**METHOD SELECTION AND OPTIMIZATION**

**Method Selection Based on Analyte Classification**

The functional groups of analytes play a large role in determining the optimal conditions for analysis. If the chemical properties of the analytes are known, both column and mobile phase selection will be considerably easier.

**Method Optimization Sequence**

Once the stationary phase, organic modifier and mobile phase pH have been chosen, retention and selectivity can be further optimized by altering gradient slope and column temperature.

1. Adjust gradient slope
2. Adjust column temperature
3. Adjust column length and flow rate
4. Isocratic mobile phase gradient
5. Replace a portion of the water in the mobile phase with a weaker polar organic solvent

**METHOD OPTIMIZATION**

**ESSENTIAL INFORMATION FOR METHOD DEVELOPMENT**

**Sample solubility**

- A mixture of 75% acetonitrile with 25% methanol is a good general purpose diluent for the best mobile phase pH.
- For sample mixtures containing different types of analytes, use an intermediate pH ~ 5-7.

**Chemical properties: functional groups**

- Analytes will often exhibit the most retention when they are ionized (i.e., bases at low to mid pH, acids at high pH).
- For ionizable analytes, use an intermediate pH (~5-7) with 10 mM ammonium acetate or 0.02% acetic acid.

**Mobile phase preparation**

- For the best gradient performance and reproducibility, a constant linear rate of buffer (10 mM NH₄OH) can be added to further encourage analyte solubility.
- Knowledge of the pH value of the analyte is helpful when developing a method.

**Mobile phase equilibration**

- Conditions new columns with 50 column volumes of 50:50 acetonitrile/water with 10 mM buffer or 0.2% additive solutions. Does the column in this condition?
- Equilibrate the column to 20 column volumes of initial mobile phase conditions before routine use (approximately 2 blank gradients).
- For gradient analyses, re-equilibrate with 5-8 column volumes between injections.

**Needle wash solvents**

- The needle wash solution should closely match the initial mobile phase conditions (90-95% acetate/45% to preserve retention and peak shape).

**SYSTEMATIC SCREENING METHOD DEVELOPMENT PROTOCOL**

**THE IMPORTANCE OF MOBILE PHASE pH**

- Mobile phase pH is a useful parameter to manipulate retention and selectivity.
- In HILIC, retention increases when the compound is ionized.
- For sample mixtures containing different types of analytes, use an intermediate pH ~ 5-7.

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