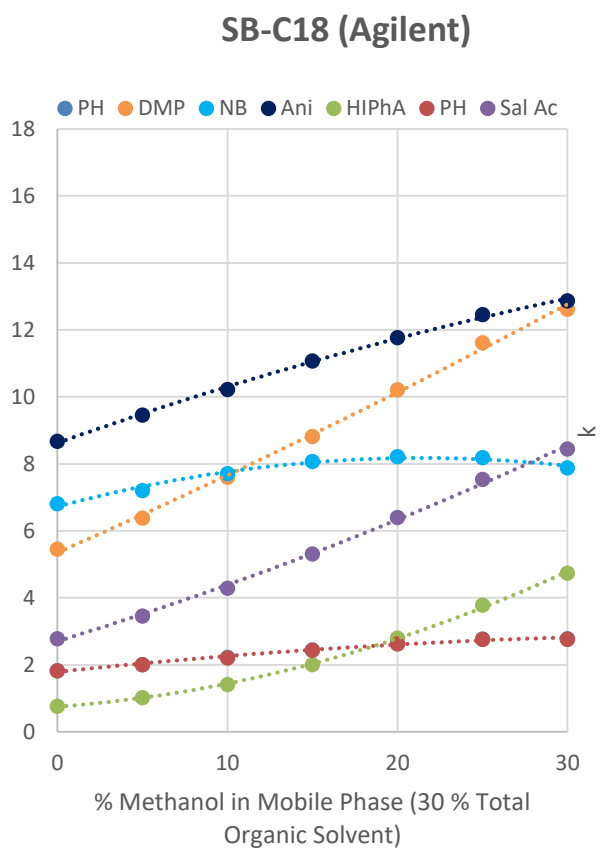
Mobile Phase: 0.1 % H_3PO_4/**Acetonitrile/Methanol** (67/**22/11**)

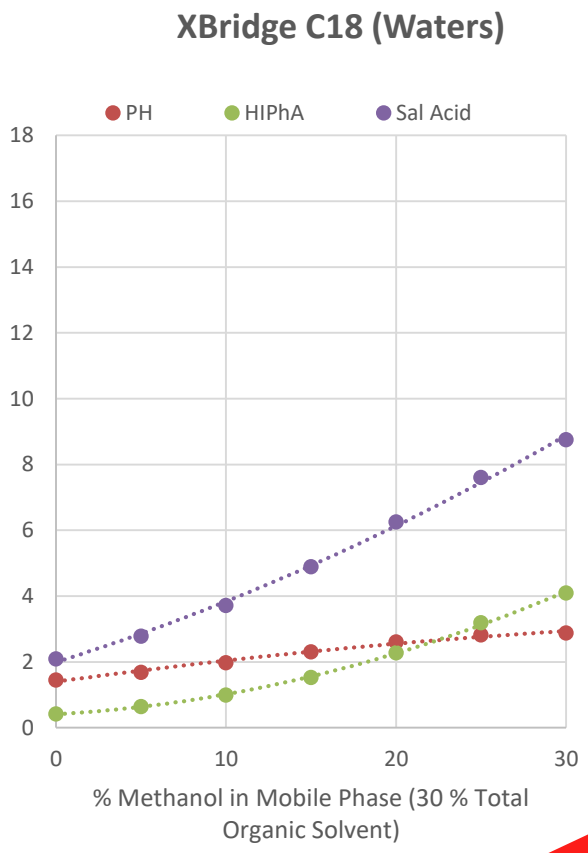
Optimized

1 Uracil
2 4-Hydroxybenzoic Acid
3 4-Hydroxyisophthalic Acid
4 Phenol
5 Salicylic Acid
6 Dimethyl phthalate
7 Nitrobenzene
8 Anisole

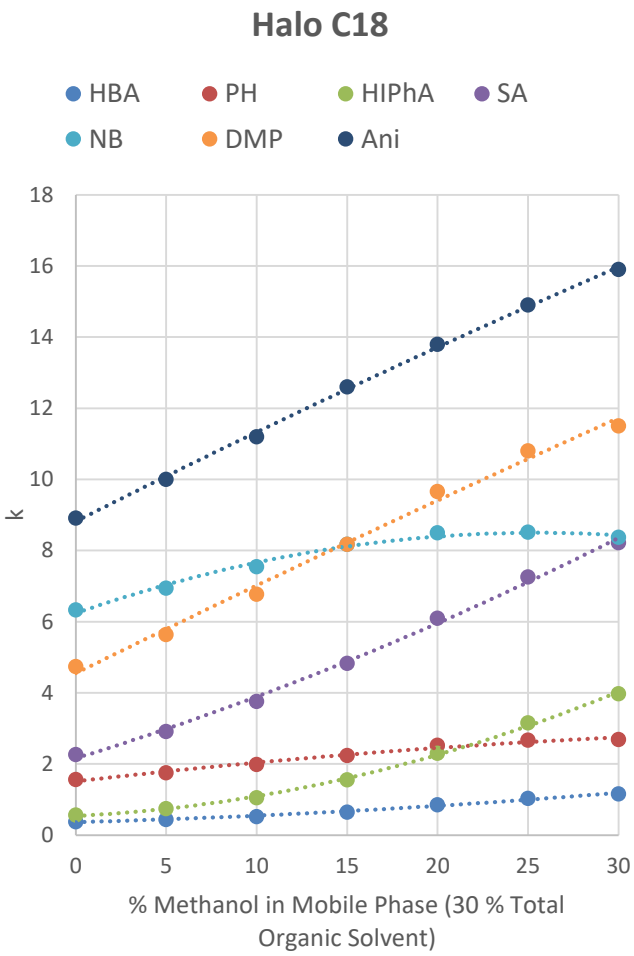
Do These Trends Apply to Other C18 Columns?

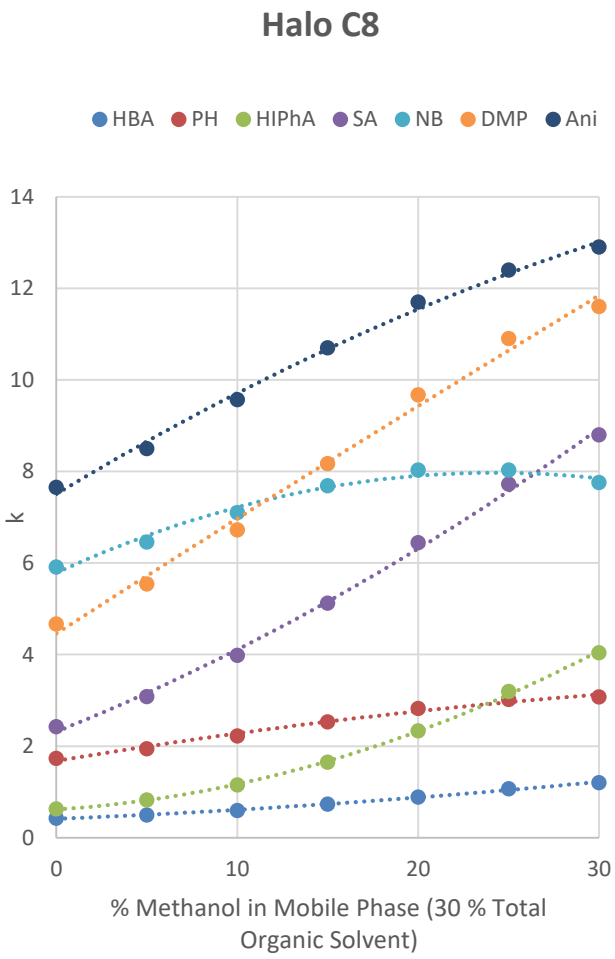


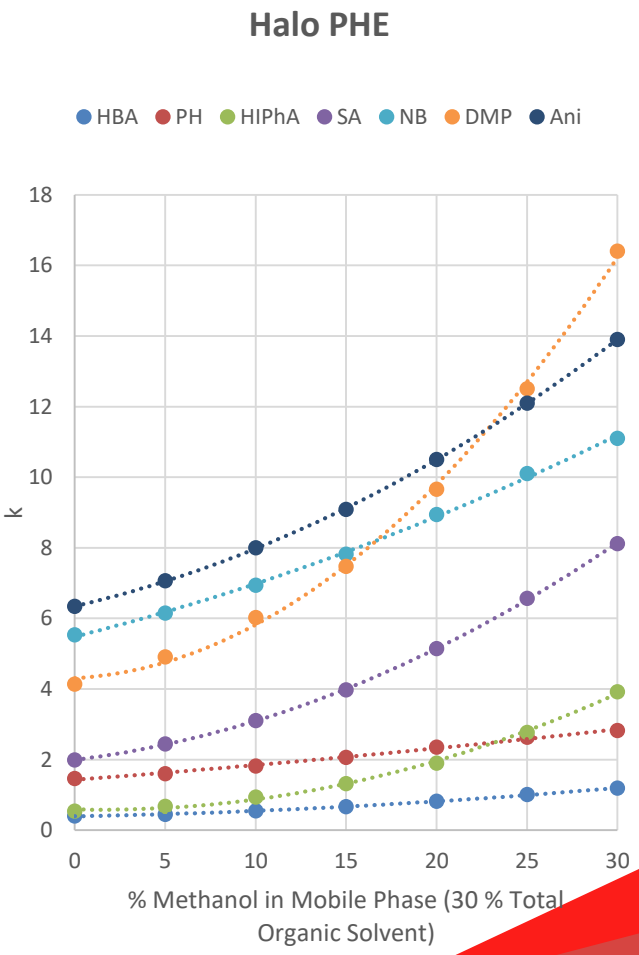




Do These Trends Apply to Other Phases?

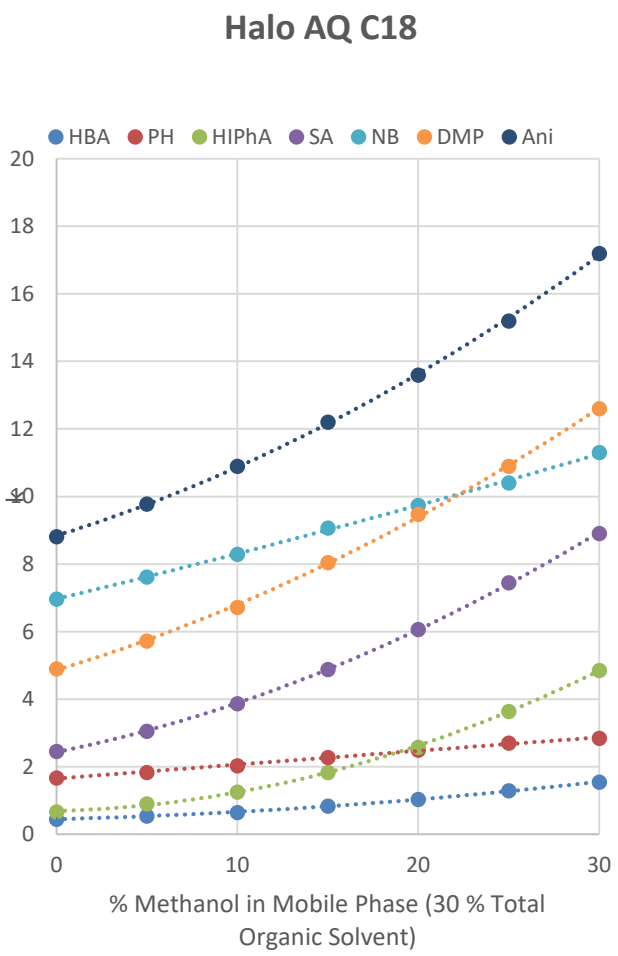


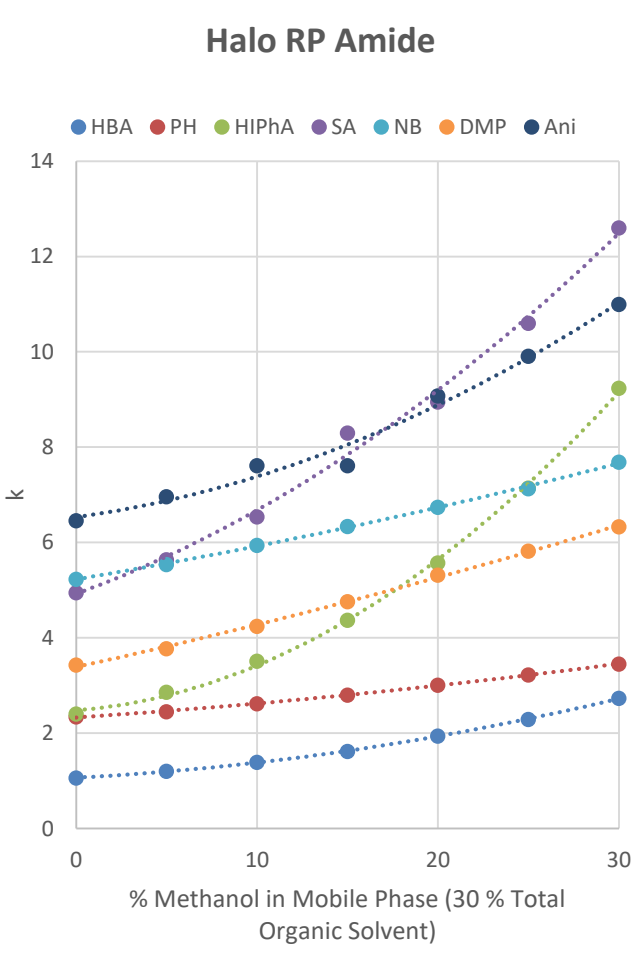


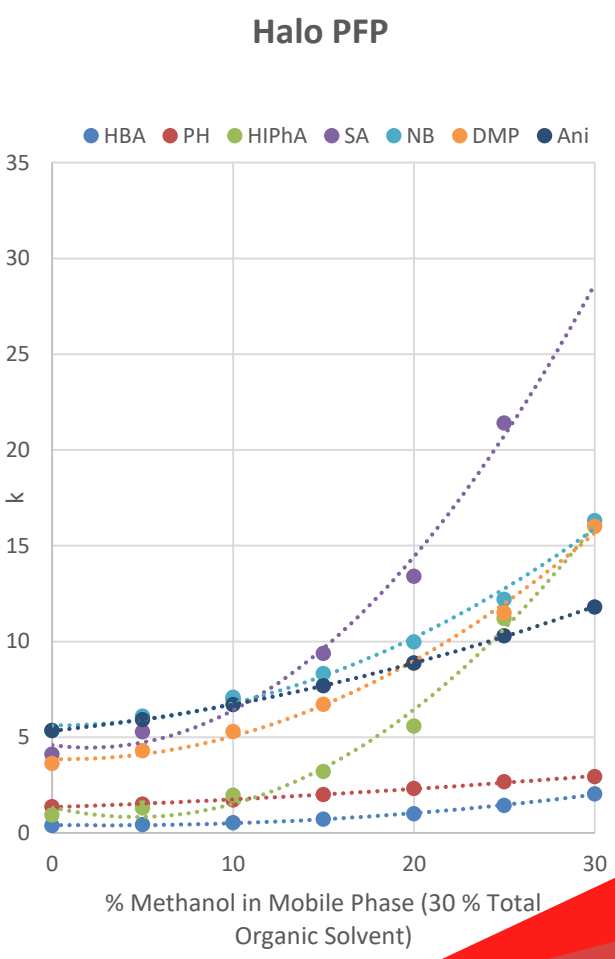


Other Phases

Trends are more complex and may be discontinuous.







Conclusions

- While binary mobile phases offer powerful selectivity changes for simple systems, ternary systems provide more tools for more complex systems, especially when selectivity changes occur.
- Aqueous/Acetonitrile/Methanol systems show a regular change in retention **when the total organic content is constant**, and the relative amounts of each organic solvent are changed.
- This change can be modelled using a 2nd order polynomial fit from only three injections.
- Selectivity differences result in elution order changes for some analytes.
  - These changes can be used to improve the overall separation.

Future Work

- Expand the available data to determine if this approach is broadly applicable to other situations (columns, compound classes, and degree of retention).
- Measure and understand the behavior of these components in ternary systems using the three common organic solvents
  - Acetonitrile-Methanol
  - THF – Methanol
  - THF-Acetonitrile
- Explore ternary gradients.

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