2. Liquid Chromatography Basics

History of Liquid Chromatography

Liquid chromatography was defined by Mikhail S. Tswett (1906–1907) who separated leaf pigments into different colored bands using chalk powder ($CaCO_3$) as adsorbent. Then reversed phase chromatography, ion exchange chromatography, size exclusion chromatography were developed. In 1971 J.J. Kirkland has succeeded in the production of chemically bonded packing material for liquid chromatography, and contributed to establish the basics of high-performance liquid chromatography, which are now one of the most important analysis methods.

HPLC Equipments

HPLC equipments are connected as shown in Figure 1 in the order of mobile phase flow from mobile phase reservoir, pump, injector, column, detector to waste solvent container. Samples are introduced into mobile phase through the injector and separated by the column. Chromatogram is drawn by recorder.

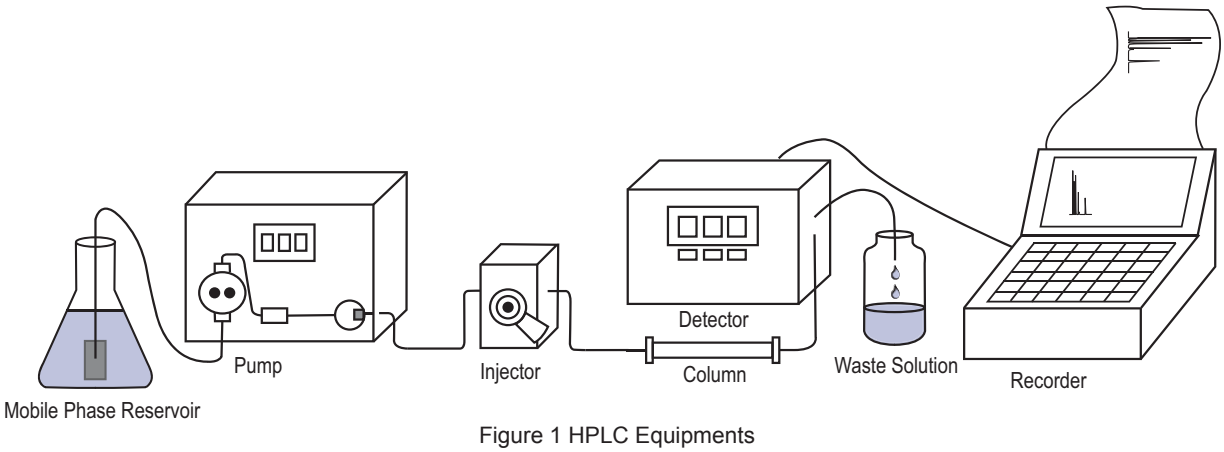

Figure 1 HPLC Equipments

- Mobile Phase Reservoir
  Glass bottle or conical flask is used as a mobile phase reservoir. To avoid clogging from insoluble compounds in the mobile phase, a suction filter (or sinker) is attached to the inlet.
- Pump
  Send mobile phase at a consistent flow rate or pressure. Connecting two pumps enables gradient elution.
- Injector
  Inject sample to column by a micro-syringe. Auto-injector is widely used for automated sample injection.
- Column
  Packing material is packed in a stainless or glass chromatogram column. To avoid the elution of packing material, a frit (2 μm) is packed on each end of the column.
- Column Thermostatic Oven
  Maintain the column at a consistent temperature. Temperature control is very important for reversed phase chromatography and ion exchange chromatography. It is desirable to keep the temperature within ±0.5°C. Water or air circulator is widely used.
- Detector
  Detect each compounds eluted from the column, and convert them into electronic signals.
- Recorder
  Process the electronic signals from detector to draw chromatograms. Retention time, peak area, and theoretical plate numbers are automatically calculated.

Chromatogram

Elution means the process to extract retained samples from a column. Retention time means the time between the sample injection and the sample extraction. Chromatogram is a two-dimension diagram (Figure 2) that reveals the retention time on the abscissa and the concentration of solute on the ordinate. The chromatogram shows following data.

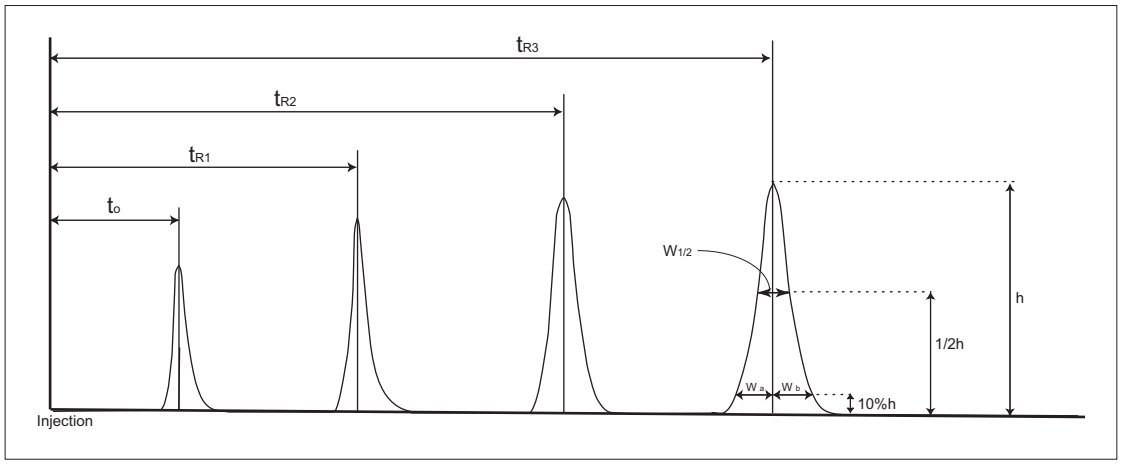

Figure 2 Chromatogram

(1) t_o : Retention time of the mobile phase

The retention time of an unretained peak. Uracil is widely used in reversed phase as a t_o marker.

(2) t_R : Retention time

Distance from the point at peak midpoint to the start of analysis.

(3) h : Peak height

Distance from the peak top perpendicular to the baseline.

(4) $W_{1/2}$: Peak half width

The peak width at half height

(5) k' (capacity factor) : Capacity factor, retention ratio of each sample, $k' = (t_R - t_o)/t_o$

A higher volume means longer retention. . The value remains consistent under the same experimental condition (packing material, mobile phase and temperature)

(6) N (theoretical plate) : theoretical plate number, $N = 5.54 (t_R/W_{1/2})^2$

A theoretical plate is an imaginary layer within a column that helps to interpret the separation process. A higher theoretical plate number corresponds to better column efficacy. The plate number depends on the packing material and experimental conditions.

(7) S (peak asymmetry) : peak asymmetrys, $S = W_b/W_a$

Wa : Distance from the leading edge of the peak to the midpoint (measured at 10% of peak height)
Wb : Distance from the point at peak midpoint to the trailing edge (measured at 10% of peak height)
The peak asymmetry S=1 indicates a perfectly symmetrical peak, and S > 1 indicates tailing, and S < 1 indicates leading. Tailing or leading occurs with deteriorated packing material, unsuitable experimental conditions or overloading.

(8) α (separation factor) : Separation factor, $\alpha = k'_2/k'_1$ (k'_1 and k'_2 : retention ratio of each sample)

The separation factor must be > 1 for peak separation. A higher α value indicates greater distance between the peaks.

(9) Rs (resolution) : Resolution, $Rs = \{\sqrt{N}/4\} \cdot (\{\alpha-1\}/\alpha) \cdot \{ k'_2 / (1+k'_2)\}$

The resolution (Rs) indicates how well two samples are separated. Rs=1.5 indicates baseline separation. If the Rs value is smaller than 1.5, peaks may overlap.

Features of Each Separation Mode of HPLC

HPLC has following separation modes.

● Normal Phase Chromatography

Adsorbent material such as silica gel or alumina is used as the packing material. Analytes are separated by the difference in adsorptive forces to the packing material, resulting in each moving at different speed. The analyte that interacts more strongly with the packing material moves at a slower rate.

● Reversed Phase Chromatography

Analytes are distributed between polar mobile phase and non-polar stationary phase, and separated by the flow speed difference due to the difference in the distribution between these two phases. If the analyte distributes more to the stationary phase, it would have a slower flow speed. Non-polar packing materials, such as octadecyl group and octyl group bonded silica gels are widely used. They are stable to heat and hydrolysis within a certain pH and temperature range, separation is influenced by the type of stationary phase, carbon rate, end capping treatment, etc.

● Ion-exchange Chromatography

Charged functional groups are bonded to the solid support to separate ionic solutes with the counter-ions. Analytes are separated by the flow speed difference due to the difference in the affinity to stationary phase. Dextran, cellulose and polystyrene are commonly used as the packing materials. Typical functional groups are Sulfopropyl (SP) and Carboxymethyl (CM) for cationic exchange, and Diethylaminoethyl (DEAE) and Quarternary ammonium (QA) for anionic exchange. The ion exchange capacity which influences separation performance depends on the type and density of the functional groups.

● Size Exclusion Chromatography

Analytes are separated by the molecular size. The analyte smaller than the pore size can penetrate the pores and migrate slowly, whereas larger analyte is excluded from the pores and migrates quickly. This mode mainly used for separation of high molecular polymer (molecular weight 2,000 and more). Organic expanded type gels (such as dextran and polyacrylamide) and inorganic gels (such as silica gel and glass) are used as packing materials.

Separation Mechanism of HPLC

The most common mode, reversed phase is used as an example here. Mixed samples are injected into a column, the lower hydrophobic analyte (A) distributes in the polar mobile phase and move faster down the column. Conversely, higher hydrophobic analyte (B) distributes in the non-polar stationary phase for a longer time and moves slower down the column. Therefore, the analytes flow out of the column in order of the polar first and the non-polar last.

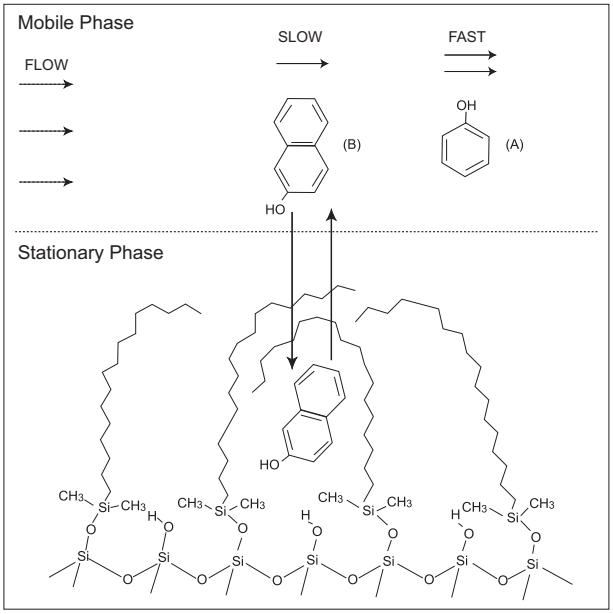

Figure 3. Separation Mechanism

Mobile Phase Solvent

The important mobile phase qualities for HPLC are shown below.

1. High solubility for the sample components
2. Good miscibility
3. No detection disturbance
4. Low viscosity
5. Higher boiling point than the operational temperature
6. Low toxicity and non-flammability
7. Low price
8. Using HPLC grade or filtered solvents.

Next, selection guide of mobile phase is shown below according to the separation mode.

● Normal Phase Chromatography

Generally, a polar solvent is mixed with a nonpolar solvent. The separation factor is adjusted by changing the mixed ratio. Please refer to the polarity and the solubility of solvent. Toluene, hexane, chloroform, ethyl acetate, and ethanol are mainly used.

● Reversed Phase Chromatography

Water, methanol, acetonitrile, and tetrahydrofuran are mainly used. Separation factor is adjusted by the mixed ratios of these solvents. When using silica-based columns for ionic analytes, it is desirable to adjust the pH range from 2 to 7.5. Generally, silica-based columns are not stable outside of this range due to cleavage of the bonded groups at pH < 2, and the dissolution of silica support at > pH 7.5. Use filtered phosphoric acid buffer solution or acetic acid buffer solution for pH control.

● Ion-exchange Chromatography

Add buffer solution in water and adjust separation factor by salt concentration (ionic strength) and pH. The more ionic strength is, the earlier the sample elutes. Lower pH decreases the separation factor on anion exchange, and increases the one on cation exchange. Cation buffer solutions, such as ammonia and amine are used for anion exchange, and anion buffer solutions, such as acetic acid salt, formic acid salt and citric acid are used for cation exchange.

● Size Exclusion Chromatography

Generally, a single solvent is used as the mobile phase, and it is not changed to adjust the separation factor. Tetrahydrofuran, chloroform, toluene anddimethylformamide are commonly used in non-aqueous mode. Add buffer solution in water for aqueous mode. Adjust pH and ionic strength to prevent adsorptionand other undesired interactions.

Quantitative Analysis

Absolute calibration curve method or internal standard method is used to calculate the amount or concentration of solute by peak area or height.

● Absolute Calibration Curve Method

1. Prepare the standard solutions in 3–4 different concentrations.
2. Inject the same volume of each standard solution, record chromatogram, and measure peak area.
3. Prepare a calibration curve by plotting the amounts of the standard on the x–axis and the peak areas on the y–axis. The calibration curve is usually a straight line through the origin.
4. Inject sample under the same conditions as the standards, and record a chromatogram. Measure the peak area (y) and use the calibration curve to determine the sample amounts.

This method should be performed exactly under a given condition. This method is also called external standard method.

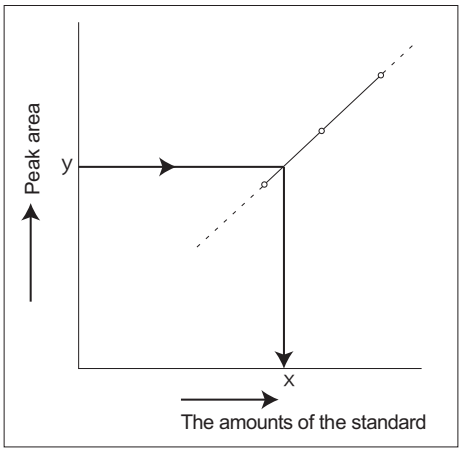

Figure 4. Absolute calibration curve

● Internal Standard Method

1. Prepare 3–4 known concentration[*1] ratios of the standards and samples[*2].
2. Inject a constant volume of each concentration, record chromatogram and measure peak areas.
3. Prepare a calibration curve (as shown on fig. 5) by plotting M_X/M_S vs. A_X/A_S ratios. M_X is the amount of the sample injected, and M_S is the amount of the standard. A_X is the peak area of the sample, and A_S is the peak area of the standard. The calibration curve is usually a straight line through the origin.
4. Then, prepare a the test solution containing a known amount of the internal standardand an unknown amount of sample[*3]. Perform the experiment under the same conditions as for obtaining the calibration curve.
5. Use the calibration curve to determine the unknown sample amount.

*1 If the calibration curve is confirmed to be a straight line through the origin, plot the calibration curve with AX/AS determined by one point of concentration of injected unknown sample.

*2 The internal standard should have similar chemical characters as the sample while completely separated from it.

*3 When the internal standard is added to the test solution, make sure the chemical reaction (e.g., precipitation) does not occur.

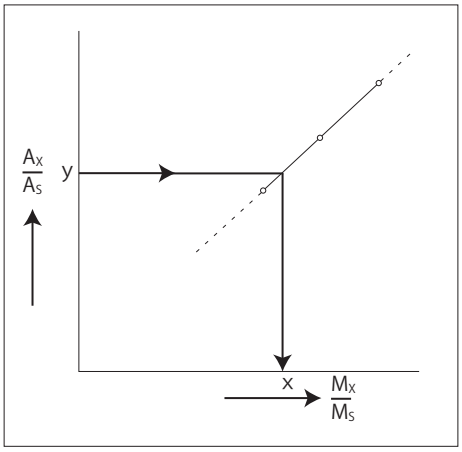

Figure 5. Calibration Curve of Internal standard method