The Do's and Don'ts of Method Development in Mixed-Mode Chromatography
Part 1. Reversed-Phase Ion-Exchange Columns

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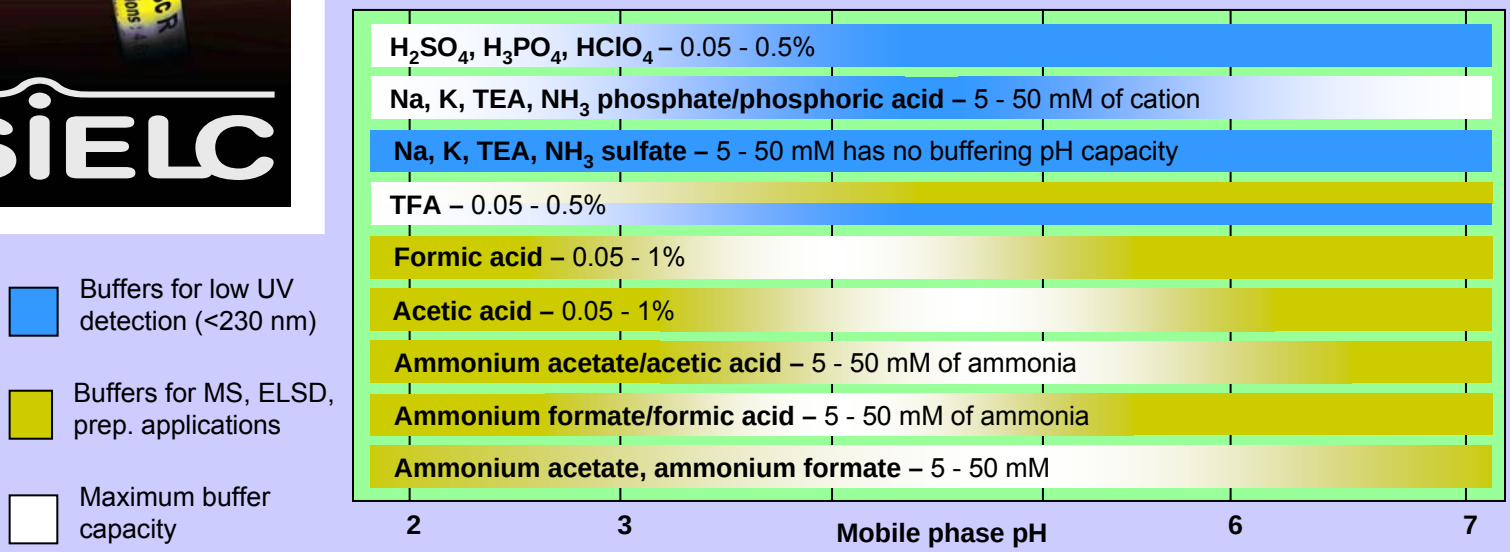
If a reversed-phase chromatography column does not work for your separation, it is most likely that a mixed-mode column is an answer. The purpose of this letter is to guide scientists through the do's and don'ts of method development in mixed-mode chromatography. So, let start from **buffer properties.**

1. Mixed-mode columns almost always work in buffered mobile phase, so it is important to understand the role and properties of common buffers (Chart 1).

Chart 1. Typical buffers and their buffering regions

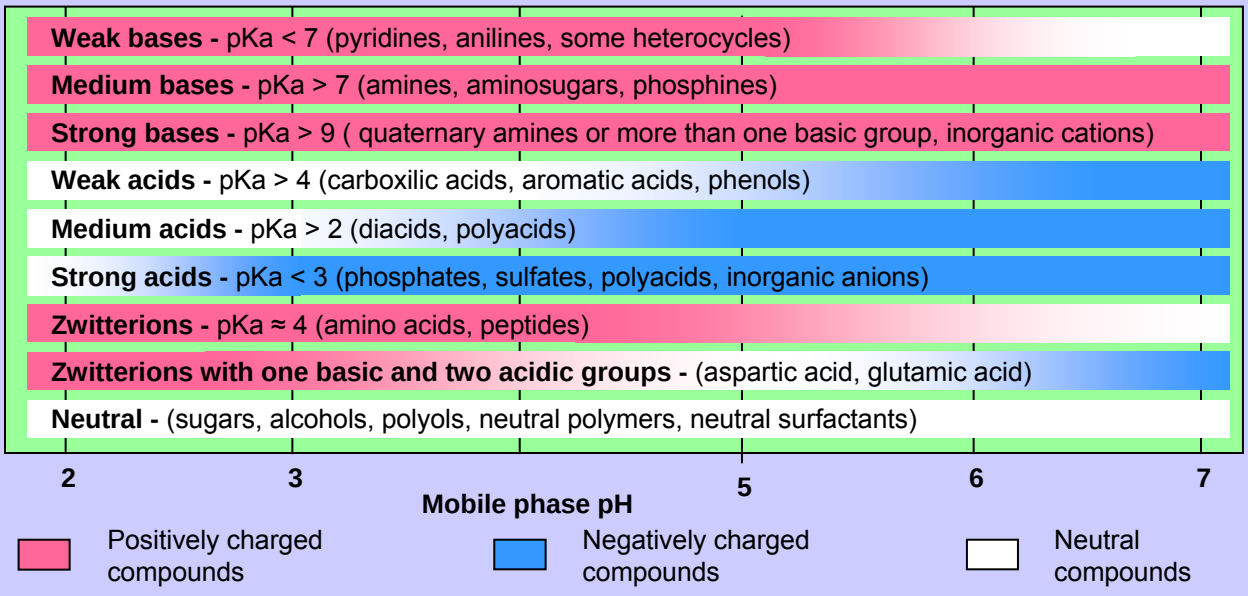


This chart indicates that low UV detection is possible only at and below pH 2 and from pHs 6 to 8. Within these two pH regions, the buffers have sufficient transparency so that low UV active compounds can be measured. For UV detection above 230 nm, the entire mobile phase pH range is available.

2. Analyte charge.

Knowing the available pH range allows to understand and select the ionization state of the analyte in the particular mobile phase. Chart 2 gives the relation of the charge of different small molecules and pH of the mobile phase.

Chart 2. pH / Charge Relation of Different Compounds



3. Column selection

Based on the properties of your compound, decide on the column (table 1) by choosing the appropriate raw for your compounds based on their pKa value or structure. Identify the problem you've are trying to address while using reverse phase column, and then choose a Primesep alternative that would address you problem. If conditions recommended in table 1 are not suitable for your detection technique or does not fully address the problem, a different column and pH can be selected based on following recommendations of the table 2 a, b.

Table 1. Mixed-mode column selection to address separation problems

Compounds	Problem with reverse phase chromatography		
	Too little retention	Peak shape problem	Resolution problem
Bases	Primesep 100, pH 2-5	Primesep D, pH 2-5	Primesep 200, pH 2-5
Acids	Primesep SB, pH 3-5	Primesep 500, pH 5-7	Primesep D, pH 2-5
Zwitterions	Primesep A, pH 2	Primesep A, pH 2	Primesep A, pH 2-5
Neutral	Primesep N, pH 3-5	Primesep D, pH 2-7	Primesep P, pH 2-5

For example, Primesep 100 column is recommended by table 1 for bases within mobile phase pH range of 2-5 to address low retention problem. However the retention is now too strong. You can then select a different column (Primesep 200 or Primesep C), according to table 2a, and get lower retention, or you can decrease pH below 2 to obtain shorter retention

Table 2a. Anion-exchange column strength

Column type	Column strength
Primesep SB	**Higher**
Primesep B	
Primesep D	
Primesep B2	**Lower**

4. Check more than one condition.

Inject your standards or markers with three different conditions: high organic (50% ACN) with high buffer concentration (50 mmol), low organic (20% ACN) with high buffer concentration (50 mmol) and high organic (50% ACN) with low buffer concentration (20 mmol). Look at retention patterns and see how compounds are eluting from the column. Adjust retention time based on the following rules:

Too Much Retention

- Increase mobile phase ionic strength
- Use buffer with lower pH
- Increase % of organic
- Try weaker column (Table 2)
- Decrease column length (columns are available as short as 10 mm)

Not Enough Retention

- Decrease mobile phase ionic strength
- Use buffer with higher pH
- Decrease % of organic. Zero organic mobile phase is acceptable too.
- Try stronger column (Table 2)
- Increase column length (columns are available as long as 250 mm)

Not Enough selectivity

- Change organic and buffer concentration. Try several different concentrations
- Try different buffer
- Try different column

Poor Peak shape

- For di- and poly-basic analytes try Primesep C
- Reduce sample injection volume

Need MS Conditions

- Use formic acid, acetic acid or ammonium formate, ammonium acetate as the buffer.
- Use TFA instead of phosphate, sulfate for low pH applications.

Table 2b. Cation-exchange column strength

Column type	Column strength
Primesep A	**Higher**
Primesep 100	
Primesep P	
Primesep 200	
Primesep C	
Primesep 500	**Lower**

5. Column care

Knowing pH limits of the column, compatible and incompatible solvents, temperature limits, long and short term storage conditions can help you in developing robust method. For validation, never use column from unknown source or old column.

Old columns are good for initial screening but a new column must be used for final optimization and method validation. Selectivity of separation and column can be altered by improper use and storage (Fig. 1). Mixed-mode columns with cation-exchange groups are not compatible with MeOH. Esterification of stationary phase can cause change selectivity of the column's stationary phase (Fig. 2)

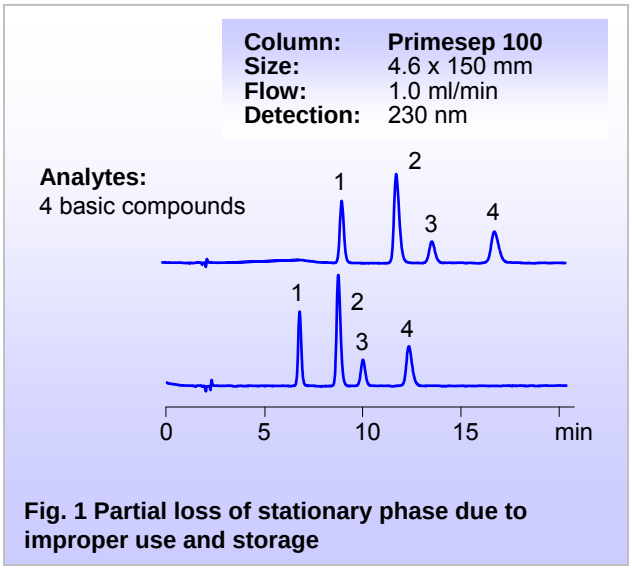


Fig. 1 Partial loss of stationary phase due to improper use and storage

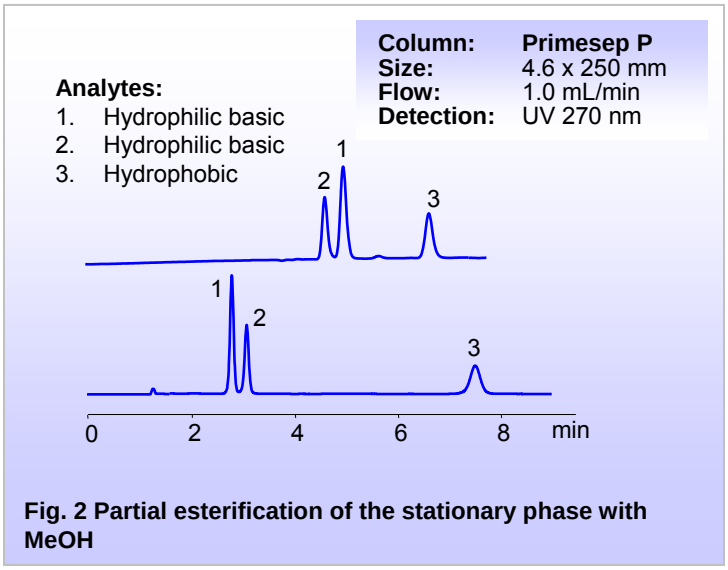


Fig. 2 Partial esterification of the stationary phase with MeOH

6. Equilibrate your column.

Column needs to be fully equilibrated with a new mobile phase. Column ion capacity needs to be consider in case of the mixed-mode columns. If a new buffer is used in the mobile phase, the amount of the ions in the mobile phase (ion strength) can be increased during equilibration to short equilibration time. If your method calls for 5 mmol buffer, use 50-100 mmol buffer instead during equilibration to remove ions from previous method or column storage mobile phase. If you are switching from strong acid to weak acid, you might need to go through the intermediate buffer. For example, replacement of TFA with acetic acid on the anion-exchange column requires a short wash with ammonium acetate (50-100 mmol) before switching to acetic acid. Otherwise it may take very long time to completely remove all TFA ions from the column (Fig. 3)

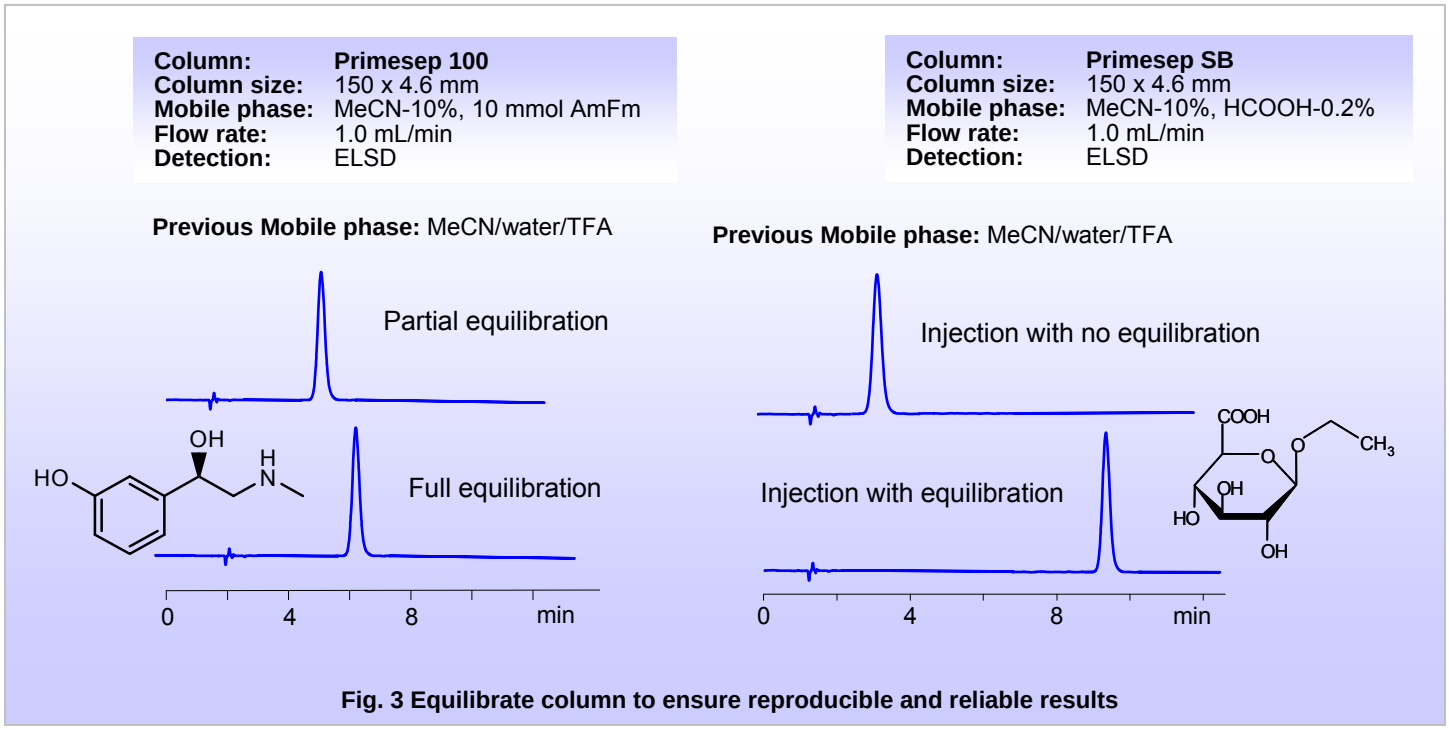


Fig. 3 Equilibrate column to ensure reproducible and reliable results

7. Peak area control.

Inject sample without column and measure peak area. This will help you to see if all of your sample eluted from the column. In most cases peak area without column and sum of peaks areas with the column should be very close (95-105%). Pay attention to "detection" properties of the analytes (Fig. 4). Very often, when people switch from low retention in RP chromatography to mixed-mode columns they can be mislead by much longer retention of parent compound (peak area 1780) and one of the possible impurities (peak area 346). They often assume that the peak they see on chromatogram is the main compound and don't realize that a stronger mobile phase is required for elution of target compound.

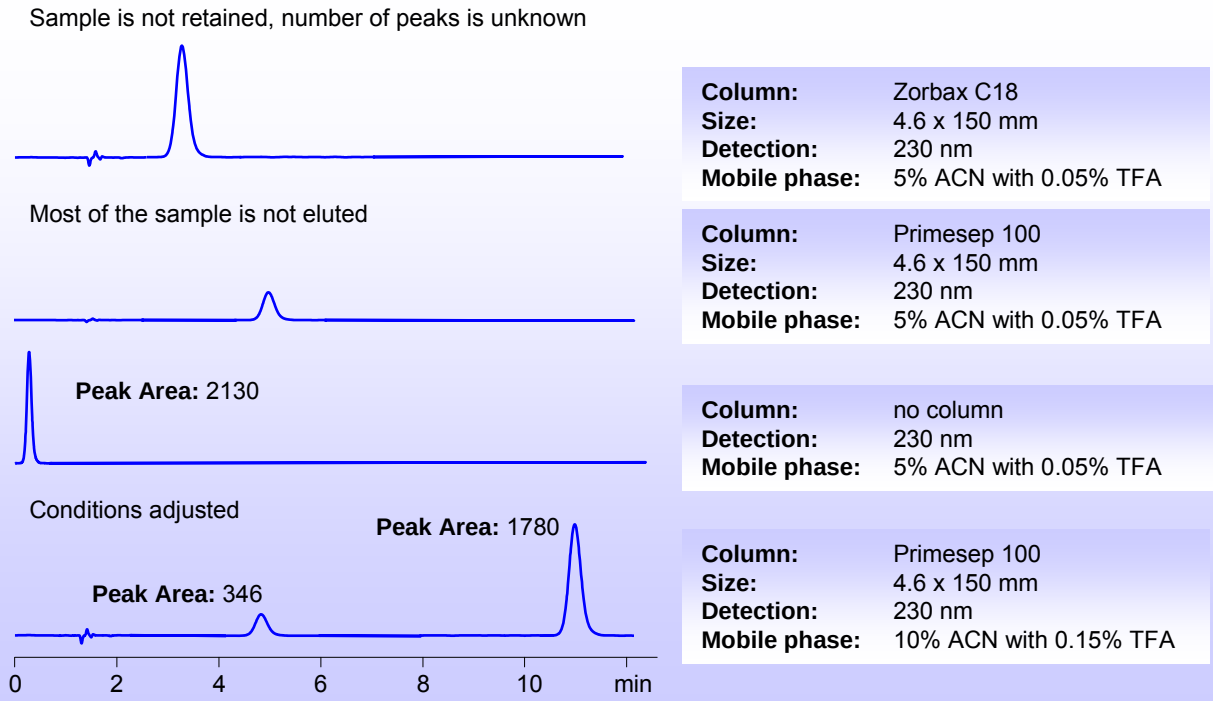


Fig. 4 Injecting sample without column to ensure elution of all compounds of the sample

8. Choose column dimensions.

Starting method development with short column can save a lot of time and solvent for initial screening (Fig. 5). When column and conditions are established, the longer column might be selected for some of the methods to increase selectivity and method robustness.

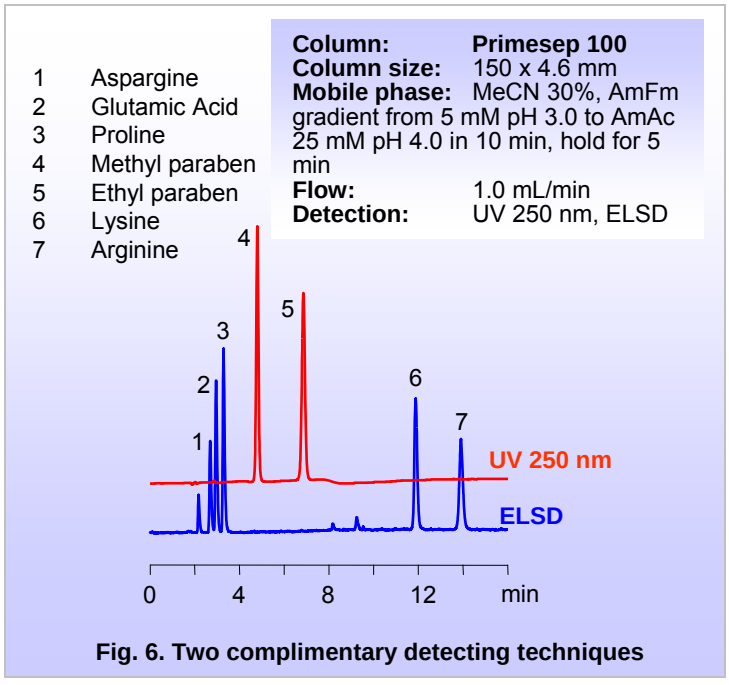


Fig. 6. Two complimentary detecting techniques

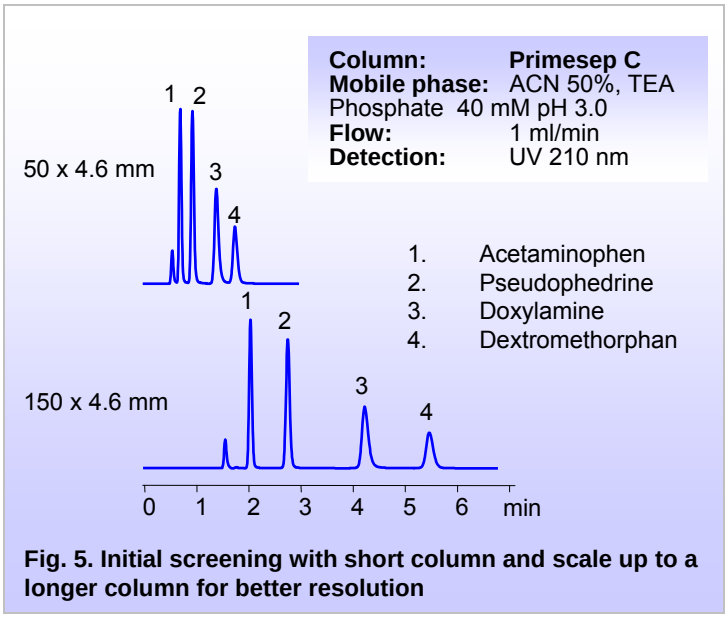


Fig. 5. Initial screening with short column and scale up to a longer column for better resolution

9. Detection choice for method development.

Use two independent detection technique. Some of the compounds can have limited UV activity and using additional detection technique, like ELSD, CAD or LC/MS will ensure that you see everything that you have in your sample and everything what comes from the column (Fig. 6)