How Does High Performance Liquid Chromatography Work?

The components of a basic high-performance liquid chromatography [HPLC] system are shown in the simple diagram in Figure E.

A reservoir holds the solvent [called the mobile phase, because it moves]. A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute. An injector [sample manager or autosampler] is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to *see* the separated compound bands as they elute from the HPLC column [most compounds have no color, so we cannot see them with our eyes]. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography [discussed in the section on HPLC Scale].

Note that high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

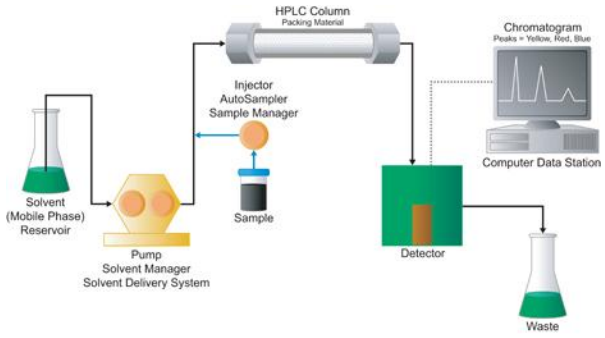

Figure E: High-Performance Liquid Chromatography [HPLC] System

The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents (see Figure F). Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector [ELSD]. The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer [MS] to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

HPLC Operation

A simple way to understand how we achieve the separation of the compounds contained in a sample is to view the diagram in Figure G.

Mobile phase enters the column from the left, passes through the particle bed, and exits at the right. Flow direction is represented by green arrows. First, consider the top image; it represents the column at time zero [the moment of injection], when the sample enters the column and begins to form a band. The sample shown here, a mixture of yellow, red, and blue dyes, appears at the inlet of the column as a single black band. [In reality, this

sample could be anything that can be dissolved in a solvent; typically the compounds would be colorless and the column wall opaque, so we would need a detector to see the separated compounds as they elute.]
After a few minutes [lower image], during which mobile phase flows continuously and steadily past the packing material particles, we can see that the individual dyes have moved in separate bands at different speeds. This is because there is a competition between the mobile phase and the stationary phase for attracting each of the dyes or analytes. Notice that the yellow dye band moves the fastest and is about to exit the column. The yellow dye likes [is attracted to] the mobile phase more than the other dyes. Therefore, it moves at a *faster* speed, closer to that of the mobile phase. The blue dye band likes the packing material more than the mobile phase. Its stronger attraction to the particles causes it to move significantly *slower.* In other words, it is the most retained compound in this sample mixture. The red dye band has an intermediate attraction for the mobile phase and therefore moves at an *intermediate* speed through the column. Since each dye band moves at different speed, we are able to separate it chromatographically.

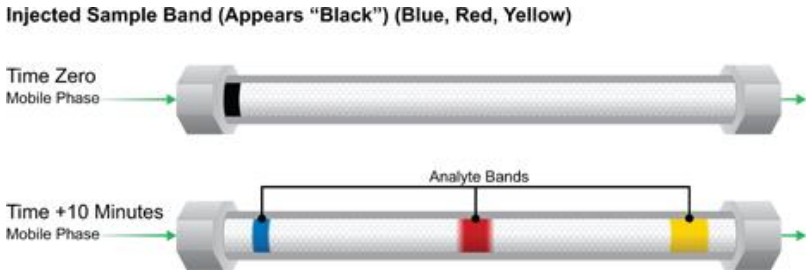

Figure G: Understanding How a Chromatographic Column Works – Bands

What Is a Detector?

As the separated dye bands leave the column, they pass immediately into the detector. The detector contains a flow cell that *sees* [detects] each separated compound band against a background of mobile phase [see Figure H]. [In reality, solutions of many compounds at typical HPLC analytical concentrations are colorless.] An appropriate detector has the ability to sense the presence of a compound and send its corresponding electrical signal to a computer data station. A choice is made among many different types of detectors, depending upon the characteristics and concentrations of the compounds that need to be separated and analyzed, as discussed earlier.

What Is a Chromatogram?

A chromatogram is a representation of the separation that has chemically [chromatographically] occurred in the HPLC system. A series of peaks rising from a baseline is drawn on a time axis. Each peak represents the detector response for a different compound. The chromatogram is plotted by the computer data station [see Figure H].

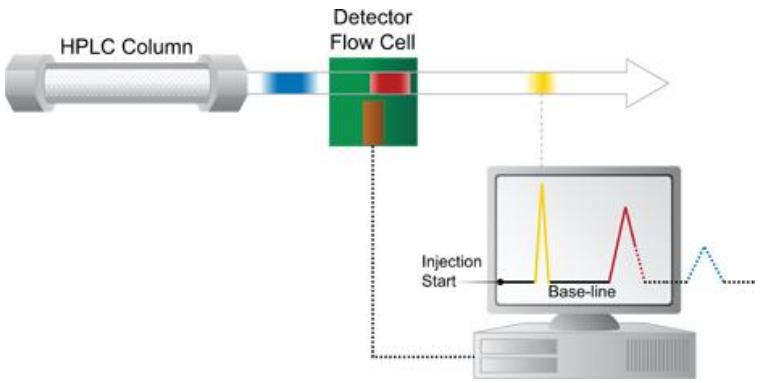

Figure H: How Peaks Are Created

In Figure H, the yellow band has completely passed through the detector flow cell; the electrical signal generated has been sent to the computer data station. The resulting chromatogram has begun to appear on screen. Note that the chromatogram begins when the sample was first injected and starts as a straight line set near the bottom

of the screen. This is called the baseline; it represents pure mobile phase passing through the flow cell over time. As the yellow analyte band passes through the flow cell, a stronger signal is sent to the computer. The line curves, first upward, and then downward, in proportion to the concentration of the yellow dye in the sample band. This creates a peak in the chromatogram. After the yellow band passes completely out of the detector cell, the signal level returns to the baseline; the flow cell now has, once again, only pure mobile phase in it. Since the yellow band moves fastest, eluting first from the column, it is the first peak drawn.

A little while later, the red band reaches the flow cell. The signal rises up from the baseline as the red band first enters the cell, and the peak representing the red band begins to be drawn. In this diagram, the red band has not fully passed through the flow cell. The diagram shows what the red band and red peak would look like if we stopped the process at this moment. Since most of the red band has passed through the cell, most of the peak has been drawn, as shown by the solid line. If we could restart, the red band would completely pass through the flow cell and the red peak would be completed [dotted line]. The blue band, the most strongly retained, travels at the slowest rate and elutes after the red band. The dotted line shows you how the completed chromatogram would appear if we had let the run continue to its conclusion. It is interesting to note that the width of the blue peak will be the broadest because the width of the blue analyte band, while narrowest on the column, becomes the widest as it elutes from the column. This is because it moves more slowly through the chromatographic packing material bed and requires more time [and mobile phase volume] to be eluted completely. Since mobile phase is continuously flowing at a fixed rate, this means that the blue band widens and is more dilute. Since the detector responds in proportion to the concentration of the band, the blue peak is lower in height, but larger in width.