

How do I Develop an HPLC Method?

Step 1. Find a reference with a similar analysis and copy it!

Step 2. If you can't find a reference to your analysis, read on...

HPLC method development is not very difficult when a literature reference for the same or similar compounds to be analyzed can be found, but what happens when references to the compounds of interest do not exist?

The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of the analytes will allow the most appropriate mode of HPLC to be selected.

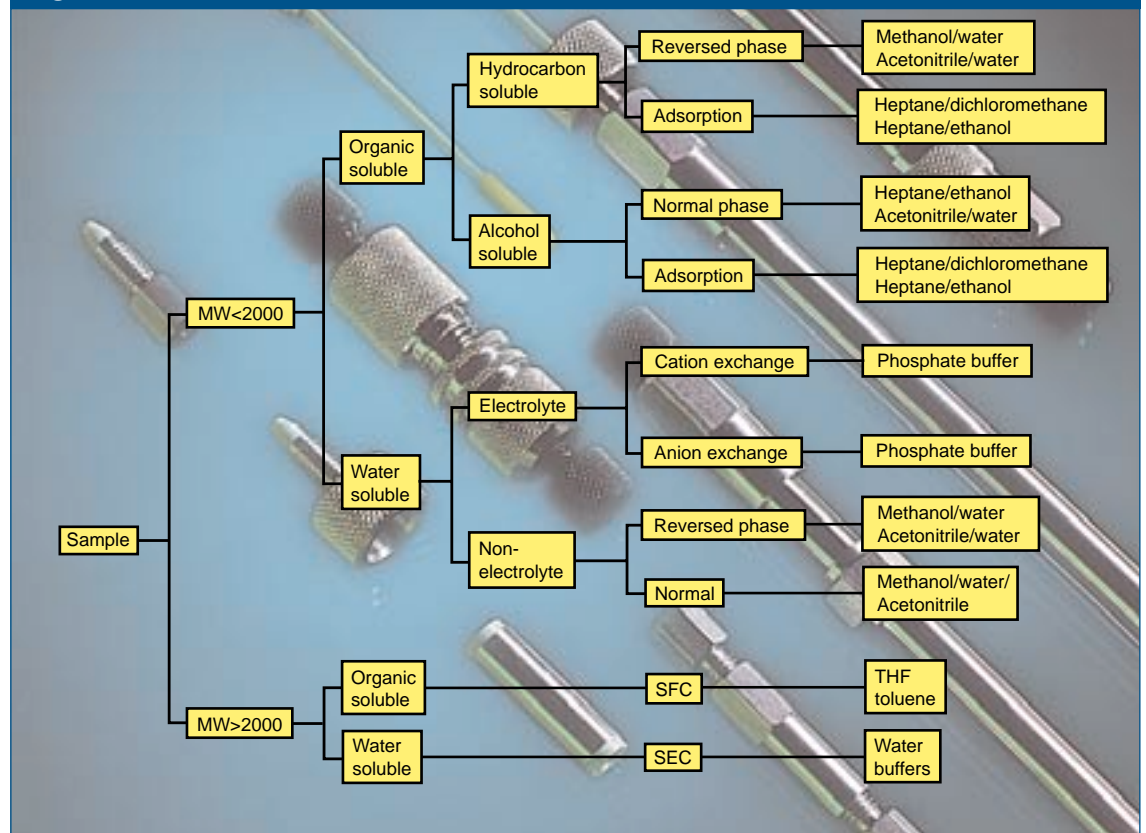
Figure 1 summarizes appropriate separation modes and mobile phases to consider for method development. (Complex samples may require an initial clean-up step to remove possible interferences.)

The Best Mobile Phase

Since the mobile phase governs solute-stationary phase interactions, its choice is critical.

- Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided.
- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column, trace impurities can easily concentrate in the column and eventually be detrimental to the results. Spectro or HPLC grade solvents are recommended.
- Volatility should be considered if sample recovery is required.
- Viscosity should be less than 0.5 centipoise, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.
- LC/MS - only volatile buffers.

Figure 1. Phase Selection Process



Given **Figure 1**, there is a temptation to quickly take an appropriate column, prepare a sample and suitable mobile phase, and run it on the HPLC system. This may work with some trial and error, but the key to efficient method development is planning.

The Best Detector

The next consideration should be the choice of detector.

There is little use in running a separation if the detector one uses cannot "see" all the components of interest, or conversely, if it "sees" too much. UV-vis detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. Unfortunately UV-vis detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chromophores, such as aromatic rings, for UV-vis detection. **Table 1** summarizes some of the available options.

The Best Column Length

Many chromatographers make the mistake of simply using what is available. Often this is a 250 x 4.6mm C18 column. These columns are able to resolve a wide variety of compounds (due to their selectivity and high plate counts) and are common to most laboratories. While many reverse phase separations can be carried out on such a column, its high resolving capabilities are often unnecessary, as illustrated in **Figure 2**. Method development can be streamlined by starting with shorter columns; 150, 100 or even 50mm long. This is simply because they have proportionally shorter run times.

The Best Stationary Phase

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C8 phase (reversed phase) can provide a further time saving over a C18, as it does not retain analytes as strongly as the C18 phase. For normal phase applications, cyano (nitrile) phases are the most versatile.



The Best Internal Diameter

By selecting a shorter column with an appropriate phase, run times can be minimized so that an elution order and an optimum mobile phase can be quickly determined. It can also be advantageous to consider the column internal diameter. Many laboratories use 4.6mm ID columns as a standard, but it is worth considering the use of 4mm ID columns as an alternative. These require only 75% of the solvent flow that a 4.6mm column uses. This translates to a 25% solvent saving over the life of the column and can be even more significant if a routine method is developed for such a column.

Figure 2. Effect of Column length

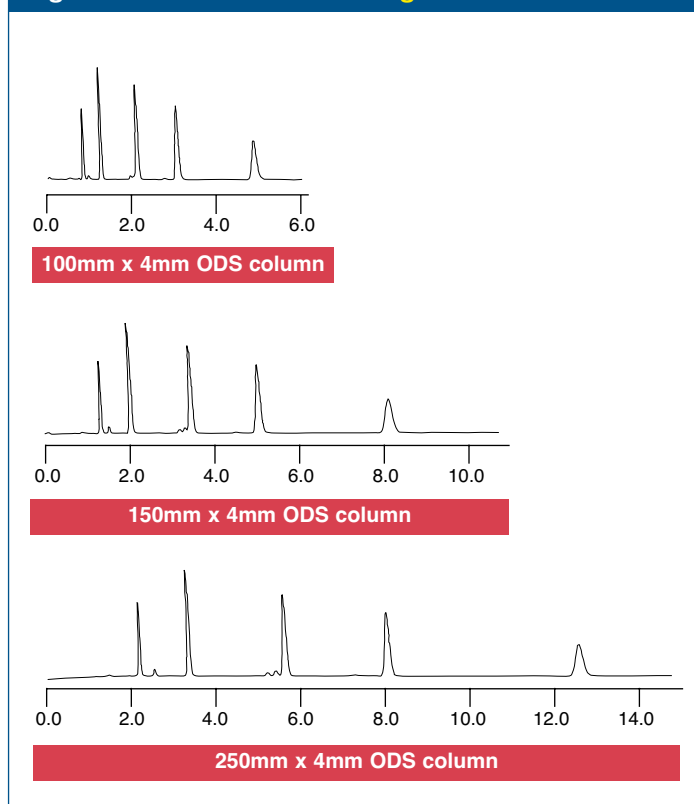


Table 1. Detector options

Detector	Analytes	Solvent Requirements	Comments
UV-vis	Any with chromophores	UV-grade non-UV absorbing solvents	Has a degree of selectivity and is useful for many HPLC applications
Fluorescence	Fluorescent compounds	UV-grade non-UV absorbing solvents	Highly selective and sensitive. Often used to analyze derivatized compounds
Refractive Index (RI)	Compounds with a different RI to the mobile phase	Cannot run mobile phase gradients	Virtually a universal detector but has limited sensitivity
Conductivity	Charged or polar compounds	Mobile phase must be conducting	Excellent for Ion Exchange methods
Electrochemical	Readily oxidized or reduced compounds, especially biological samples	Mobile phase must be conducting	Very Selective and sensitive
Evaporative Light Scattering (ELSD)	Virtually all compounds	Must use volatile solvents and volatile buffers	A universal detector which is highly sensitive. Not selective
Mass Spectrometer (MS)	Broad range of compounds	Must use volatile solvents and volatile buffers	Highly sensitive and is a powerful 2nd dimensional analytical tool. Many modes available. Needs trained operators