# CHAPTER II

# THEORY

## 2.1 LC Instrument

The HPLC equipment comprises mobile reservoir, pump, injector, column, detector and data system. Mobile phase is retained in mobile reservoir and introduced into system by pump which is the flow rate controller and generates pressure to drive the mobile phase. The injector is used to retain and place the sample on the column. There are many types of column. The responsibility of column is the sample separation, so the suitable column should be considered for each sample groups. The detector is used to respond the change of sample concentration. Data system provides the data processing from the detector. [33, 34]

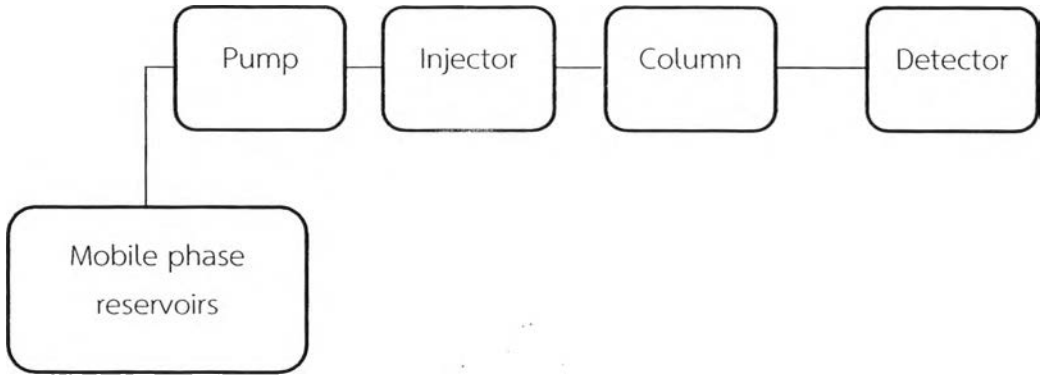



**Figure 2.1** HPLC instrument diagram.

### 2.1.1 Mobile phase reservoirs

The mobile phase reservoir is basically equipped in HPLC system. The most reservoirs were made from glass or stainless steel. For isocratic system, only one reservoir is used but for gradient system, more than one reservoir is required. The cleanliness of reservoir is important for the system, so the mobile phase should be filtered before use. The reservoirs should be covered to protect the mobile phase evaporation and the contamination which cause blocked column. The cover should

not be the polymer such as parafilm because it might be dissolved and contaminated in mobile phase. There is an inlet-line frit at the end of tube which is the load to keep the tube at the bottom. The degassing should be done before let the mobile phase through the pump to eliminate the bubbles which cause the spurious peak in detector output.

### 2.1.2 Pump

Pump is the important component for HPLC system which is high pressure system. The high resisted pump is required for fast analysis with small particle packed column. The precision and accuracy of the pump is the key of success. The pump at high pressure was found the leak problem. For gradient system, the buffer often left in the pump which caused blocked pump, so before shut the pump off, the buffer should be flushed out the pump for more than one hour at the minimum flow rates.

### 2.1.3 Injector

Injector serves to introduce required sample volume accurately into the HPLC system. The sample is introduced by a syringe into a constant volume loop. In the LOAD position, the loop is not in the path of the mobile phase. The amount of sample that over the sample loop was wasted. By rotating to the INJECT position, the sample in the loop is moved by the mobile phase stream into the column. It is important to allow some sample to flow into waste from loop because the new sample will help to wash the previous sample to prevent memory effect. Before use, the injector should be primed with solvents to be used. The needle also should be cleaned to prevent the injector contamination.

### 2.1.4 Column

Column is the heart of analysis with HPLC system. The high efficiency separation and speed can be gained from the column development. There are many types of stationary phase which developed for a wider range of sample. Column

comprises tube which is filled with small particle (1.5-5.0 μm ID.) or a single piece of monolithic (silica or polymer based) as described below (Section 2.2).

### 2.1.5 Detector

The first detector for HPLC separation was the human eyes. Then, the detector was developed in many types to suit for each sample. The chromatographic detector is a transducer that converts a physical or chemical property of an analyte into an electrical signal that can be related to analyte concentration. The detector should have some of these: the high sensitivity, respond to all solutes, stable even change of temperature or pressure, respond independently of the mobile phase. HPLC detector should have a minimum volume to reduce the extra-column band broadening. The most common detector used for HPLC is a UV detector.

## 2.2 LC column

The column is the heart of analysis in HPLC system. The high efficiency separation and speed can be gained from the column development. There are many types of stationary phase which was developed for a wider range of sample.

### 2.2.1 Particle packed column

The column consists of the tube which is filled with small particle (1.5-5.0 μm I.D.). Porous silica particles are the most commonly used. The porous and surface areas of particles are related with the retention time, in other word, the retention increase when the surface area increase and the pore diameter decrease. The pore size selection should be determined from the sample size and to gain the best efficiency and fast separation. Moreover, the particle sizes are also important. The variation of particle sizes influenced peak shape, so the narrow particle size distribution is preferred. It provides the large of theoretical plate number (*N*). So Particle surface area, average pore diameter and particle size distribution are important to selection stationary phase that suited with sample. There are many types of particles for HPLC as seen below.

#### 2.2.1.1 Pellicular particles

Pellicular particles are the solid which covered with a very thin surface layer. These are presently 1.5 -2.5 μm in diameter which provide the large value of *N* for macromolecules. They are suited for the separation of large biomolecules because the low surface area of these particles reduced retention and injection volume of sample.

#### 2.2.1.2 Superficially porous particles

Superficially porous particles are also called fused-core™ particles, shell particles or controlled-surface-porosity particles which have a solid core with porous outer. The surface areas of these particles are higher than those pellicular particles because superficially porous particles have diameters of 2-5 μm with porous shell of 0.25-0.5 μm in thickness. The high surface areas influenced the longer retention.

#### 2.2.1.3 Perfusion particles

These particles have a very large pores (through pore; 400-800 nm) connected to a small pores (30-100 nm). Because of their feature, the solutes distribute into the stationary phase by the diffusion and flow of mobile phase. Thus, band broadening is reduced for large molecules at high flow rate.

As described above, there are many types of particles which are available in HPLC system. The decreased particles size provides the good efficiency, narrow peak shapes and reduced extra-column peak broadening. However, the separation with small particle packed stationary phase was operated under high pressure and require high resistance of pump.

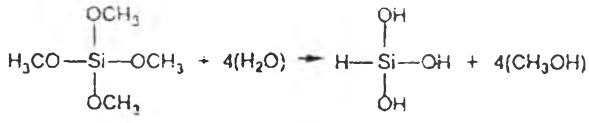
### 2.2.2 Monolithic column

Monolith becomes the interesting column in analytical separation. Because of their characteristic, the monoliths have an interconnected porous bed.

The monoliths have the feature as either column or disk. There are 2 types of monoliths which divided from the material (silica and polymer based). The polymer based monoliths have excellent pH stability but the drawback of these monoliths is the swell/shrinkage of column when exposed to various organic solvents.

#### 2.2.2.1 Silica-based monoliths

Silica monoliths are a single piece with interconnected skeleton, which provide the 2 types of pore (through pores/macropores and mesopores). Macropores has ~2 μm of diameter, whereas mesopores showed ~10 nm of diameter. The large through pores and small skeleton size of silica monolith can reduced the diffusion pathlength which caused the narrow peak. These features (high through pore size/skeleton size ratio) of silica based monolith columns provide high column efficiency under low pressure which result 10 times of improvement in pressure over a particle packed column [12, 13]. The silica monolith column can be prepared in capillary column and was prepared from a mixture of tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMS) via sol-gel process. The mixture of precursors is used to prevent the space that might occur between the silica skeleton and column wall [11]. The sol-gel process comprises a hydrolysis and a polycondensation reaction. The hydrolysis would take place first, and then the latter will occur. Although the first step will be occur, these reaction are known to continue in parallel. The hydrolysis occurred from the cleavage of chemical bonds (Si-OR) by the addition of water as seen in the equation (2.1) [35]. The product (Si-OH) from the hydrolysis reaction can condenses with another Si-OH to produce Si-O-Si linkage and water via polycondensation reaction. The siloxane oligomers link together to form gel network [36].



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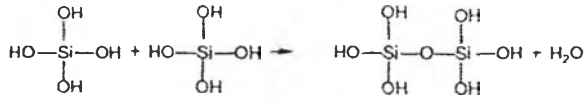

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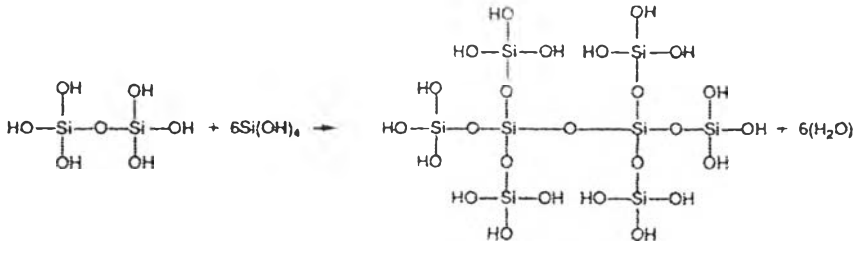

(2.3)

The cross-linking density of gel network depends on the amount of water in hydrolysis reaction. The gel networks with low cross-linking density are formed, when the amount of water is low (water/alkoxide ≤ 2). While the amount of water is high (water/alkoxide ≥ 4), the gel networks with high cross-linking density are formed. Moreover, the concentration and kind of catalyst, the composition are important parameters that influence the morphology. Phase separation mechanism known for materials with relatively slow dynamics is spinodal decomposition. The spinodal decomposition starts with a small composition fluctuation and the phase domain continuously grows. This mechanism requires slow dynamics of the rate of physical and chemical change which affected the phase separation. The silica rich domain grows to be silica skeleton and the solvent rich domain grows to be macropores. The final morphology depends on the freezing time relate to the development of phase separating domain.

The domain size is competitive between the phase separation and sol-gel transition. The macropore size can be controlled by PEG/silica ratio because the glycol forms hydrogen bonds with the silanol of growing silicate

polymer. The solvent volume in starting solution becomes the pore space after drying. Figure 2.2 shows a diagram of solvent, silica and PEG.

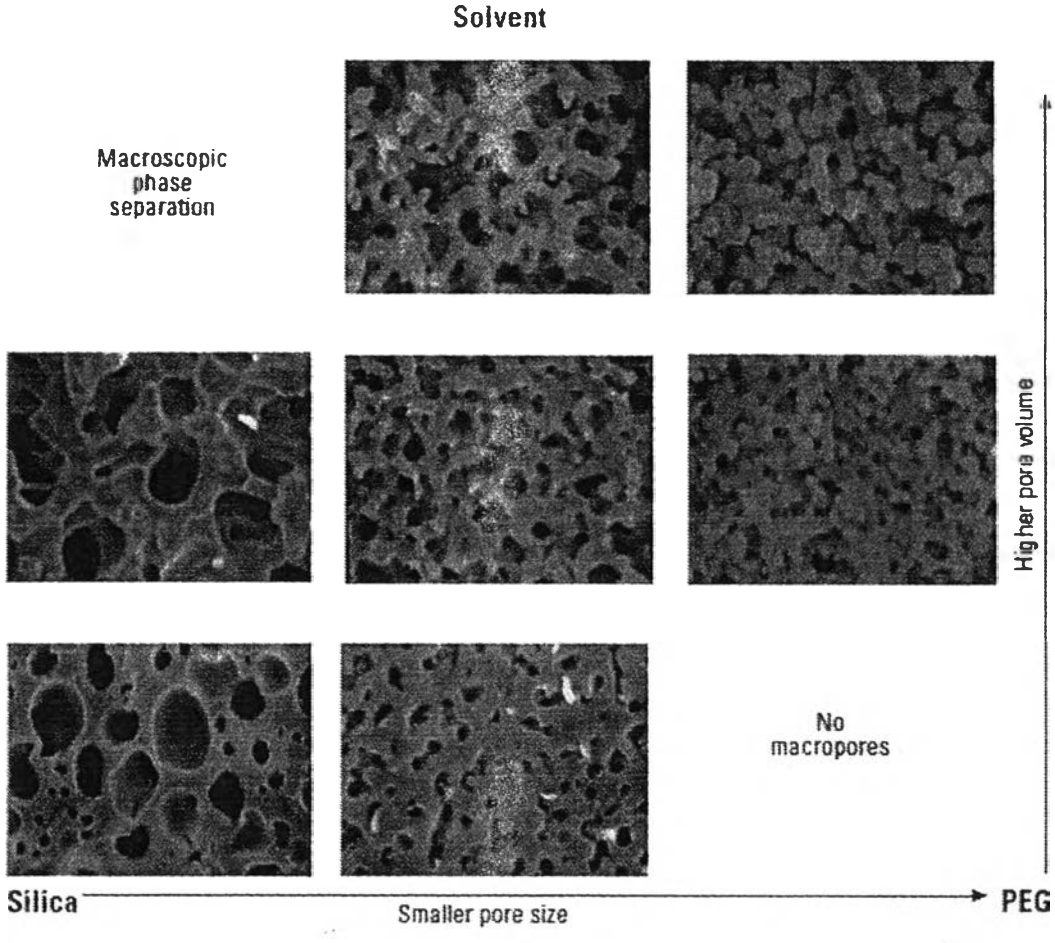


**Figure 2.2** The composition triangle representing the fractions of silica, PEG and solvent [7].

The monolith possess a network of pores defined as macropore (> 50 nm), which provide ~80% of total porosity and mesopore (2-50 nm), which is 10-15% of total porosity [37].

#### 2.2.2.2 Organic polymer-based monoliths

The organic polymer based monoliths can be made from polymerization reaction of a mixture comprising monomers, initiator, and porogenic

solvent. The morphology of organic polymer is the clusters of little organized microglobules. This type of monolith is suitable for a biological molecules such as peptides, protiens, etc.

## 2.3 Mode of Liquid Chromatography

### 2.3.1 Normal phase

Normal phase is a classical mode of chromatographic separation. Normal-phase can be used for compounds that are too hydrophobic or hydrophilic. The stationary phase is more polar than the mobile phase. There are a number of stationary phases available for normal-phase chromatography. Silica is the most common of the non-bonded phases and can provide very high selectivity for many applications.

### 2.3.2 Reversed phase

Reversed Phase Chromatography (RPC) is usually a first selection for the separation of both neutral and ionic samples, using a column which is coated with a less polar bonded phase such as $C_8$ or $C_{18}$. The mobile phase is in most cases a mixture of water and either acetonitrile (ACN) or methanol (MeOH). The solvents used for RPC are less flammable or toxic, water miscible and are more compatible with UV detection at wavelengths below 230 nm for increased detection sensitivity. The advantage of RPC is fast equilibration after change the mobile phase when using gradient elution.

### 2.3.3 Ion exchange chromatography

Ion-exchange chromatography is one of the most widely used for analysis. It is used in research, analysis, and process-scale purification of proteins. Ion exchange is the first choice for capture of proteins because of its high capacity, relatively low cost and the buffer conditions can be adapted to suit a broad range of proteins. The separation of Ion-exchange chromatography is based on charge

characteristics. Charged groups on the surface of analytes with oppositely charge immobilized on the ion-exchange column.

### 2.3.4 Mixed-mode reversed phase

Mixed-mode chromatography is a chromatographic method that used more than one interaction to retain the solutes. There are two interactions which are reversed mechanism and ion exchange in mixed-mode reversed phase. The advantage of mixed-mode chromatography is salt-independent adsorption, facile elution by charge repulsion, and unique selectivity [38]. It is definitely different from normal mode chromatography (only one interaction) because the second interaction cannot be too weak, and the second interactions should both contribute to retain the solutes. The peak tailing problem which take place from the secondary interaction was found in normal mode and then the researcher have tried to eliminate or minimize. Mixed-mode in early is the connection of the different functional group between two columns [39]. However it is difficult to operate, then the mixed-mode in one column has received much attention.

## 2.4 Theory in LC

The successful use of HPLC requires an understanding of how separation is affected by experimental conditions: the column, solvent, temperature and flow rate.

### 2.4.1 Retention and Capacity factor

The retention time of the unretained compound is referred to the column dead-time ($t_0$). The retention time ($t_R$) for each solute is the time from sample injection to the top of the peak in the chromatogram. It can be present in term of capacity factor ($k'$) as seen in Equation 2.4. The capacity factor indicates the ability of sample separation.

$$k' = \frac{t_R - t_0}{t_0} \qquad (2.4)$$

The column with high capacity factor shows the great ability of solute retention. The resolution of a separation will be improved by using the column with high capacity factor due to the longer time to analyse, the resolution and analysis time must be achieved with a compromise. Normally the capacity factor in the range of 2-5 shows a good balance between the resolution and analysis time.

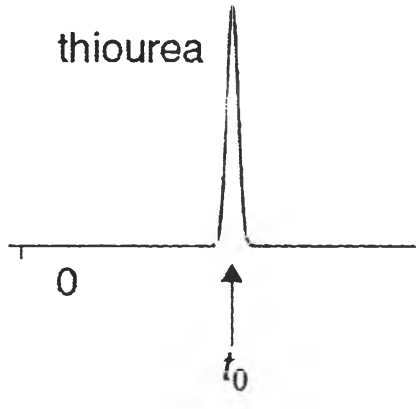


**Figure 2.3** The column dead time ($t_0$) acquired from an unretained compound (thiourea) in reversed-phase LC.

### 2.4.2 Plate theory and Rate theory

The plate theory assumes that a sudden equilibrium is set up for the solutes. The chromatographic column is considered with a number of plate. The more column efficiency is the greater number of theoretical plates.

$$N = \frac{L}{H} \qquad (2.5)$$

L is the length of column, therefore the greater efficiency of column (high N) can be achieve by the small plate height (H). Normally, the small H value

can get by the small particle size of stationary phase, low flow rate of mobile phase, higher separation temperature and less viscous mobile phase.

The rate theory was developed by Van Deemter. There are three outstanding terms represent three different processes. The general form of the presented dependence is well known as the Van Deemter equation, which has following below.

$$H = A + \frac{B}{u} + Cu \qquad (2.6)$$

Three terms represent three processes that support for band broadening, which A is eddy diffusion, B is longtitudinal diffusion, C is resistant to mass transfer and *u* is linear velocity which related to flow rate.

Eddy diffusion (A); the column is packed with different paths around the stationary phase particles. The mobile phase flows through the column and transports the molecules with it. Some of the solutes will arrive at the outlet of the column at different time. The band broadening from eddy diffusion can be decreased when the column is packed with uniform particles.

Longtitudinal diffusion (B) is band broadening process in which solutes diffuse from the concentrated center of a zone to the direction of flow of the mobile phase.

Resistance to mass transfer (C); the pores of stationary phase particles are filled with mobile phase. A sample molecule can enter into a pore termination to be transported by solvent flux and changes its position by diffusion only. Mass transfer between the mobile phase and stationary phase has adsorptive which attract the molecule around them.

### 2.4.3 Resolution

HPLC method development refers to the selection of separation conditions that provide an acceptable separation of a given sample. The separation of two peaks is usually described in terms of their resolution ($R_s$) as equation 2.7 [33].

$$R_S = \frac{2\left[t_{R1} - t_{R2}\right]}{W_1 - W_2} \qquad (2.7)$$

$W_1$ and $W_2$ are the baseline widths for peaks 1 and 2, respectively. Increased resolution gains from a larger difference in peak retention times and/or narrower peaks.

### 2.4.4 Selectivity

The selectivity ($\alpha$) is defined as the column capacity to separate between two peaks of analytes. It can characterize from the ratio of retention factor of an examined band (solute B) and adjacent band (solute A) in which solute B is a strongly retained species and solute A is a rapidly eluted species. The solute retention is determined by various interactions among the solute, mobile phase, and stationary phase (column) which can affect the column selectivity.

$$\alpha = \frac{k'_B}{k'_A} \qquad (2.8)$$

Shape selectivity has been described as the two separate manifestations of steric interaction [40]. The basis of shape selectivity is illustrated in Figure 2.4 for a narrow molecule (i), a wide molecule (j). The j molecule is excluded from part of the stationary phase because its minimum cross-section exceeds the spacing between ligands, in other word, the j molecule cannot squeeze between the

ligands. Thus, the solute that can be penetrated through the ligands was higher retention than the other.

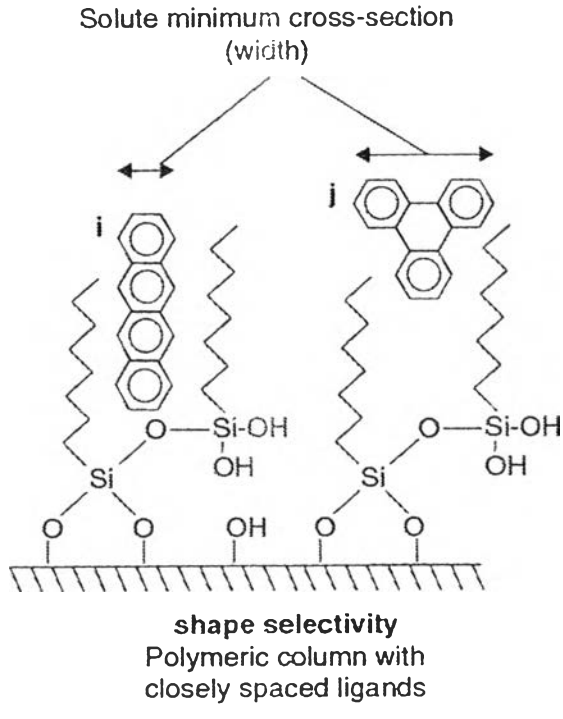


Figure 2.4 The manifestation of shape selectivity [41].