Purification by Liquid Chromatography

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Chromatography Application Note AN04

Application Overview

This document provides basic information on chromatographic separations work. This knowledge can then be applied to producing satisfactory results when using Isco Redi*Sep*® Normal Phase and reusable Redi*Sep* Reversed Phase C18 columns for purification using Isco Combi*Flash*® instruments. The main purpose of Combi*Flash* systems is to preserve the fraction for later use. By contrast, analytical HPLCs are for analysis and the analyzed sample is not recovered. For more information on specific applications refer to Isco's library of application notes.

Liquid Chromatography is a useful tool for purification when:

1. *The compound to be purified into fractions is in liquid form or can be dissolved.* Compounds that are difficult to dissolve can be loaded via a solid sample load cartridge or injected directly onto the top of the column. See Isco AN13 on injection for more information.
2. *It is desirable to non-destructively separate the compound so that it may be conserved for later use.* Liquid chromatography has the capability to separate a compound into fractions. Isco's Combi*Flash* instruments are particularly useful for this purpose.
3. *The compound can be separated based on affinities of individual components.* If the compound can be separated by thin-layer chromatography (TLC) then it is very likely that it can be separated on a column. The advantage of column systems is that larger quantities of compounds can be purified automatically.

There are several possible reasons to separate a sample into its components:

- Purification is desired so that the contents of an individual compound or fraction can be studied.
- A certain desired component is to be separated away or purified from unwanted components.
- The mixture of components is too complex, or similar to each other, for direct analysis.

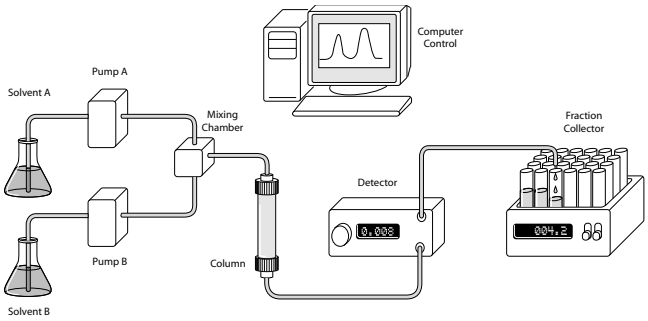

Figure 1: Chromatographic System Components

General Theory

In a chromatographic system, a mixture of compounds is introduced or "injected" onto the column. The solvent delivery system then pumps liquid mobile phase through the column. After passing through the column, the separated fraction is passed through a detector. The data the detector produces is in the form of peaks, the amplitude of which is based on the amount of light absorbed in the flowcell.

A chromatographic system consists of four basic components:

1. A chromatographic column:
   a. Supporting medium is column packing, typically made up of beads, that does not move. The basic material is silica and this provides the surface on which the stationary phase is bound.
   b. Stationary phase is the layer or coating that is bound to the supporting medium and interacts with the compounds carried along in the mobile phase.
   c. Mobile phase is the solvent mixture that moves through the column, solubilizing the sample and exposing it to the stationary phase. This mixture can be isocratic, where the mixture is fixed during the entire run. A solvent gradient is when the mixture is changed periodically or continually through the run.
2. A pumping system is required to control the flow of mobile phase through the column.
   a. Isocratic systems contain a single mobile phase mixture.
   b. Gradient systems combine two or more mobile phases allowing more control over elution. The terms "A" and "B" are used to identify the solvents that make up the gradient.

   i. Solvent A is the weaker solvent and is used for equilibration and normally makes up the bulk of the mobile phase.
   ii. Solvent B is the stronger solvent and is used to control retention time in a gradient system.
3. A detector capable of identifying relevant peaks for purposes of collection.
4. A fraction collector system for collection of peaks of interest. Frequently all fractions are collected regardless of detected peaks.
   a. Collection criteria can be either time based or peak based (via a detector). When the absorbance of the species passing through the flow-cell exceeds certain parameters, the column effluent is collected.
      i. Threshold—an absorbance value that must be exceeded for the column effluent to be collected as a peak.
      ii. Slope—a certain value based on a Gaussian distribution that must be exceeded for the column effluent to be collected as a peak.

Operation

The order of elution through the column depends largely on the compound's affinity for the stationary phase versus its affinity for the mobile phase. The components of the sample that interact most strongly with the stationary phase will take longer to pass through the column and will collected in the last fractions. Components with weaker interactions will pass through more quickly and will be collected earlier.

In isocratic systems it is merely a matter of time before the mobile phase carries the fraction off of the column. In a gradient system the mobile phase reaches certain strength (%B) before the fraction is carried off the column.

For normal phase columns, the molecule interacts directly with the silica which is polar. The polar character of this supporting media will have an affinity for compounds that are more polar, ionic, or aromatic.

For C18 reversed phase the compound interacts with a C18 group that is attached to the silica. The C18 separation is largely a function of polarity where the more polar compounds come off earlier, and the less polar compounds come off later.

In normal phase chromatography, the stationary phase is relatively polar and the mobile phase is nonpolar. A common mobile phase in normal phase chromatography is hexane, which is fairly nonpolar. Adding ethyl acetate to the hexane mobile phase will make more polar. This will cause compounds to be eluted that are bound too tightly for hexane alone to elute. This addition of the more polar solvent can be done isocratically with a fixed A solvent blend. "B" mobile phase can be gradually added via a gradient system to elute the compounds more quickly with a higher purity.

In reversed phase there are several differences from that of normal phase. Reversed phase is more commonly performed on a gradient system and less likely to be isocratic. In reversed phase the A solvent is the more polar solvent, commonly water (very polar). The B solvent will be a less polar and more organic solvent like acetonitrile or methanol. Redi*Sep* reversed phase columns utilize a C18 hydrocarbon group attached to the supporting silica.

Certain compounds will respond differently when injected onto reversed or normal phase. As a general rule reversed phase should be used for more polar molecules and normal phase for more nonpolar molecules. If the substance to be tested is soluble in water then it is fairly polar. If the substance is soluble in hexane then it is fairly nonpolar.

References

Bidlingmeyer, B. A. *Practical HPLC Methodology and Applications*; John Wiley & Sons: New York, 1992; Chapter 1.

Poole, C. F. and Poole, S. K. *Chromatography Today*; Elsevier Science Publishing Company: New York, 1991.

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