Fast Separation of Vastly Different Compounds by Isocratic HPLC

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Abstract

The separation of vastly different compounds by HPLC, although often necessary, is challenging and complex; requiring either gradient elution or multiple assays for adequate analysis. When reversed-phase techniques are employed for such samples the very hydrophilic compounds are often difficult to retain even in highly aqueous mobile phases while the very hydrophobic compounds are retained indefinitely under these conditions, requiring a gradient to higher percent organic to elute. It is often difficult to quantitate the early eluting compounds due to lack of selectivity while much time is lost waiting for the late eluting compounds. Gradient elution has several limitations when compared to isocratic techniques; requiring more complex pumping systems, less rugged and reproducible, and long equilibration times. These limitations can result in increased cost and analysis time often precluding its use in quality control and high-throughput applications.

Abstract Con't.

We will present a new column technology which allows for the rapid separation of samples containing compounds ranging from very hydrophilic to very hydrophobic with simple, isocratic mobile phase conditions. This is achieved by incorporating several distinct and controlled analyte-bonded phase interactions including anion and cation exchange, hydrophobic mechanisms, and complex formations. By adjusting the ionic strength of the mobile phase, the ion-exchange interactions can be controlled so that the very hydrophilic, ionizable compounds can be retained, while not being greatly effected by the organic modifier concentration. Conversely, the retention of hydrophobic species will be affected by the organic modifier, but not by ionic strength. By independently adjusting these two mobile phase parameters, the retention of both hydrophobic and hydrophilic compounds can be controlled independently.

This technology will be compared to conventional silica-based, alkyl type chemistries for more effective separations of complex mixtures. Measurable improvements in retention, selectivity, and analysis time will be shown for common cold relief formulas and other complex samples.

Objective

- To develop an HPLC method for complex mixtures offering the following improvements over conventional reversed-phase bonded phases:
  - Shorter analysis time
  - Better selectivity for polar compounds
  - Better quantitation for early eluting compounds

Background

- Frequently samples which are to be analyzed by HPLC contain a wide variety of compounds some of which may be extremely hydrophobic while others are extremely polar.
- Such samples often require gradient elution in order to have adequate retention and selectivity for polar compounds and reasonable retention times for hydrophobic compounds. Gradient techniques can be less than desirable in may instances as they:
  - Require more complex equipment which is often not available in Quality Control Environments
  - Require long equilibration times
  - Are not compatible with ion-pairing techniques
  - Are not compatible wit certain detection methods

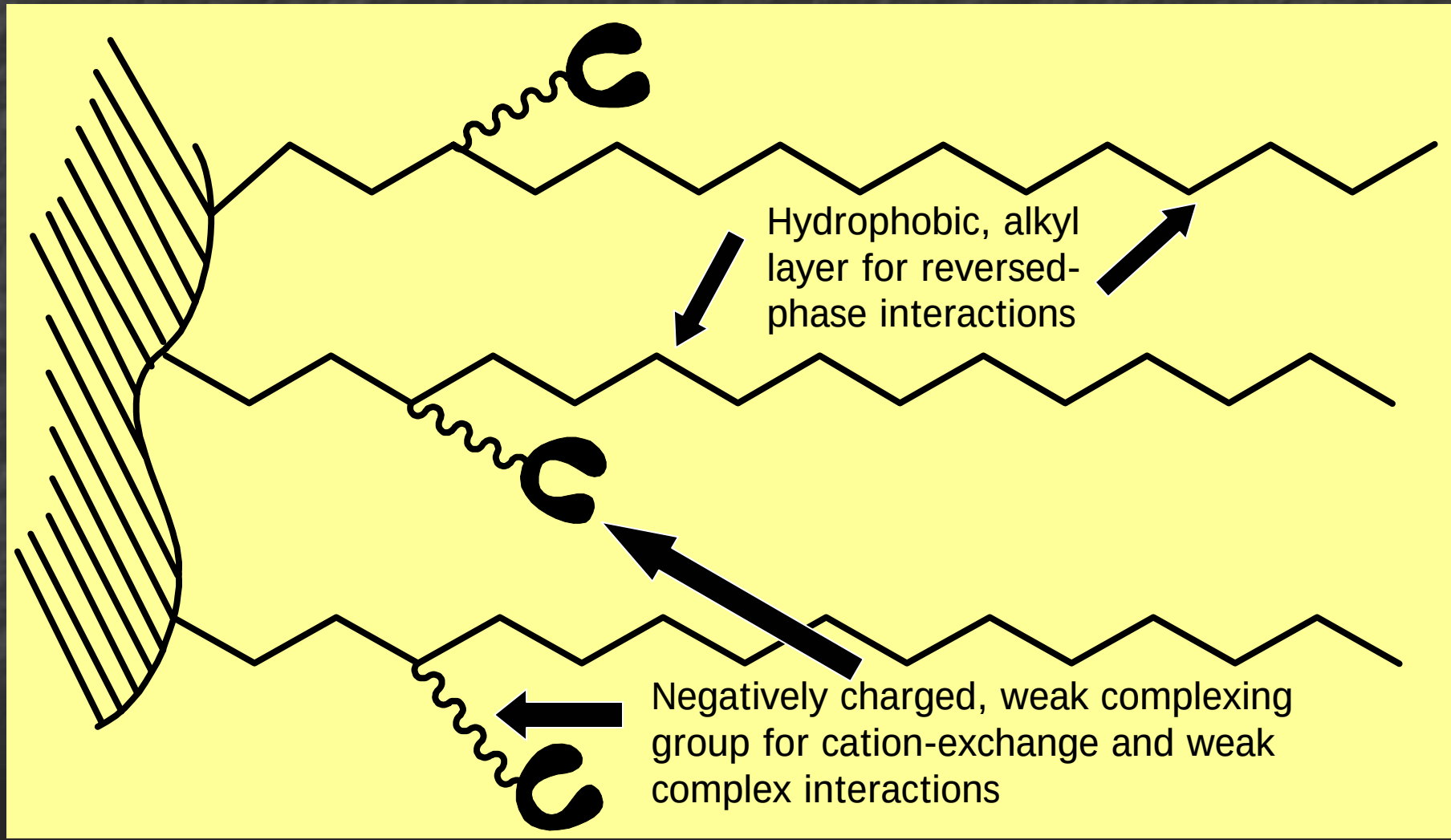
Background Con't

- Traditionally secondary, mixed-mode interactions between the analyte and silanol groups vary from lot-lot of bonded phase and result in poor peak shape for basic compounds.
- Due to their negative effects these secondary interactions are minimized by:
  - Secondary end-capping
  - Base-deactivated pure silica
  - Buffered mobile phases
- By masking all secondary interactions, the only mode of interaction between analyte and bonded-phase is hydrophobic.
  - The only variable of consequence in this system is the organic modifier in the mobile phase
  - In this system, all analytes are effected by changing the percent organic modifier in the mobile phase.
    - If isocratic conditions are selected so that the hydrophilic compounds are retained, the retention time for the hydrophobic compounds will be unacceptably long, peaks will be broad, and the compounds may not elute at all
    - If isocratic conditions are selected so that the hydrophobic compounds elute rapidly, the hydrophilic compounds will be un-retained and not quantifiable

Background, Con't

- A mixed-mode, ion-exchange, complexing, reversed-phase ligand was created to offer multiple, independent retention mechanisms. This new bonded-phase is illustrated in Figure 1.
  - A typical reversed-phase retention profile is observed for neutral compounds
  - Amines with equal hydrophobicity retain on Primesep C in the following order:
    - Tertiary<secondary<primary
  - Alkali metals are in the following order:
    - $K^+ < Na^+ < Li^+$
    - Reverse order compared to classical ion-exchange

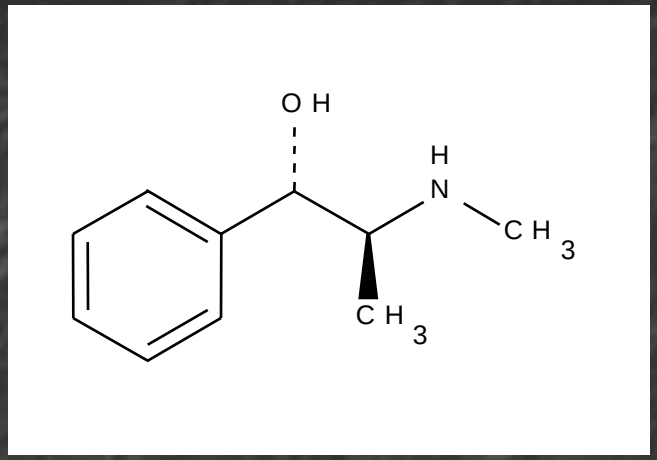
Figure 1. Design of Primesep C



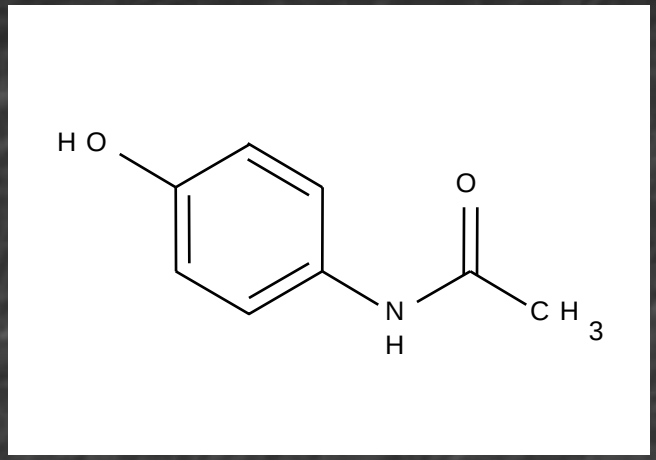
Experimental, Figures 2, 3, 4

- Column: 5um, 150x4.6mm
- Mobile Phase: As indicated on figures
- Injection: 5uL
- Flow: 1.0mL/min
- Detection: UV@205nm
- Sample: NyQuil®
  - 1. Pseudoephedrine
  - 2. Acetaminophen
  - 3. Doxylamine
  - 4. Dextromethorphan

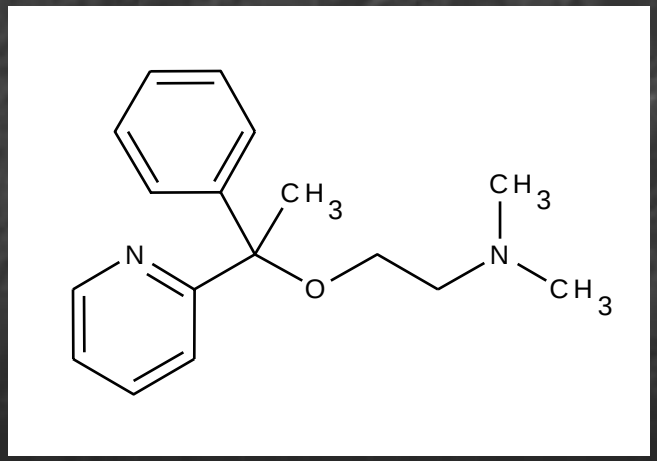
Figure 2: Structures of Active Ingredients in NyQuil ®



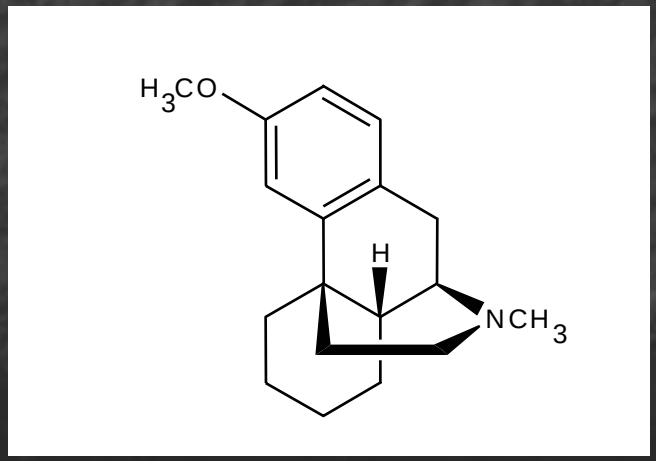
1. Pseudoephedrine



2. Acetaminophen

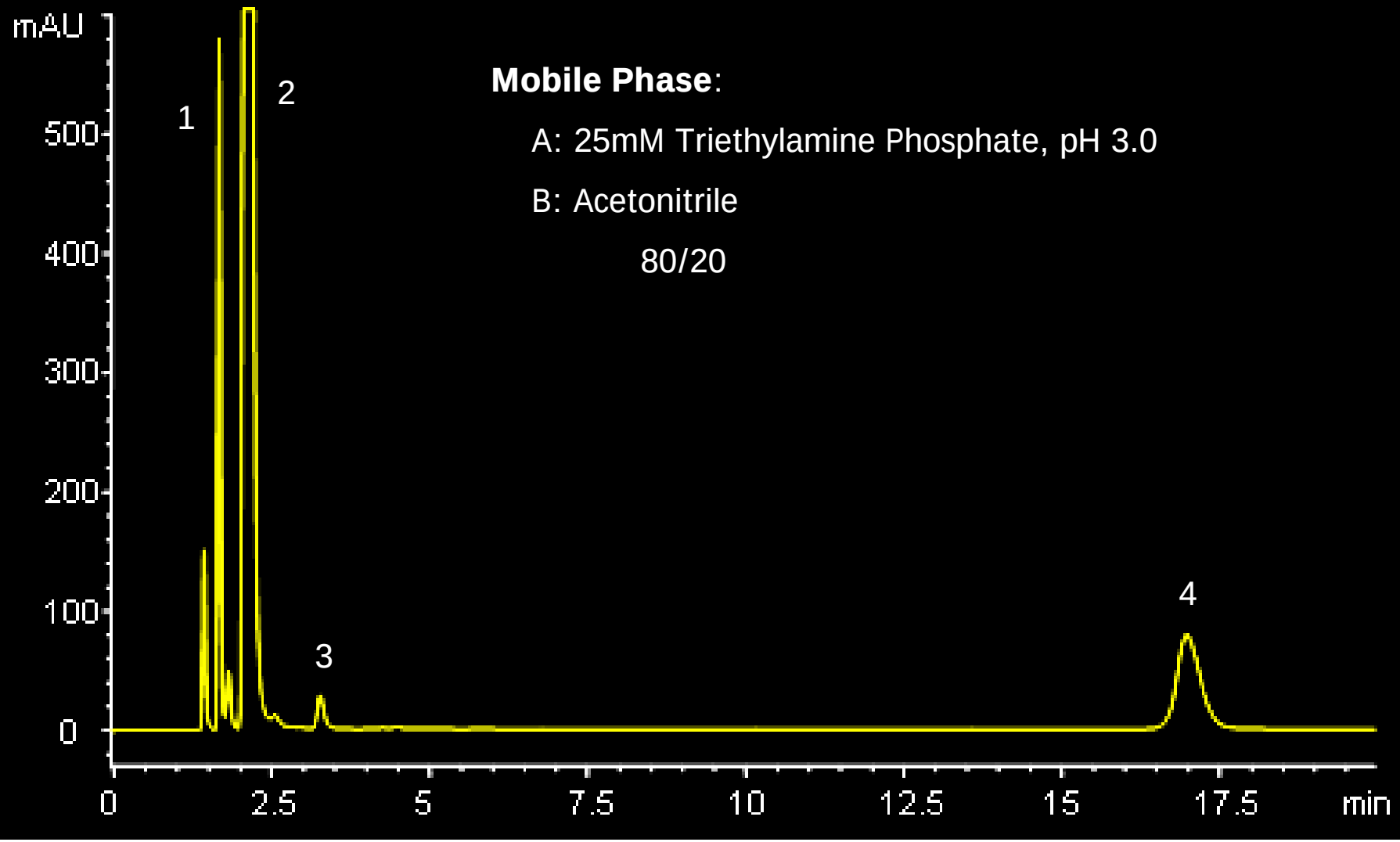


3. Doxylamine



4. Dextromethorphan

Figure 3: Separation of NyQuil® Active Ingredients on Leading Brand C18



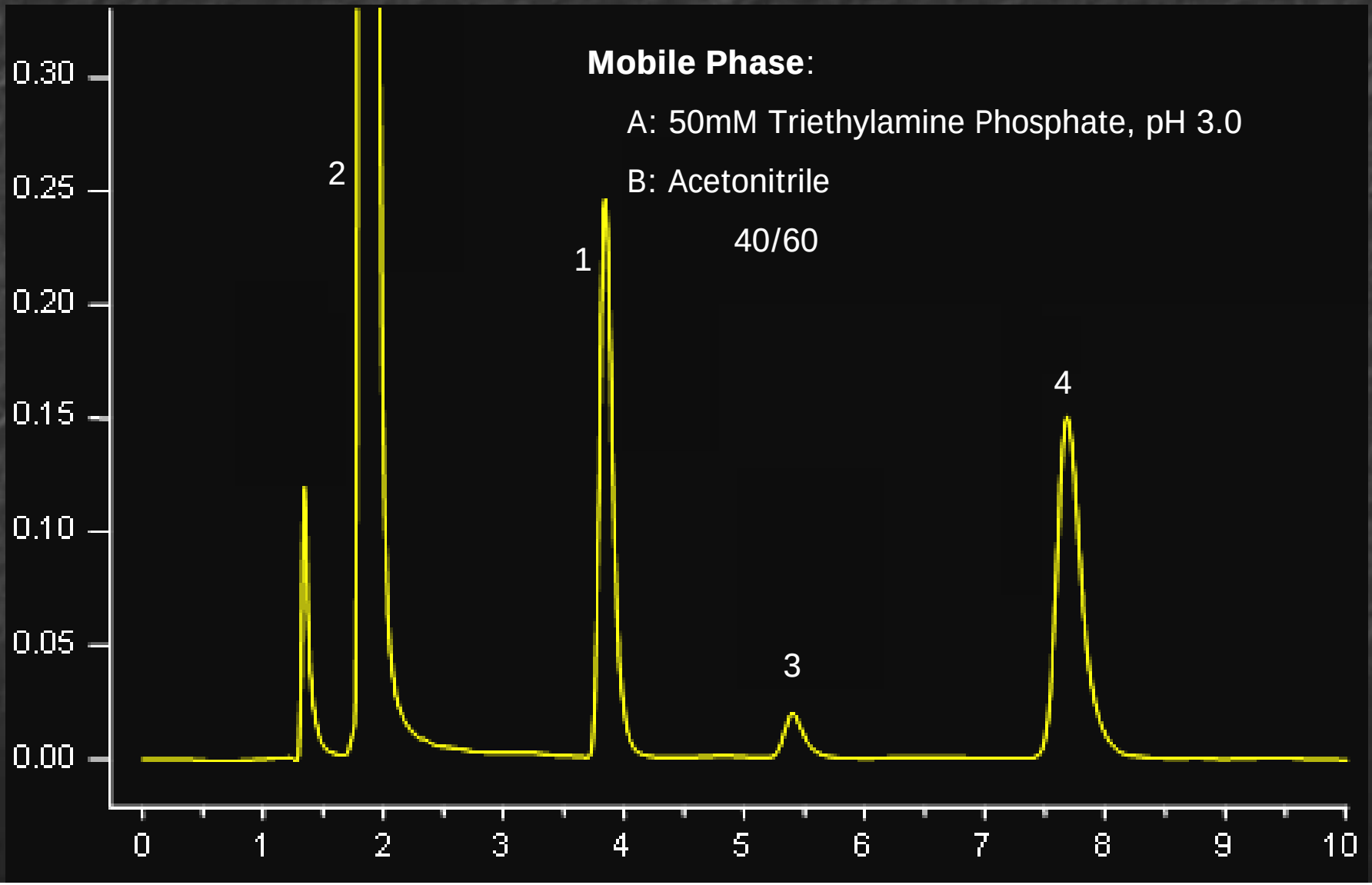
Mobile Phase:

A: 25mM Triethylamine Phosphate, pH 3.0

B: Acetonitrile

80/20

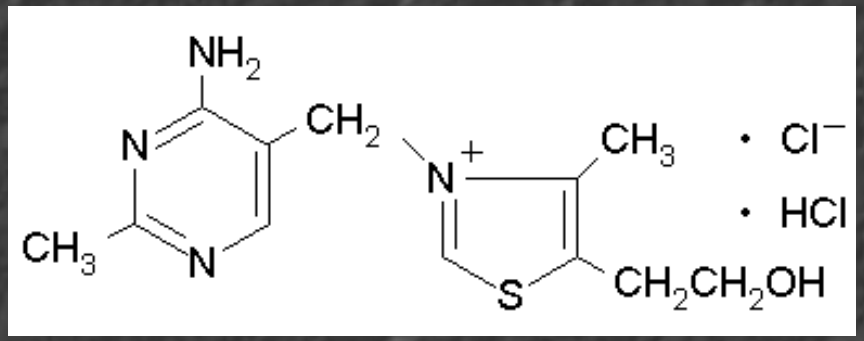
Figure 4: Separation of NyQuil® Active Ingredients on Primesep C



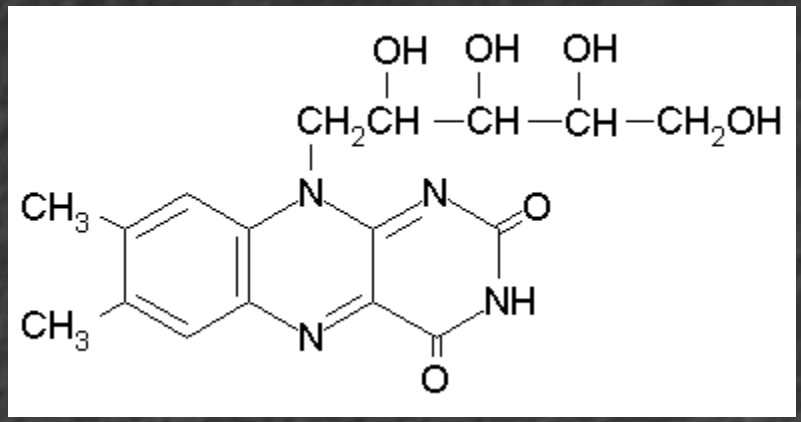
Experimental, Figures 5, 6, 7

- Column: 5um, 150x4.6mm
- Mobile Phase: As indicated on figures
- Injection: 5uL
- Flow: 1.0mL/min
- Detection: UV@280nm
- Sample:
  - 1. Vitamin B1-Thiamine
  - 2. Vitamin B2-Riboflavin
  - 3. Vitamin B6-Pyridoxine

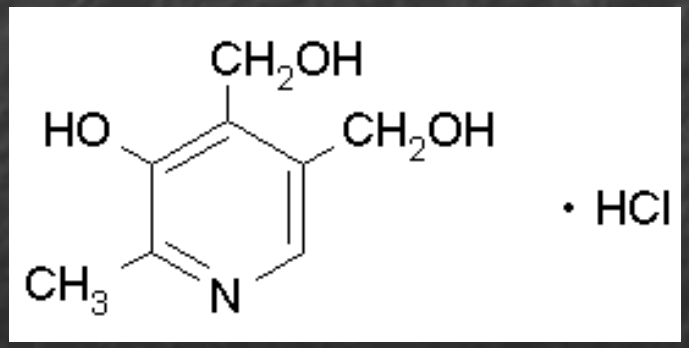
Figure 5: B Vitamin Structures



1. B1-Thiamine

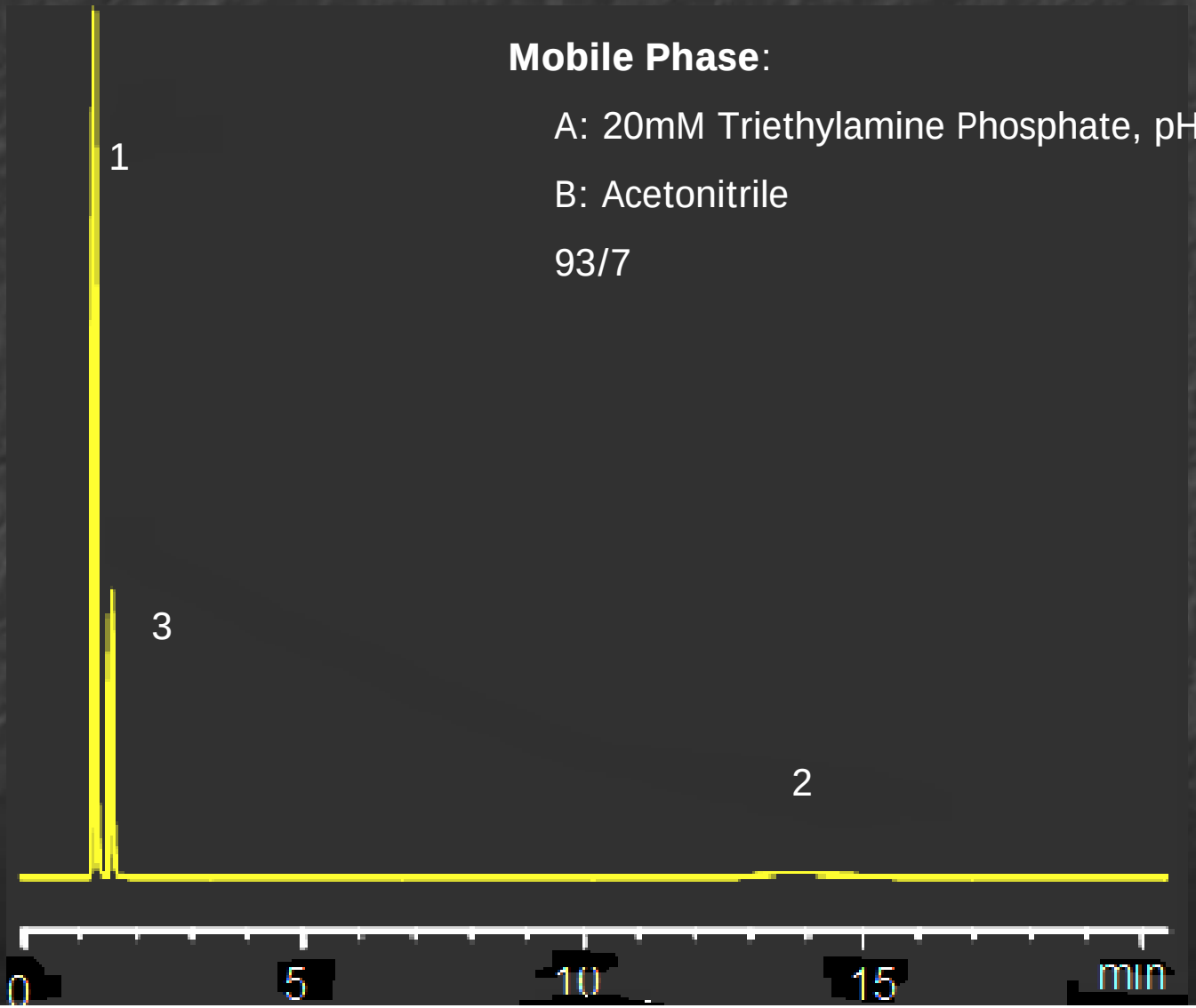


2. B2-Riboflavin



3. B6-Pyroxidine

Figure 6: Separation of B Vitamins on Leading Brand C18



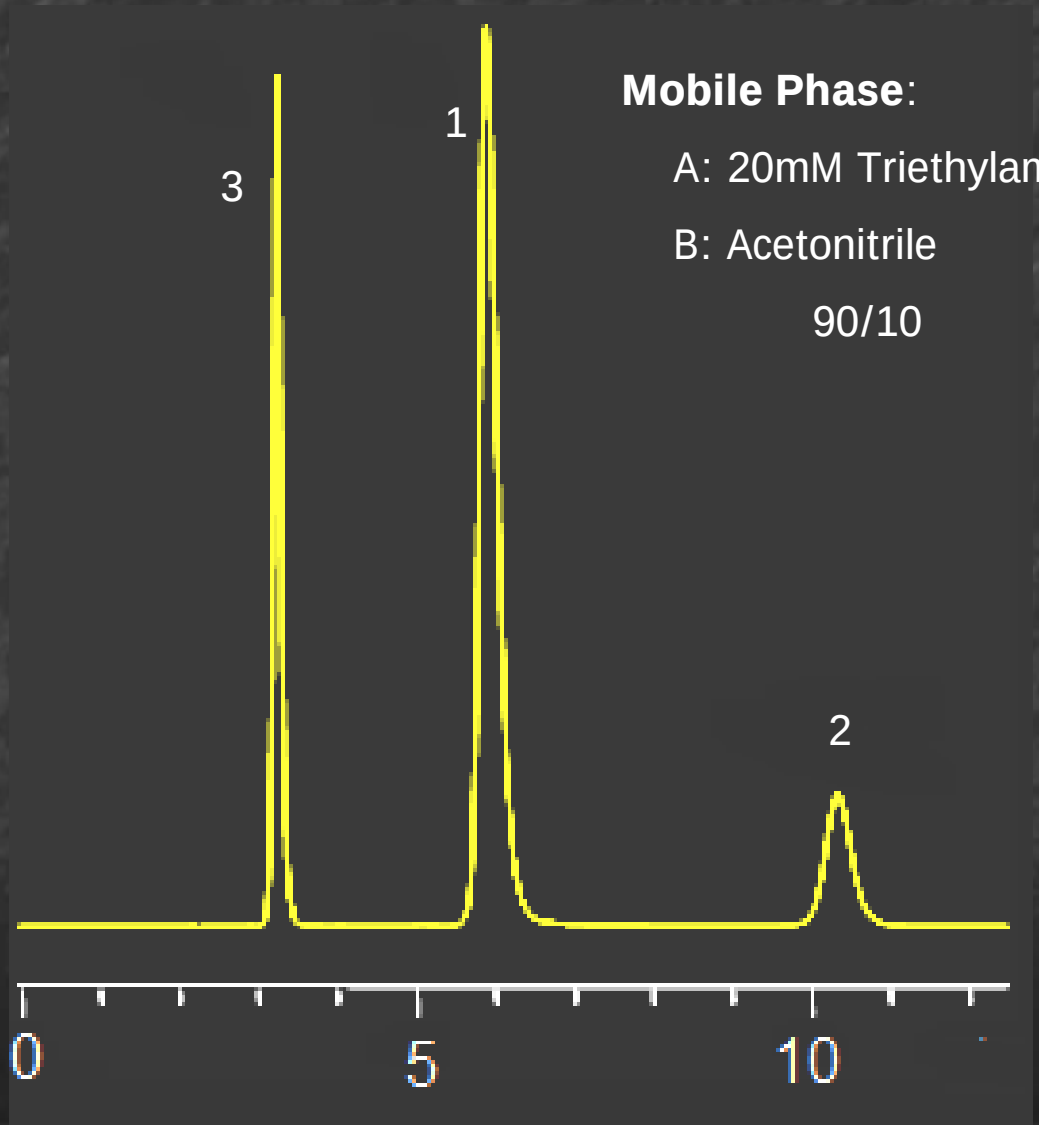
Mobile Phase:

A: 20mM Triethylamine Phosphate, pH 3.0

B: Acetonitrile

93/7

Figure 7: Separation of B Vitamins on Primesep C



Mobile Phase:

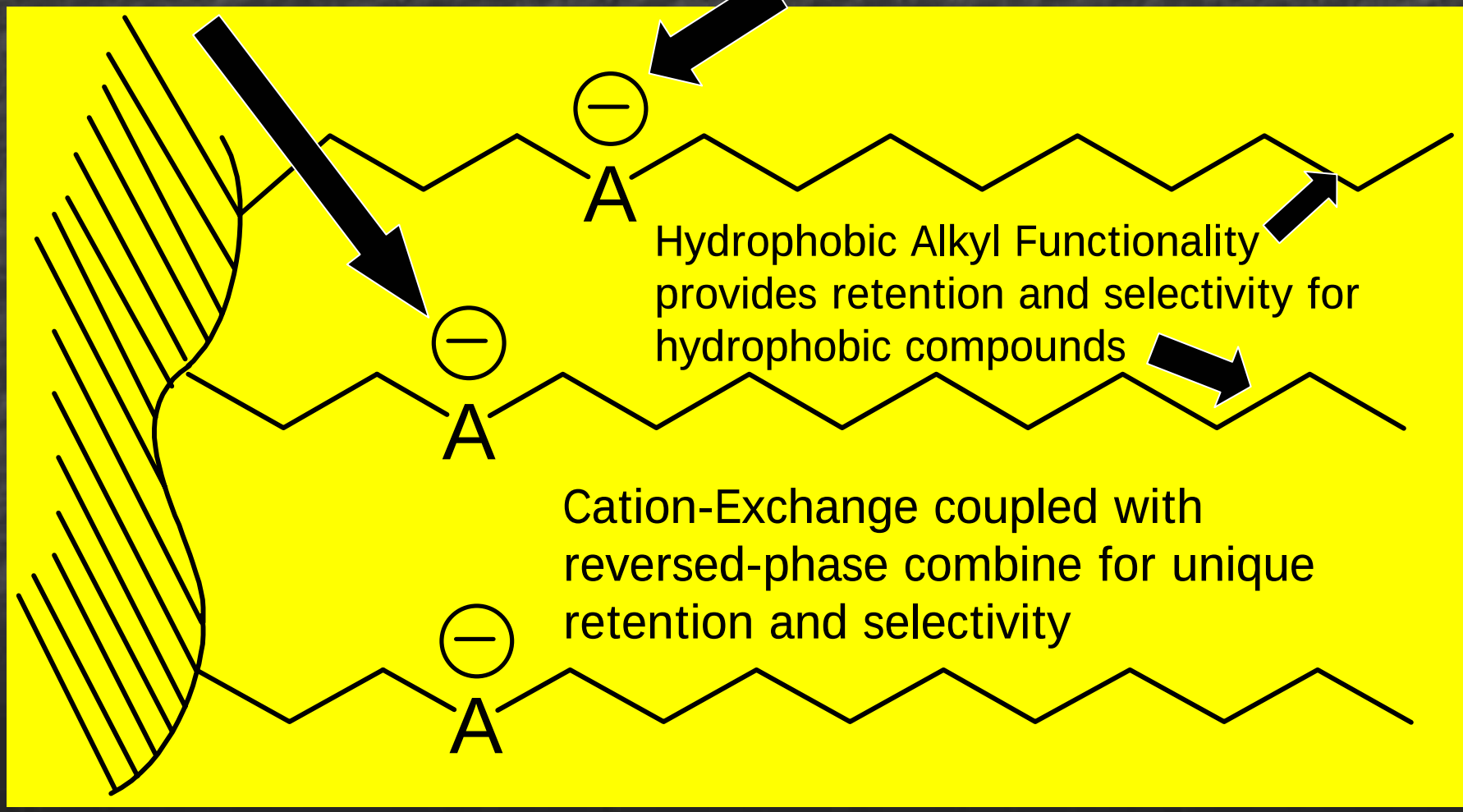
A: 20mM Triethylamine Phosphate, pH 3.0

B: Acetonitrile

90/10

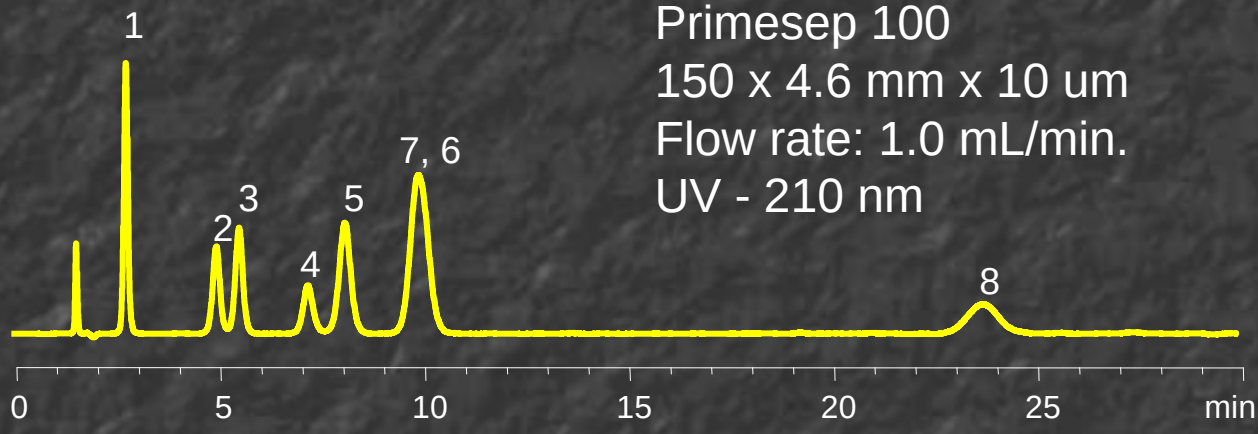
Figure 8: Design of Primesep 100

Cation Ion-Exchange Functionality provides retention and selectivity for polar neutral and polar-basic analytes



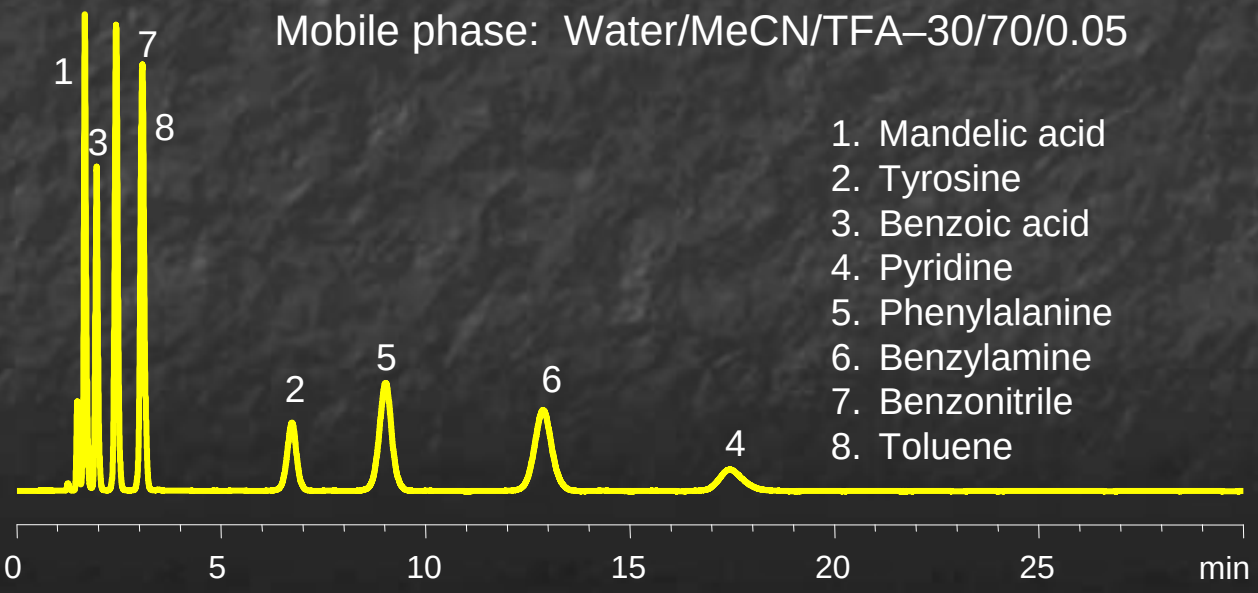
Experimental Figure 9

Mobile phase: Water/MeCN/TFA–70/30/0.2



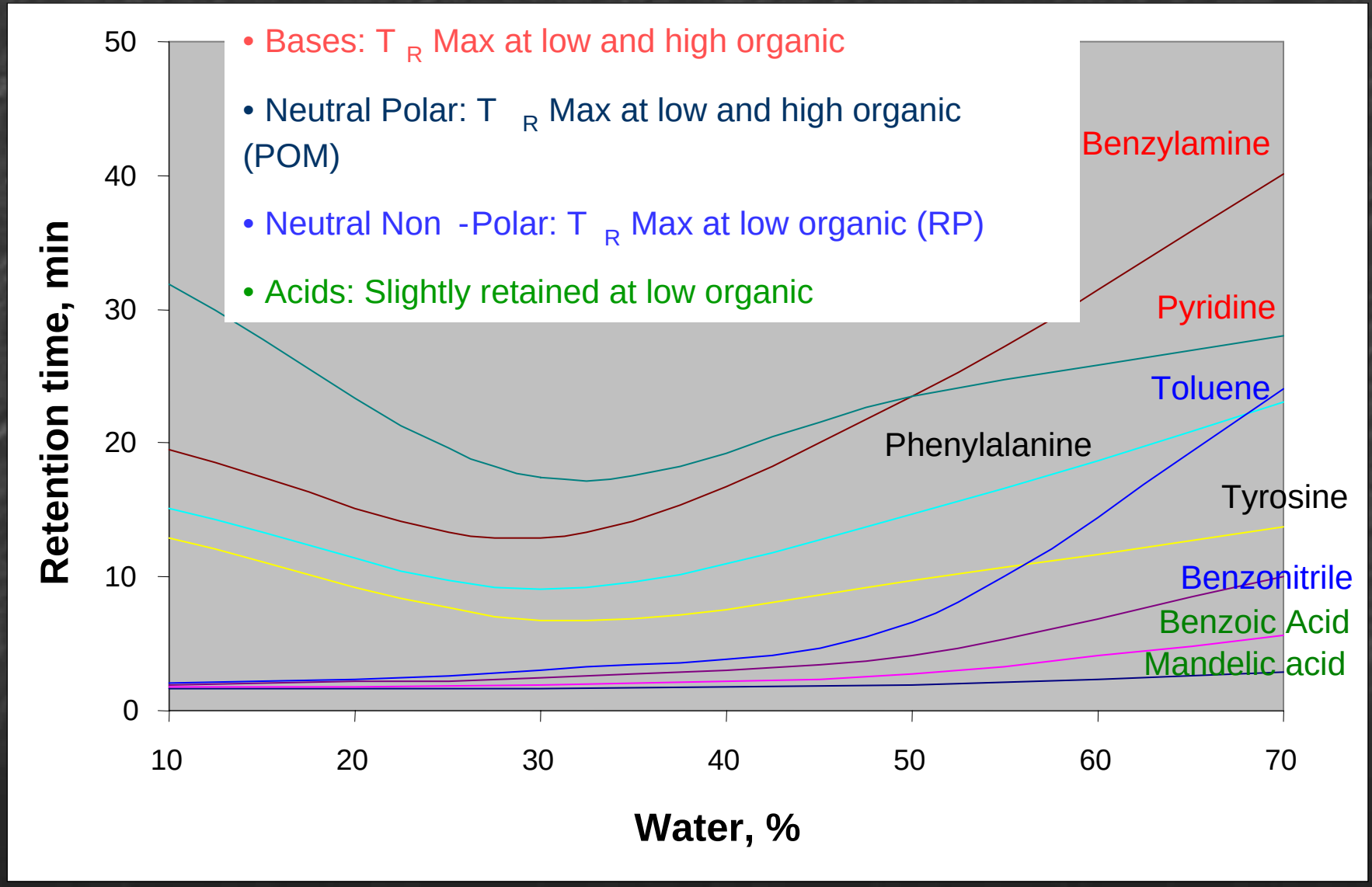
Primesep 100
150 x 4.6 mm x 10 um
Flow rate: 1.0 mL/min.
UV - 210 nm

Mobile phase: Water/MeCN/TFA–30/70/0.05



1. Mandelic acid
2. Tyrosine
3. Benzoic acid
4. Pyridine
5. Phenylalanine
6. Benzylamine
7. Benzonitrile
8. Toluene

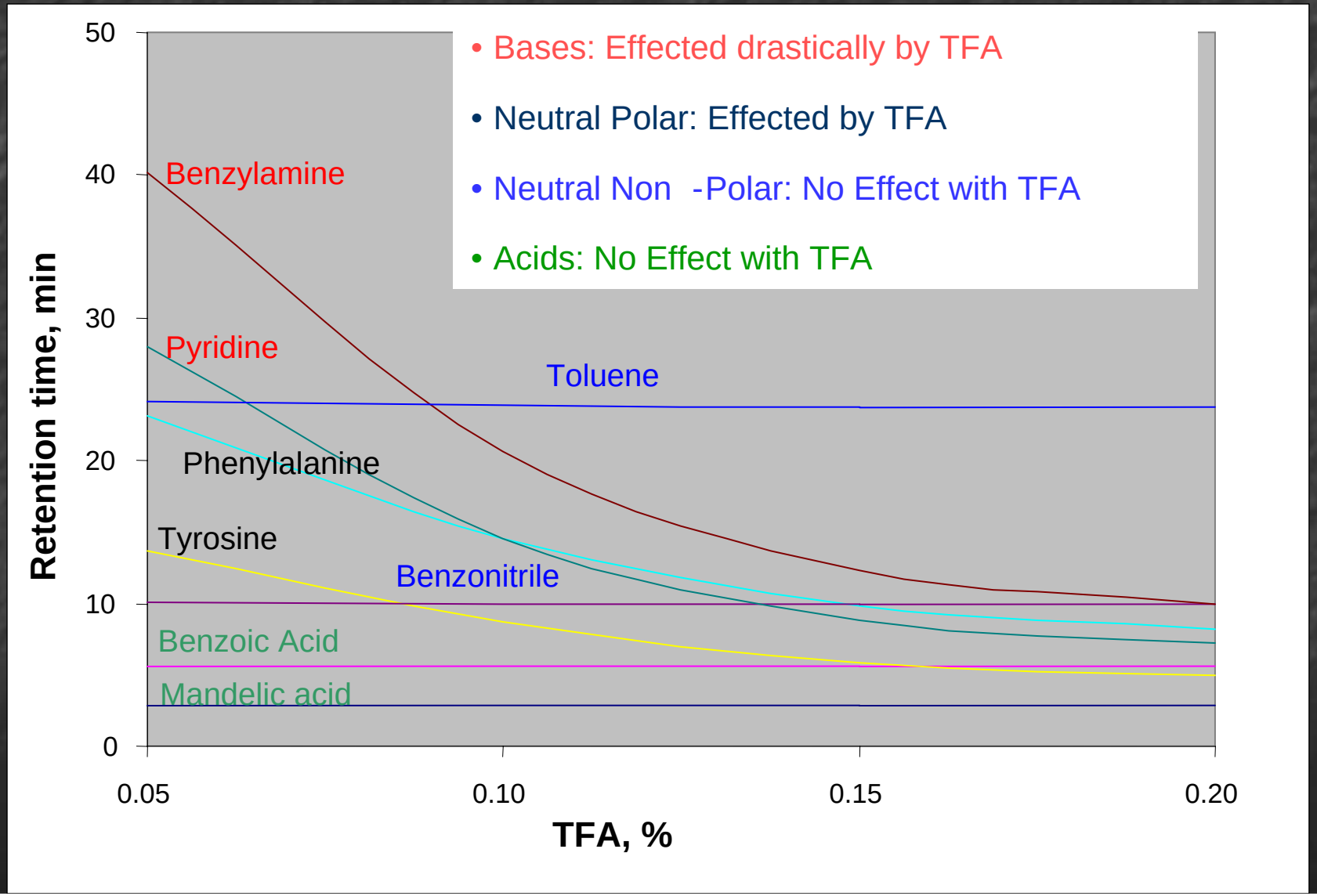
Figure 10: Effect of Water Concentration on Retention Time



Experimental Figure 11

- Column: Primesep 100, 10um, 150x4.6mm
- Mobile Phase: H_2O/Acetonitile/TFA, 70/30
  - TFA was varied as indicated on graph
- Flow: 1.0mL/min
- Detection: UV@210nm

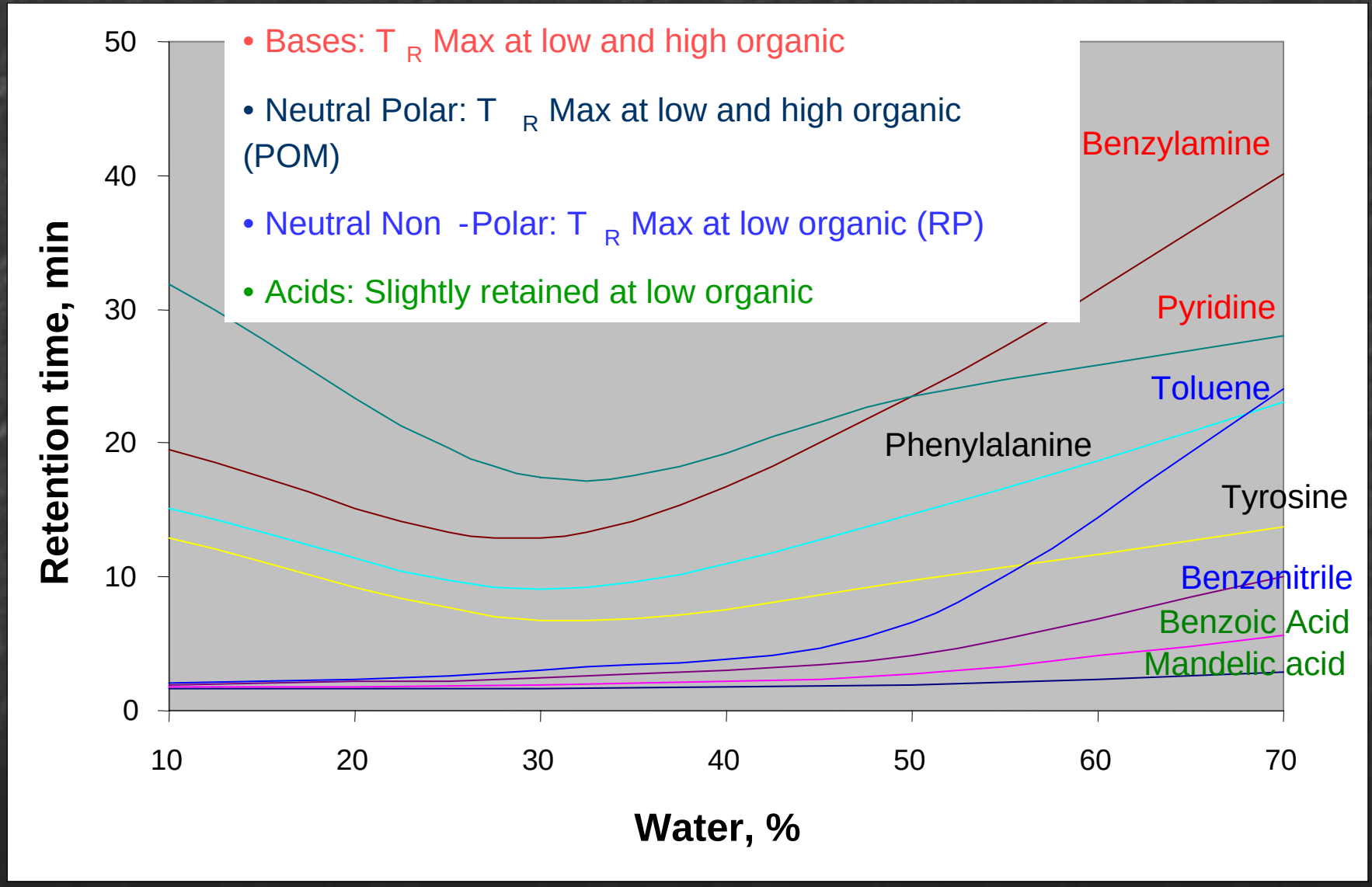
Figure 12: Effect of TFA Concentration on Retention Time



Experimental Figure 13

- Column: Primesep 100, 10um, 150x4.6mm
- Mobile Phase: H_2O/Acetonitile with 0.05% TFA
  - Acetonitrile concentration was varied as indicated on graph
- Flow: 1.0mL/min
- Detection: UV@210nm

Figure 14: Effect of Water Concentration on Retention Time



Results

- Figures 3,4 and 6,7 demonstrate the effectiveness of mixed-mode bonding technology for improving overall chromatography for vastly different compounds.
  - For both sample sets overall analysis was decreased significantly
  - For both sample sets selectivity and retention for polar, early eluting compounds was improved

Results, Con't.

- Figure 9 illustrates the effect of TFA on various types of compounds:
  - Retention of basic compounds is dominated by ion-exchange interactions as demonstrated by the dramatic decrease in retention as TFA concentration is increased
  - Retention of polar-neutral compounds is also affected by the concentration of TFA due to polar-polar interactions
  - Retention of non-polar-neutral and acidic compounds is not affected by concentration of TFA and their retention is dominated by hydrophobic interactions

Results Con't.

- Figure 10 illustrates the effect of water concentration on the retention of various types of compounds
  - All compounds are affected to certain degree by water concentration
    - Basic and polar-neutral compounds demonstrate maximum retention at both high and low water concentration as they transition between reversed-phase and normal phase modes similar to CN type chemistries
    - Neutral non-polar and acidic compounds due not undergo this transition and exhibit hydrobphic, reversed phase retention exclusively.

Discussion

- Primesep C and Primesep 100 mixed-mode HPLC bonded phases offer multiple, independent modes of interaction between analyte and bonded phase.
- Multiple, independent modes of interaction can be utilized to develop optimum separation conditions
  - Ionizable Compounds interact with stationary phase by:
    - Reversed-phase
    - Complex Formation
    - Ion-exchange or ion-exclusion
  - Neutral Compounds participate in different polar interactions
- Amount of acid/buffer in mobile phase influences ion-exchange
- Amount of Organic modifier influences hydrophobic interactions
- Type of acid/buffer affects neutral-polar interactions

Conclusion

When mixed-mode type bonded phases are employed, different analyte properties can be independently controlled. By adjusting the ionic strength, pH and buffer type of the mobile phase. The retention and selectivity for polar, ionic compounds can be established without drastically affecting the retention of very hydrophobic compounds. The amount of organic modifier in the mobile phase can then by varied so that the retention of hydrophobic compounds can be minimized without drastically affecting the retention of early eluting compounds due to the dominating ion-exchange interactions controlling their retention.

This is not feasible with single-mode bonded phases such as C18 chemistries. Because there is only one mode of interaction, hydrophobic interactions, polar and non-polar compounds are affected equally by the amount of organic modifier in the mobile phase due to the lack of dominating ion-exchange and complex forming interactions.

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