CHAPTER II

THEORY

Sample preparation procedure is one of the most important parts of the analysis to influence the analytical results. The objective of sample preparation process is to isolate target analytes from various matrices and convert the analytes into a more suitable form for separation and detection. Matrix effects are considered as a major problem in extracting analytes as they may lead to low recovery. These effects depend on sample properties and the concentration of analytes in the sample. Several studies have attempted to develop sample preparation procedures to remove interferences, increase the concentration of analytes, and provide a simple, inexpensive, robust, and reproducible method. The traditional sample preparation method, liquid-liquid extraction (LLE) is still in use because of its simplicity. LLE uses an organic solvent to extract analytes from aqueous sample in the principle of phase partition. With LLE, it is possible to achieve both analyte enrichment and sample clean-up, but the main disadvantage is the consumption of large quantities of organic solvent, which may result in environmental impacts and potential health hazards.

Solid-phase extraction (SPE) has gradually replaced classical LLE and become the common sample preparation technique. SPE utilized a solid sorbent or bonded organic phase material to preconcentrate and clean-up analytes from sample. Analytes are extracted and partitioned between a solid stationary phase and a liquid sample phase. These analytes must have greater affinity for the solid phase than the sample matrix. Choice of sorbents and elution solvents are the essential parameters affecting recovery and LOD of target compounds. SPE is used for extraction, preconcentration, and clean-up purposes. Compared with LLE, this technique offers high recovery, specificity, automation possibility, less organic solvent usage, and SPE sorbents are commercially available in form of disposable cartridges. However, SPE technique is time-consuming, expensive, labor-intensive, has limited selectivity, and low preconcentration ability.

An optional technique, which provides some distinctive advantages over LLE and SPE, especially in case of selectivity, enrichment power, and automation potential, is membrane extraction, where the attempt is to use LLE advantages by avoiding its disadvantages.

2.1 Membrane extraction *(21,22,23,24,25)*

Membrane extraction was introduced in 1999 by Jönsson et al.. *(21)* A membrane was applied as a selective barrier between two aqueous phases. The phase in which the transfer of analytes occurrs is called feeding or donor phase, and the phase into which the analytes are extracted is called stripping or acceptor phase. The membrane is a synthetic product of different chemical natures and displays different properties. Common membrane characterization is based on the porosity of the membrane, which can be porous or non-porous. In porous membranes, two liquid phases are in contacted through the membrane pores and only particles smaller than the pore size can pass through the membrane. These membranes are used for the separation of analytes from matrix with particle size selection, which leads to an efficient clean-up without enrichment of analytes. Thus, the separation by porous membranes is a function of analyte molecular size and pore size distribution of the membrane. Porous membranes are often applied in filtration, reverse osmosis, and dialysis process. On the other hand, non-porous membranes have been widely used for extraction. Analytes are transported from donor to acceptor phase by diffusion under the driving force of pressure, concentration, or electrical potential gradient. Non-porous membranes, which act as interface between two liquid solutions, can consist of a liquid or a solid phase. It can be a liquid-impregnating porous membrane or an absolute solid membrane. The extraction from non-porous membrane provides efficient clean-up with high enrichment factors. Non-porous membranes offer a powerful membrane extraction technique without significance use of organic solvents.

One remarkable non-porous membrane extraction is supported liquid membrane (SLM). This technique employs a polymeric pore membrane as support for an organic solvent and creates three-phase system. The organic solvent within the membrane is a barrier between the aqueous donor and the acceptor solution. If the acceptor solution is

also organic solvent, it is called two-phase system, which is also named microporous membrane liquid-liquid extraction (MMLLE). Both SLM and MMLLE principles are frequently applied rather for on-line membrane system than off-line. However, the off-line membrane configuration, which is a versatile non-porous membrane extraction technique, was developed by Rasmussen et al. and is termed liquid-phase microextraction (LPME). *(22)* Nowadays, LPME has been widely used for many applications not only its simplicity, low-cost, and elimination of carry-over effects, but also for its fast and almost solvent-free use.

2.2 Liquid-Phase Microextraction (LPME) *(26,27)*

LPME, a miniaturized LLE, was firstly based on the extraction of analytes from aqueous sample into a small droplet of organic solvent hanging at the end of microsyringe needle. The organic solvent droplet was placed into aqueous solution and analytes were extracted into the organic hanging droplet by passive diffusion. After extraction, the droplet was withdrawn into the syringe and transferred to inject into gas chromatography (GC). Afterwards, this technique has been separately termed from LPME as single-drop microextraction (SDME). Due to the instability of organic droplet in SDME, LPME was improved to a more robust configuration, which utilized disposable low-cost hollow fiber membranes to stabilize the extracting phase. This technique is called hollow-fiber liquid-phase microextraction (HF-LPME).

2.2.1 Hollow-Fiber Liquid-Phase Microextraction (HF-LPME) *(28,29,30,31)*

HF-LPME utilizes porous, hydrophobic, hollow fibers impregnated with an organic phase to perform both SLM and MMLLE systems. A hollow fiber membrane employed in HF-LPME is shown in Figure 2.1. The basic principle of HF-LPME is to fill the aqueous sample into vial and then a piece of hollow fiber impregnated with organic solvent in the pores is placed into the sample solution. The solvent must be immiscible with water to remain in the fiber pores. Analytes are extracted from the aqueous sample through the organic solvent in pores of the hollow fiber and further into an acceptor solution. Figure 2.2 illustrates the basic diagram of extraction in HF-LPME. Similar to

the principle of MMLLE and SLM, membrane extraction technique can be divided into two modes of extraction depending on the extracting or acceptor phase type. In HF-LPME, there are called two-phase and three-phase systems.

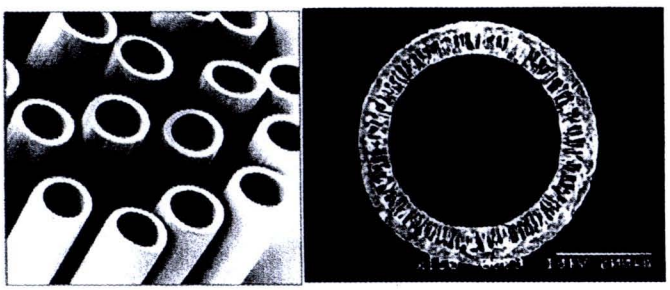

Figure 2.1 Hollow fiber membrane. *(32)*

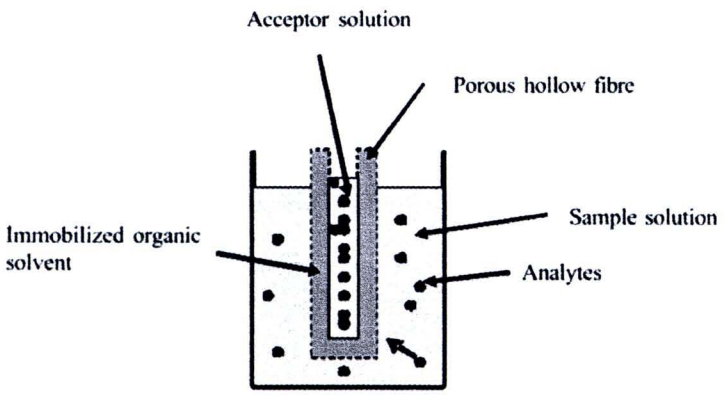

Figure 2.2 Diagram of basic HF-LPME principle. *(28)*

2.2.1.1 Two-phase HF-LPME

In two-phase LPME, the extraction principle is similar to MMLLE in membrane extraction. The analytes are extracted from aqueous sample (donor) phase into organic solvent presented in both the porous wall and lumen of the hollow fiber. A two-phase cross-section diagram of hollow fiber inside the aqueous sample is shown in Figure 2.3. In this case, the acceptor solution is the same organic solvent as impregnated in the fiber porous wall. Two-phase systems are applied for analytes with a high solubility in non-polar organic solvents.

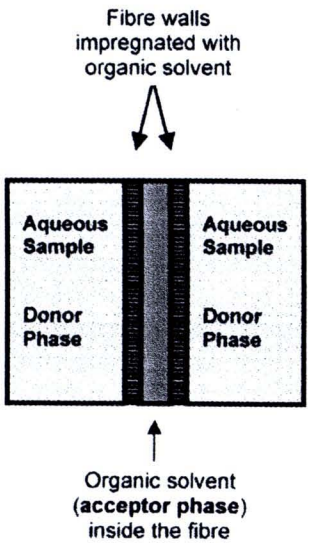

Figure 2.3 Two-phase cross-section diagram of HF-LPME in the aqueous sample. *(26)*

The extraction process of two-phase extraction is shown in Eq. 1.

$$A_{donor} \longleftrightarrow A_{organic\ acceptor} \qquad \text{(Eq. 1)}$$

where A refers to target analyte in sample (donor) and in organic acceptor. The partition coefficient (K) of analytes between acceptor and donor phase is defined in Eq. 2.

$$K_{acceptor/donor} = \frac{C_{eq,acceptor}}{C_{eq,donor}} \quad \text{(Eq. 2)}$$

where $C_{eq,acceptor}$ is the concentration of analytes in the acceptor solution (organic phase) at equilibrium and $C_{eq,donor}$ is the concentration of analytes in the sample (donor phase) at equilibrium.

From Eq. 1 and Eq. 2, the two-phase recovery of analytes at equilibrium (R), the extraction efficiency (EE), and enrichment factor (EF) are calculated by the following equations (Eq. 3-5).

$$R(\%) = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + V_{donor}} \times 100 \quad \text{(Eq. 3)}$$

$$EE = \frac{R}{100} = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + V_{donor}} = \frac{C_{eq,acceptor} V_{acceptor}}{C_{donor} V_{donor}} \quad \text{(Eq. 4)}$$

$$EF = EE \frac{V_{donor}}{V_{acceptor}} = \frac{C_{eq,acceptor}}{C_{donor}} \quad \text{(Eq. 5)}$$

where $V_{acceptor}$ is the volume of acceptor solution, V_{donor} is the volume of sample (donor) solution, and C_{donor} is the initial analyte concentration in the aqueous donor solution.

It can be predicted that the recovery is related to the partition coefficient, the volume of organic solvent in acceptor phase, and the volume of sample (donor). To obtain high recovery, sample volume should be low and the partition coefficient ($K_{acceptor/donor}$)

should be high. The value of partition coefficient depends on the selection of organic solvent and the selection of pH in aqueous solution for acidic or basic analytes in order to obtain non-ionic species. In two-phase systems, the extracted analytes must be more miscible with the organic solvent than aqueous medium to obtain high partition coefficient (K) values for analytes between organic acceptor and aqueous donor phase ($K_{acceptor/donor}$). High $K_{acceptor/donor}$ for analytes are obtained for moderately or highly hydrophobic compounds containing acidic or basic groups, and neutral compounds with hydrophobic properties.

The enrichment factor provided by two-phase extraction is noticeable high because the ratio ($V_{donor}/V_{acceptor}$) is frequently high. While the donor volume is in mL-level, the volume of acceptor is in μL-level. This is the main advantage of HF-LPME because high enrichment of analyte is achieved.

After extraction with two-phase system, the organic acceptor solution is compatible with GC and normal-phase HPLC detection. For reversed-phase HPLC analysis, the solvent should be evaporated and the analyte dissolved in aqueous medium prior to injection.

2.2.1.2 Three-phase HF-LPME

In three-phase LPME, the extraction principle is similar to SLM in membrane extraction technique. It differs from two-phase system in the type of acceptor solution used. The analytes are extracted from the aqueous sample (donor) phase, through organic solvent immobilized in hollow fiber pores, which acts as a barrier between the two phases. Analytes are further extracted into aqueous acceptor solution inside the lumen of hollow fiber. A three-phase cross-section diagram of hollow fiber inside the aqueous sample is shown in Figure 2.4. In this case, the acceptor solution is another aqueous solution.

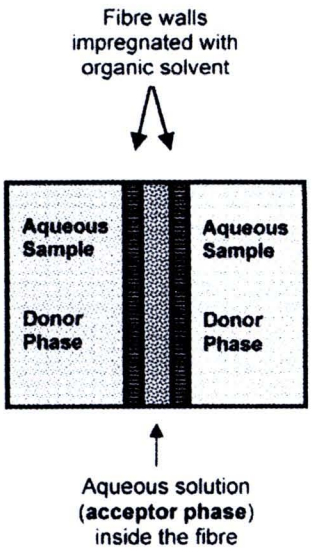

Figure 2.4 Three-phase cross-section diagram of HF-LPME in the aqueous sample *(26)*

Three-phase extraction is suitable for acidic or basic analytes with ionizable functionalities and its process is described in Eq. 6.

$$A_{donor} \longleftrightarrow A_{organic} \longleftrightarrow A_{aqueous\ acceptor} \qquad \text{(Eq. 6)}$$

where A refers to target analyte in the sample (donor), the organic, and in the aqueous acceptor phase. Among the partitioning of analytes in the three phases, the partition coefficient of analytes between the acceptor and donor phase ($K_{acceptor/donor}$) is considered as the overall driving force of three-phase extraction and defined in Eq. 7.

$$K_{acceptor/donor} = K_{organic/donor} \times K_{acceptor/organic} = \frac{C_{eq,acceptor}}{C_{eq,donor}} \qquad \text{(Eq. 7)}$$

where $$K_{organic/donor} = \frac{C_{eq,organic}}{C_{eq,donor}}$$

and $$K_{acceptor/organic} = \frac{C_{eq,acceptor}}{C_{eq,organic}}$$

where $C_{eq,donor}$, $C_{eq,organic}$, and $C_{eq,acceptor}$ refer to the concentration of analytes in the sample (donor phase), organic phase, and acceptor solution (organic phase) at equilibrium, respectively.

From Eq. 6 and Eq. 7, the three-phase recovery of analytes at equilibrium (R), the extraction efficiency (EE), and enrichment factor (EF) are calculated by the following equations (Eq. 8-10).

$$R(\%) = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + K_{organic/donor} V_{organic} + V_{donor}} \times 100 \qquad \text{(Eq. 8)}$$

$$EE = \frac{R}{100} = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + K_{organic/donor} V_{organic} + V_{donor}}$$

$$= \frac{C_{eq,acceptor} V_{acceptor}}{C_{donor} V_{donor}} \qquad \text{(Eq. 9)}$$

$$EF = EE \frac{V_{donor}}{V_{acceptor}} = \frac{C_{eq,acceptor}}{C_{donor}} \qquad \text{(Eq. 10)}$$

where V_{donor}, $V_{organic}$, and $V_{acceptor}$ are the volume of sample (donor) solution, organic phase, and acceptor solution, respectively, and C_{donor} is the initial analyte concentration in aqueous donor solution.

The extraction mechanism of three-phase system is shown in Figure 2.5, where pH is the critical driving force to promote the extraction. For basic analyte compounds, the pH of donor solution should be adjusted to alkaline to promote only basic analyte in deionized form partitioning into organic phase, while other acidic compounds which is ionized in the donor solution cannot partition into organic phase. Meanwhile, pH of acceptor solution should be acidic to promote high extraction efficiency from organic phase into acceptor phase. On the other hand, for acidic analytes, the donor pH should adjust to be acidic to allow analyte in deionized form extraction into organic phase, and the acceptor pH is adjusted to alkaline in order to prevent analyte back-extraction into organic phase.

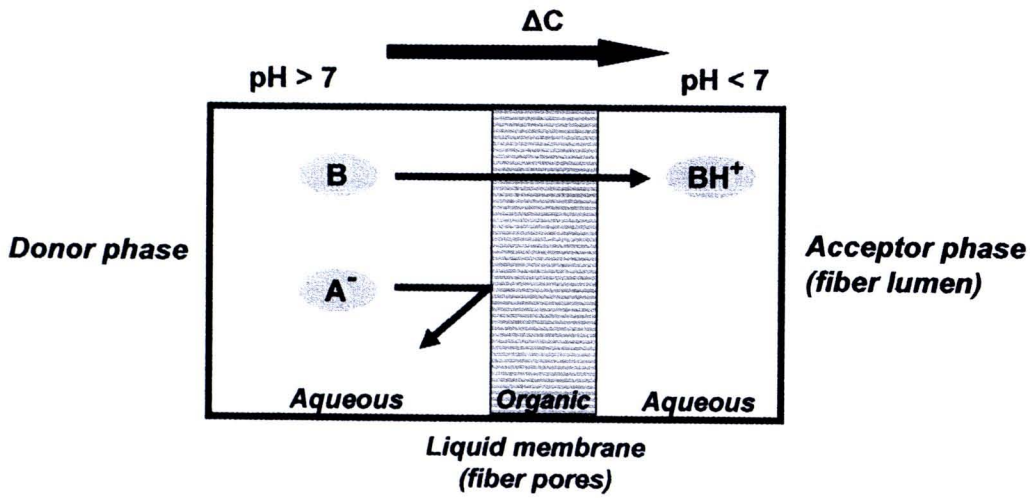

Figure 2.5 Three-phase extraction mechanism in HF-LPME for basic analyte.

(B = basic species , A = acidic species) (adapted from *(25)*)

The recovery of analyte in three-phase system is controlled by two individual partition coefficients ($K_{organic/donor}$ and $K_{acceptor/organic}$) to reach high recoveries. High partition coefficients are performed by proper selection of organic solvent to create SLM and proper selection of pH conditions in both aqueous solutions (donor and acceptor). In

addition, another parameter affecting the recovery is the volume of sample and organic, which should be low to increase recovery.

Similar way to two-phase systems, the enrichment of three-phase systems depends on the volume ratio of donor and acceptor ($V_{donor}/V_{acceptor}$), which should be low and then the enrichment factor is normally found to be high.

Following three-phase extraction, the acceptor solution can directly be injected into HPLC or capillary electrophoresis without prior treatment.

Besides high analyte enrichment ability, both two-phase and three-phase provide efficient clean-up from matrix components by excluding the acceptor phase from macromolecules and other compounds in the sample that could interfere with analysis.

Two-phase and three-phase extractions are based on diffusion, which means that the extraction can be promoted by high partition coefficients. However, for very hydrophilic compounds that have low partition coefficients, the extraction is not possible with both two- or three-phase modes. Low partition coefficients indicate that analytes cannot be extracted based on diffusion alone. To solve this problem, HF-LPME has further been developed into carrier-mediated HF-LPME.

2.2.1.3 Carrier-mediated HF-LPME *(33,34)*

Carrier-mediated HF-LPME utilizes ion-pairing agents to transfer analytes from donor solution to acceptor solution. This method was developed by Ho et al. *(34),* for the attempt to extract high hydrophilic or polar analytes with HF-LPME. This ion-pair HF-LPME is well-known as carrier-mediated membrane transport or carrier-mediated HF-LPME.

The carriers employed in carrier-mediated HF-LPME mode are various types of compounds and can be cationic, neutral, and anionic carrier. Some carriers are illustrated in Figure 2.6.

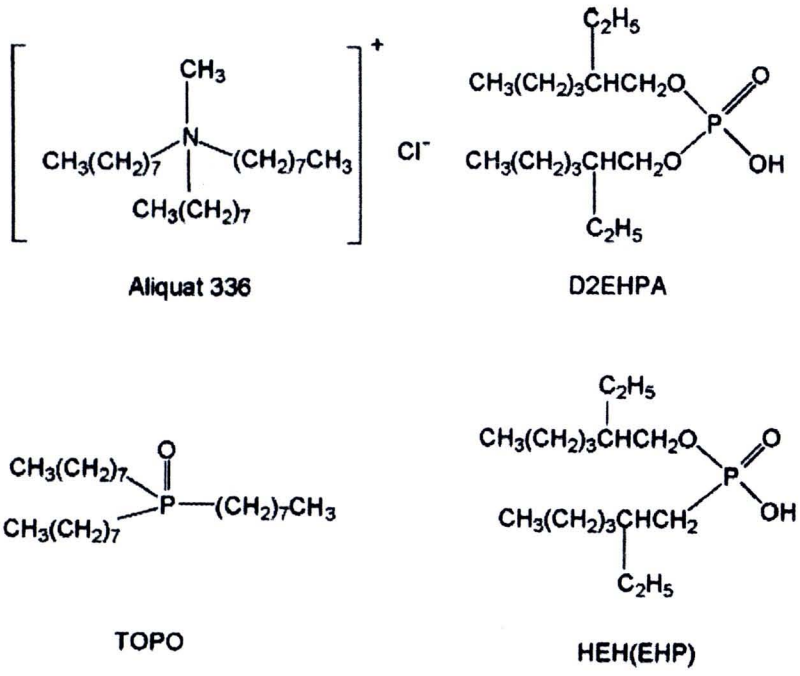

Figure 2.6 Some carriers in carrier-mediated HF-LPME. *(35)*

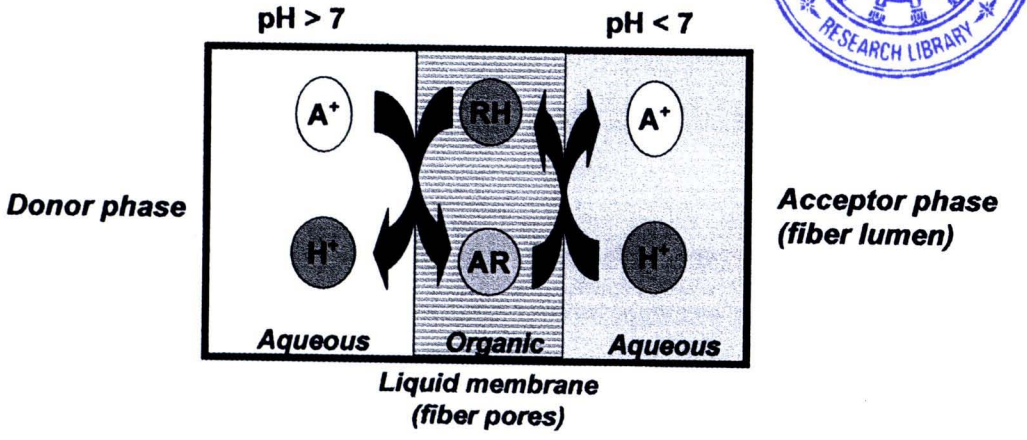

Figure 2.7 Carrier-mediated HF-LPME mechanism.

(A^+ = hydrophilic analyte species , RH = carrier , AR = ion-pairs between analyte and carrier) (adapted from*(31)*)

The principle of carrier-mediated HF-LPME is to add a carrier, an ion-pairing agent into sample solution or organic solvent in order to form ion-pair complex with hydrophilic analytes. The ion-pairs between carrier and target analyte offer a higher partition possibility into organic solvent than the native analyte; therefore, target analytes can be transfered from sample solution through organic solvent, and subsequently extracted into acceptor solution in their native forms. Carrier-mediated HF-LPME is usually applied in three-phase extraction and the analytes in the acceptor solution should be performed in a condition suitable for detection with analytical methods such as HPLC or CE.

In three-phase systems, the carrier-mediated process is controlled by two individual partition coefficients ($K_{organic/donor}$ and $K_{acceptor/organic}$). Besides diffusion and ion-pairing effects, the counter-ions present in acceptor solution and pH gradient between donor and acceptor phase are the essential driving forces to promote extraction. As seen in the carrier-mediated mechanism from Figure 2.7, the counter-ions in acceptor solution should be in sufficient quantity to form ion-pair complexes with carrier in the contact area and then these counter-ions are back-extracted into sample solution to allow the carrier to form new ion-pair complex with analyte, and the carrier-mediated extraction process of analyte is repeated again. For basic analytes, the adjustment of sample pH is to ensure target analytes are in their ionized state, whereas the acceptor pH should be adjusted to acidic to have sufficient protons, which behaved as counter-ions and to release the carrier within acceptor phase.

In present, carrier-mediated HF-LPME has been efficiently applied for the extraction and determination of polar analytes from complex matrices such as environmental and biological samples with high enrichment characteristics and remarkable clean-up efficiency.

2.2.1.4 Parameters affecting HF-LPME procedure

2.2.1.4.1 Hollow fiber membrane

Hollow fiber is a synthetic membrane classified based on the geometry. HF-LPME hollow fiber membranes are porous and mainly made of polypropylene polymer. Nowadays, hollow fibers are commercially available with inner diameter of 600 μm, wall thickness of 200 μm, and average pore size of 0.2 μm. The character of hollow fiber membrane for HF-LPME should be hydrophobic and inert. For extraction, the hollow fiber should be compatible and resist in the choice of organic solvent. Because the hollow fiber size affects the mechanical stability, the inner diameter size is important to have a proper volume to contain the acceptor solution in the fiber lumen. Additionally, the wall thickness should be convenient to create a thin layer of SLM and provide a short diffusion distance. Besides all, the fiber porosity should be high enough to promote extraction speed by providing large surface area of impregnated organic solvent and to be in contact with the sample solution. Pore sizes of 0.2 μm are suitable for the penetration of small molecules of target analytes through the fiber pores. Compared with the structure and ability of flat sheet membranes, hollow fibers allow low-cost extraction with reducing carry-over effects, provide higher surface area per unit volume, and have lower solvent usage. For their advantages, hollow fibers are more extensively used for LPME than flat sheet membranes.

2.2.1.4.2 Organic solvent

The selection of organic solvent in HF-LPME is an essential step for two-phase and three-phase systems. The organic solvent chosen for extraction must be immiscible with water, strongly immobilized within the fiber pores to prevent leakage of analytes into sample solution, and provide appropriate extraction selectivity related to extraction recoveries. Due to the fact that the partition coefficients of analyte in aqueous and organic phase control the extraction efficiency in HF-LPME process, the organic solvent type and composition are necessarily optimized parameter.

In two-phase extraction, the organic solvent should be selected to provide high solubility of hydrophobic analytes in organic phase (high $K_{organic/donor}$) and should have

suitable properties to be compatible with GC analysis. 1-octanol is the most popular organic solvent used in two-phase HF-LPME, but some organic solvents used in three-phase system can also be applied in many two-phase systems.

In three-phase extraction, organic solvent immobilized within fiber pores serve as a barrier between the two aqueous phases (donor and acceptor). Therefore, selected solvent should offer high $K_{organic/donor}$ and high $K_{acceptor/organic}$ for target analytes together with proper polarity when combined with polypropylene hollow fibers. The volume of organic solvent is related to recovery, extraction efficiency, and enrichment factor in three-phase system as seen in Eq. 8-10. However, the volume of organic solvent employment depends on hollow fiber porosity, which is difficult to optimize. For three-phase mode, 1-octanol and dihexyl ether are extensively used as organic solvent.

The composition of organic solvent is another choice to improve extraction of target analytes. In case that one organic solvent cannot extract a large group of analytes with different polarity, mixed solvent systems are applied to cover the dissimilar properties. In carrier-mediated HF-LPME, the addition of ion-pairing agent into organic solvent has been proven to effectively enhance extraction of very hydrophilic analytes. Therefore, carrier type and its composition in organic solvent are alternative parameters to increase the extraction efficiency.

2.2.1.4.3 Extraction kinetics

To obtain high recovery and enrichment, high extraction speed is required in HF-LPME and agitation or stirring are effective ways to achieve that. These techniques are related the increase extraction kinetics. Agitation or stirring of sample solution cause faster diffusion of analytes into organic or acceptor solution. With enhanced extraction kinetics, the extraction time is reduced and the repeatability of extraction method is improved. However, improper agitation can affect the organic solvent immobilized in fiber pores. Hence, magnetic stirring can properly promote the diffusion of analytes. To date, there are multi-stirrer devices that are convenient for extraction. Many sample

solutions can be extracted simultaneously and help to decrease time and labor for extraction.

2.2.1.4.4 Donor solutions

Donor or aqueous sample solution has three main parameters to be optimized such as pH, volume, and composition. For donor volume, it directly related with acceptor volume to create the volume ratio ($V_{donor}/V_{acceptor}$) affecting the enrichment factor and recovery of analytes. From Eq. 5 and Eq. 10, the donor volume should be relatively high to provide large volume ratio in both two-phase and three-phase systems corresponding with mL-level of the sample volume. The pH of donor solution is associated with the extraction enhancement and donor pH changes may lead to higher analyte preconcentration. pH in the donor solution should be adjusted as such deionized analytes are obtained in order to reduce their solubility in the sample solution and to promote their transport to organic phase. In addition, a carrier can be added to the sample solution instead of organic solvent to efficiently transfer analytes. Besides carrier which is the one additive in sample solution, the solution filled to adjust donor pH is another consideration. The pH adjustment solutions should not react with analytes or carrier and must not interfere the extraction process.

2.2.1.4.5 Acceptor solutions

Acceptor solutions in two-phase and three-phase extraction are different. It can be defined that the acceptor phase is a parameter that separates the two modes of extraction. While in two-phase systems the acceptor solution is an organic solvent, the acceptor solution in a three-phase system is aqueous. Two-phase systems properly extract hydrophobic analytes into organic acceptor phases, whereas hydrophilic analytes are to be extracted with three-phase systems. Like the donor solution, the three considerations of acceptor solution are volume, type, and composition. The acceptor volume is relatively low in μL-level to be easily directly injected into HPLC analysis. Low amounts of acceptor combined with the large donor volume in solution are also inducing high volume ratio of donor and acceptor solution ($V_{donor}/V_{acceptor}$). Besides

high recovery and enrichment factor obtained, the sensitivity of method is increased by a high volume ratio. In three-phase mode, pH of the aqueous acceptor solution be adjusted to ensure efficient extraction of analytes from organic phase and to prevent analytes to be trapped in the organic phase. Hence, acceptor pH should be adjusted to obtain analytes in their ionized form. The composition of acceptor phase is determined by the analytical method chosen. For two-phase mode, organic acceptor should match with GC behavior, while aqueous acceptor in three-phase mode should be appropriate for HPLC or CE detection.

2.2.1.4.6 Extraction time

In HF-LPME, mass transfer is based on time-dependent equilibrium process. When the extraction system is close to equilibrium, the mass transfer rate is reduced. In other words, HF-LPME is defined as a non-exhaustive method. It, therefore, may consume long time for the system to reach equilibrium. Even the longer extraction times result in increased extraction efficiencies; short time is strongly required in practical analysis. During the experiment, consistent and precise timing is necessary for good precision in simultaneous extraction of a large number of samples. High sample throughput capacities compensate long extraction time.

2.2.1.4.7 HF-LPME configuration

There are several configuration utilized in HF-LPME. The U-shaped configuration seems to extensively used compared to other configurations. The technical set-up of this configuration is illustrated in Figure 2.8.

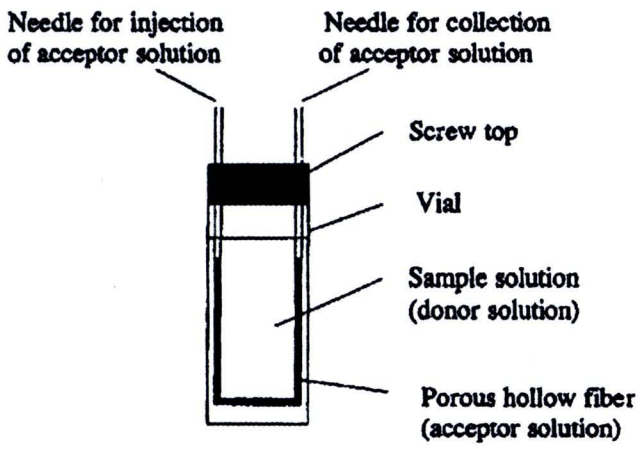

Figure 2.8 HF-LPME technical set-up in U-shaped configuration. *(28)*

In U-shaped configuration, the two porous hollow fiber ends are connected to syringe needles to hold the fiber in U-shape within the sample solution. One fiber end is used to fill acceptor solution into fiber lumen, while the other end is employed to collect acceptor solution after extraction. This configuration provides excellent extractions, but it has some drawbacks in transferring the acceptor solution into the instrument, which leads to difficulties in automation.

There is another U-shaped configuration, where one end of the fiber is connected to a funnel-shaped injection guide, while the other end is held by a small dent in the injection guide. This configuration is shown in Figure 2.9. The set-up decreases air-bubble formation in the acceptor solution and the device can directly be transferred to an autosampler for further analysis.

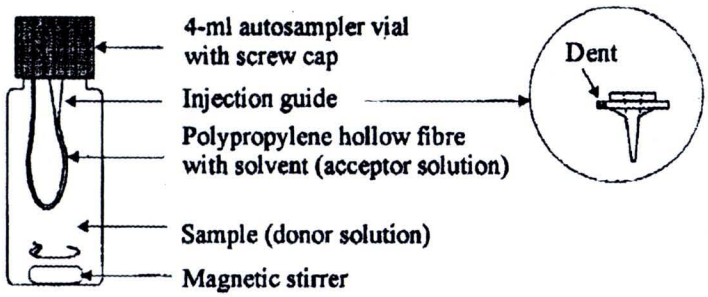

Figure 2.9 Alternative HF-LPME technical set-up in U-shaped configuration. *(28)*

Besides U-shaped, HF-LPME has rod-like configuration as shown in Figure 2.10. This configuration has resolved the U-shaped problem with the application of one tip for both addition and removal of acceptor solution and lead to a more convenient automated system.

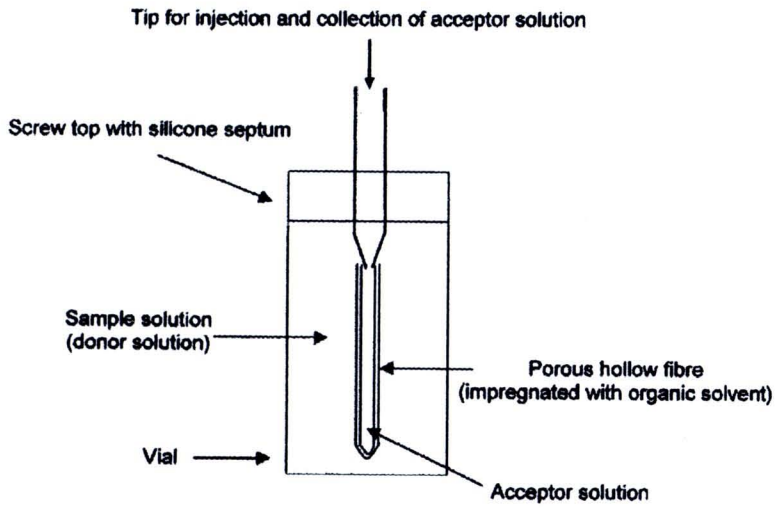

Figure 2.10 HF-LPME technical set-up in rod-like configuration. *(28)*

However, the hollow fiber configuration is crucial to extraction efficiency and enrichment factor of HF-LPME. The selected configuration may have to be additionally optimized (e.g., length of hollow fiber, the volume of acceptor, and suitable supplementary devices).

2.3 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) *(36, 37, 38, 39, 40)*

Currently, high-performance liquid-chromatography (HPLC) has been widely applied as determination step for residual analysis. HPLC accurately and precisely provides capabilities in separation and quantification of polar, non-volatile, and thermally unstable analytes. However, HPLC cannot provide enough information regarding the identity of compounds. Mass spectrometry (MS) is the detection system that can overcome this limitation and also offers high sensitivity and selectivity of analysis. MS can provide absolute identification by providing information about the molecular weight, structure, identity, and quantity of specific sample components. As a result of the resolving power of LC and the detection specificity of MS, LC is coupled with MS and has become the method of choice for routine qualitative and quantitative analysis.

2.3.1 High Performance Liquid Chromatography (HPLC)

Chromatography is a technique to separate mixtures into their individual components, so they can be identified and measured. In liquid chromatography (LC), the separation principle is based on the interaction of a solute with a stationary and a mobile phase. These interactions can be controlled through different choices of both stationary and mobile phases. A schematic diagram of a typical HPLC instrument is shown in Figure 2.11.

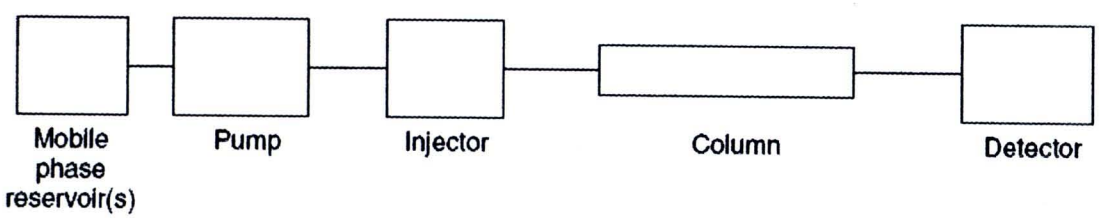

Figure 2.11 Schematic diagram of a typical HPLC instrument. *(36)*

In HPLC, the chromatographic process begins when the solute is injected into the injector, then the mobile phase, which is forced by a pumping system, carries the solute and flows through a chromatographic column. In the column, the mixture is separated into its components by the individual interaction of each component with mobile and stationary phase; and then the components are determined at the detector. The result of separation is shown in forms of chromatogram. From the HPLC diagram, the instrument consists of five parts (i.e., mobile phase, pump, injector, column, and detector).

2.3.1.1 Mobile phase and mobile phase reservoir

The most common type of mobile phase reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing, and filters to connect the bottles to the pumping system. The mobile phase reservoir should be placed away from sunlight and temperature gradients should be avoided.

Mobile phases in HPLC are usually mixtures of two or more individual solvents with or without additional additives or modifiers. The solvents chosen affects the elution of solute. In column HPLC there are two elution types such as isocratic and gradient mode. The selection of elution mode depends on the polarities of analytes. In isocratic elution, the mobile phase is employed at constant composition, while change in mobile phase compositions during the separation is called gradient elution. Gradient elution mode reduces analysis time and increases resolution for complex mixtures.

Solvents used must be high purity, most often HPLC grade because impurities in solvents or reagents can react with solute. Besides purity, there are other considerations to be made in solvent selection such as viscosity, polarity, toxicity, boiling point, and detector compatibility. Mobile phases must be filtered and degassed before used because the dissolved gases in solvents can be collected in the columns, pumps, and detectors and, therefore, affect the reproducibility of the volume delivered. Additionally, large bubbles may stop the pump from working.

2.3.1.2 Pump

The mobile-phase solvents are delivered from their reservoirs by toa pump. High pressure pumps are needed to force solvents flow through column with a controlled flow rate because the particles in column are packed with high density. For HPLC, typical flow rates of 0.5-5.0 mL/min are produced by pumps operating at 300-6000 psi. The two major categories of pumps applied are constant flow or volume and constant pressure. Constant flow pumps generate a certain flow rate of mobile phase, while constant pressure pumps apply a constant pressure to the mobile phase flowing through column. Most HPLC instruments use a reciprocating pump for both maintaining a constant flow rate up to several milliliters per minute and obtaining high output pressure to push the mobile phase through the chromatographic column. Reciprocating pump results in a pulsed flow that induces noise to the chromatogram. To eliminate this problem a pulse damper is placed at the outlet of the pump.

2.3.1.3 Injector

The purpose of the injection system is to apply the sample extract onto the column in a narrow band. The three available techniques of injection are direct syringe injection, stop flow syringe injection, and injection valve. The sample injected should be in solution, so solid samples need to be dissolve in an appropriate solvent, which must not be the same type as mobile phase prior to injection. The injection valve is widely used as injection device for reproducibly introducing sample extracts into pressurized columns without flow interruption. After the valve is loaded with sample, it switches mode sample and mobile phase flow to the column.

2.3.1.4 Column

In HPLC, the two columns typically utilized are an analytical and a guard column. An analytical column is used to separate the sample, while the guard column is placed before the analytical column to protect the analytical column from contamination.

Typical analytical columns are 10, 15, or 25 cm in length and are fitted with extremely small diameter particles (3, 5, or 10 μm). The internal diameter of the columns is usually between 1 and 4.6 mm. The major advantages of these shorter columns are faster separations and improved sensitivity of detection. The most widely used columns contain chemically modified silica stationary phase with the chemical modification determining the polarity of the column. The stationary phase selection is based on the surface interactions and the adsorption sites. Modern HPLC adsorbents are small rigid porous particles with high surface area. A very popular stationary phase is C18 alkyl group, which is bonded to the silica surface.

The guard column is employed to eliminate two threats to the analytical column. Firstly, solutes binding irreversibly to the stationary phase will degrade the analytical column's performance by decreasing the available of the stationary phase. Secondly, particulate material injected with the sample may clog the analytical column. Guard columns usually contain the same particulate packing material and stationary phase as the analytical column but are significantly shorter and less expensive.

2.3.1.5 Detector

The function of an HPLC detector is to continuously and instantaneously monitor the components emerging from the column. The most popular HPLC detectors based on spectroscopic measurements are UV/Visible and fluorescence detectors. The analytical wavelength is selected in a modified spectrophotometer equipped with a flow cell. When using a UV/Visible detector, the resulting chromatogram is a plot of absorbance as a function of elution time. An instrument utilizing a diode array detector (DAD) is giving a three-dimensional chromatogram showing absorbance as a function of wavelength and elution time. One limitation in using absorbance is that the mobile phases must not have absorbance at the chosen wavelength. Fluorescence detectors

provide additional selectivity when solutes can fluorescence. The resulting chromatogram is a plot of fluorescence intensity as a function of time. Another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. Nowadays, mass spectrometry (MS) is commonly used as a chromatographic detector. MS determination can be definitive, providing information on analyte retention, and concentration, while simultaneously confirming analyte identity.

2.3.2 Ultra Performance Liquid Chromatography (UPLC)

UPLC has been developed with the same practicality and principles as HPLC. With smaller size of packing materials from HPLC, UPLC offers greater resolution, speed, and selectivity. Owing to the efficiency of HPLC increased as particle sizes of the column packing decreased, the UPLC, which has particle size of 1.7 μm, provides efficiency three times greater when compared with 5 μm particle sizes of typical HPLC. In addition, the resolution can be increased up to 70%. Because of the small particle packing, the UPLC column length can be reduced by three times and the flow rate can grow up three times compared to HPLC. For these characteristics, UPLC provides high speed separation with low injection volume and proposes high sensitivity from less band spreading during migration through a column. A UPLC column is illustrated in Figure 2.12.

The Acquity system from Waters is the only UPLC system that is commercially available. UPLC is operated at high pressure of around 8000 psi due to the small size particle packing in column. Therefore, UPLC application requires a better pumping system than HPLC and the detector for UPLC must have a high sampling rate for sensitive detection and reliable quantification of the narrow peaks produced.

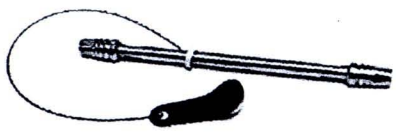

Figure 2.12 Acquity™ UPLC column *(41)*

2.3.3 Mass Spectrometry (MS)

Mass spectrometry is one of the most important analytical tools, in order to obtain information about the chemical composition and abundance of isotopes. A mass spectrometer produces ions from the substance, separates them according to their mass to charge ratio (m/z), and records the relative abundance of each ionic species present. The three major components of a MS instrument are ion source, mass analyzer, and detector. Figure 2.13 shows a schematic diagram of the mass spectrometry process.

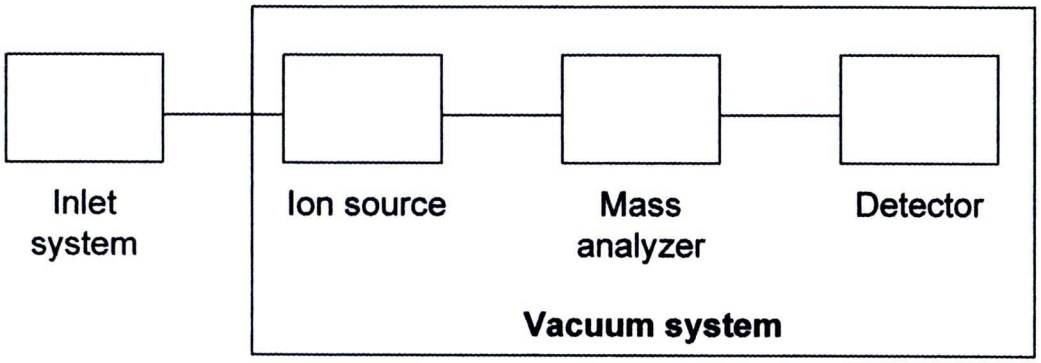

Figure 2.13 Schematic diagram of MS system.

In MS, samples are transferred through the introduction system into the vacuum area of the mass spectrometer. In the ion source region, sample molecules are ionized to gas phase ions and accelerated into mass analyzer, where all ions are separated according to their mass to charge ratio. Finally, separated ions are determined with a detector and signals are delivered to data system analysis. All MS instruments have a high vacuum system to minimize the collision between ions, prevent the loss of ions, and increase the mean free path of ions.

2.3.3.1 Ion source

Ion source is the region, where ionization of analytes occurs. In hyphenated systems of LC and MS, the ionization appears on the interface area of LC and MS, where the separated components from LC are introduced. The LC-MS interface is utilized to eliminate the mobile phase from LC and produce gas phase ions of analytes for further separation and detection in the MS system. Extensive ionization techniques in LC-MS are atmospheric pressure electrospray ionization (AP-ESI) and atmospheric pressure

chemical ionization (APCI). The ionization technique is selected based on analyte properties. Most mass spectrometers use positive ions, which are easily created. However, sometimes negative ions are required.

2.3.3.1.1 Atmospheric pressure electrospray ionization (AP-ESI)

AP-ESI is a useful ionization technique to analyze samples that become single or multiple charged depending on their molecular structures. It can be used to create either positive or negative ions, and it also ionizes high molecular weight components. AP-ESI ionization process is followed by evaporation. The three basic steps of AP-ESI are nebulization and charging, desolvation, and ion evaporation. These steps are shown in Figure 2.14.

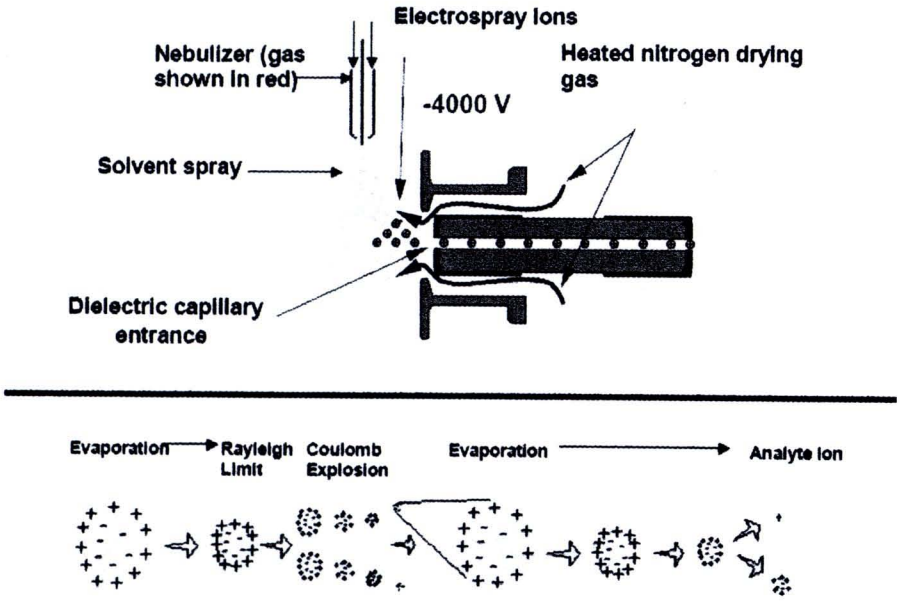

Figure 2.14 Atmospheric pressure electrospray ionization process. *(38)*

Firstly, the HPLC effluent is pumped through a nebulizing needle, which is set at ground potential. The spray passes an electrode, which is held at high potential. The potential difference between the needle and the electrode produces a strong electrical field. This field charges the surface of the liquid and forms a spray of charged droplets. During the desolvation step, the droplets are attracted to the capillary and dried with a

heated nitrogen gas flow and uncharged species are eliminated. After the charged droplet size is reduced, the repulsive force within charges overcomes the cohesive force of surface tension and creates coulombic explosion. This process is repeated until the analyte ions are desorbed into the gas phase. These gas-phase ions are then continuously passed to the mass analyzer.

AP-ESI is a concentration dependent technique and has many advantages such as high sensitivity to polar compounds, it produces multiply charged ions, and is suitable to reverse phase solvents.

2.3.3.1.2 Atmospheric pressure chemical ionization (APCI)

APCI is an ionization technique that is applicable to a wide range of polar and nonpolar analytes of moderate molecular weight. APCI differs from AP-ESI as evaporation process occurs and is followed by ionization. APCI also has three basic steps; nebulization, desolvation, and ionization. These steps are shown in Figure 2.15.

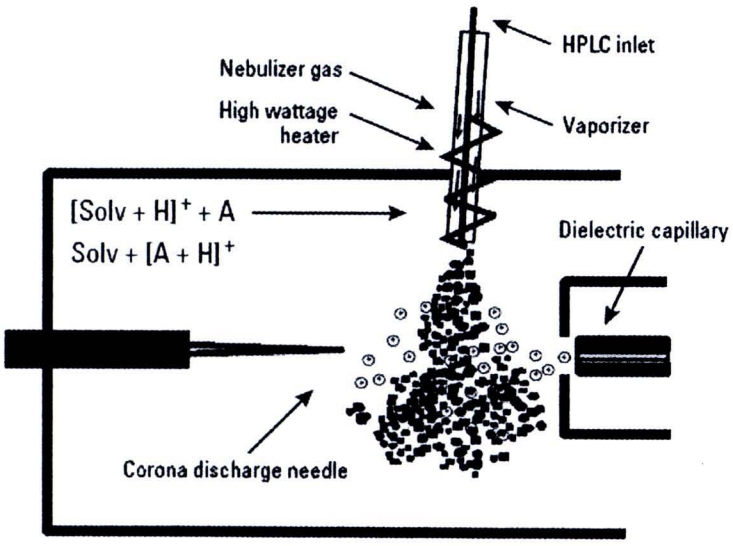

Figure 2.15 Atmospheric pressure chemical ionization process. *(38)*

APCI nebulization is similar to API-ES, but APCI nebulization occurs in a hot vaporizer chamber (typically 250°C–400°C). The effluents from HPLC are evaporated to spray droplets of solvent and analytes in gas phase. The gas-phase solvent molecules

are ionized by a corona needle discharge. Then, the charge is transfered from the ionized solvent species to the analyte molecules, and the charged analytes are delivered to the mass analyzer.

APCI can handle HPLC flow rates up to 2 mL/min, efficiently works with many compounds, especially non-polar, and produces only single charged ions. Nevertheless, possible thermal degradation is of concern in APCI; furthermore, compounds require a certain vapor pressure.

2.3.3.2 Mass analyzer

The mass Analyzer separates ions by their mass to charge ratio (m/z) in space or in time. After ions are formed in the ion source region, they are accelerated into the mass analyzer. The mechanism is performed with electric and magnetic fields, sometimes including RF fields. There should have some ion focusing device to prevent the spread of ions from ion source. The selection of mass analyzer depends on the resolution, mass range, scan rate, and detection limits required for the application. Each analyzer has different operating characteristics, and an additional instrument. In hyphenated LC-MS, quadrupole and time-of-flight (TOF) are widely used mass analyzers. Both techniques are considered as ion transmission system.

2.3.3.2.1 Quadrupole mass analyzer

The quadrupole mass spectrometer is the most common mass analyzer because of its compact size, fast scan rate, high transmission efficiency, and moderate vacuum requirements. In the mass spectrometer, the quadrupole analyzer consists of four parallel metal rods or electrodes. Two parallel rods are connected to direct current (DC), while the others are connected to radio frequency (RF). Both DC and RF are chosen to filtered ions. When the ions travel through the quadrupole, they are selected by DC and RF according to their m/z, only ion of selected m/z or resonance ion pass through quadrupole analyzer. A quadrupole mass analyzer is schematically shown in Figure 2.16.

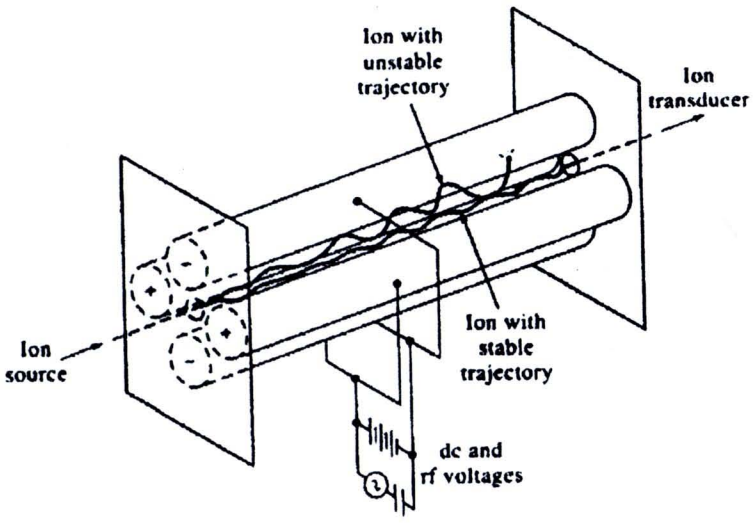

Figure 2.16 Quadrupole mass analyzer. *(39)*

2.3.3.2.2 Time-of-flight mass analyzer (TOF)

The time-of-flight mass analyzer (TOF) is the simplest configuration of the mass separation devices. The selection of ions is based on the movement of ion through the flight tube (Figure 2.17). TOF is usually applied to separate macromolecules with large m/z. The separation is based on the principle that ion of different masses experience individual velocities in the flight tube, and, in conclusion, have different flying time to the end of the tube, where transferred to the detector.

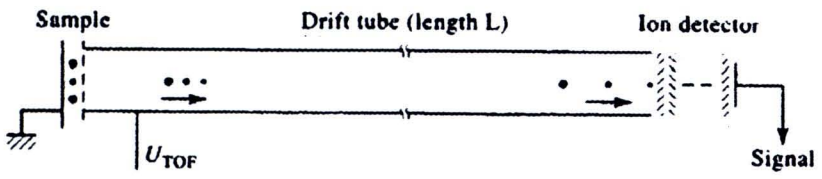

Figure 2.17 Time-of-flight mass analyzer. *(39)*

2.3.3.3 Detector

The detector is used to measure the ions leaving from the mass analyzer by converting ions into an electrical current or other forms of signal, processing and recording into mass spectrum. A detector is selected by speed, dynamic range, gain, and geometry. Most detectors currently used to amplify the ion signal are electron multiplier tube (Figure 2.18) and photo multiplier tube (Figure 2.18). Electron multiplier tube offers electron from surface of tube for analyte ions. The entrance of tube is held with potential charge opposite from the analyte ions. Analyte ions are attracted to the entrance of tube and collide with tube surface, then the inner surface coated with electron-emissive material releases electrons. These electrons are accelerated to hit another portion of tube by electrostatic force and the surface loses more electrons in every collision. Amplified electrons are counted by an electrical circuit and displayed as signal intensity. The photo multiplier tube comprises a photocathode and a series of dynodes. In the high voltage tube, incident photon strikes the photo cathode and emits electrons due to the photoelectric effect. These electrons are accelerated towards a series of additional electrodes called dynodes. At the dynodes, the amount of electrons is increased at every collision. This creates an amplified signal that is finally collected and measured at the anode.

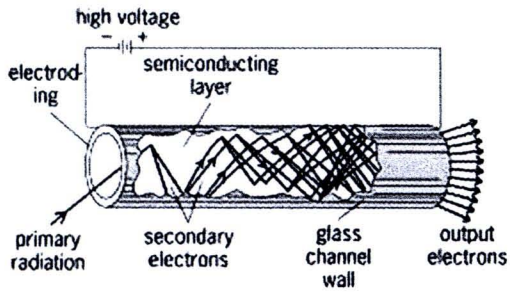

Figure 2.18 Electron multiplier tube. *(42)*

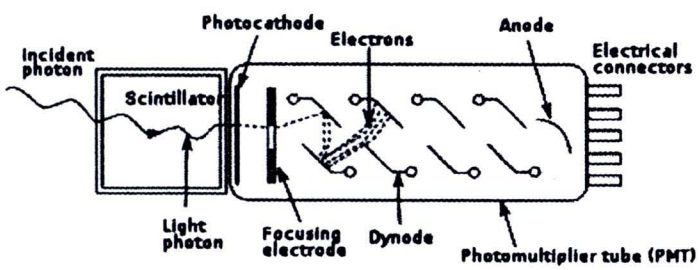

Figure 2.19 Photo multiplier tube. *(43)*

2.3.4 Tandem Mass spectrometry (MS/MS)

Tandem mass spectrometry uses two or more sequential mass spectrometers. MS/MS is a powerful technique that provides both the molecular weight of an analyte and information about the structure of the molecule involved. Therefore, MS/MS has been applied for many qualitative and quantitative applications. MS/MS is used to isolate an ion of interest in first mass analyzer (MS1) and then chemically or energetically modifies these ions with second mass analyzer (MS2). MS/MS process involves the determination of mass relationship between a precursor or parent ion in MS1 and a product or fragmented ion in MS2. The most commonly used tandem mass spectrometer is the triple quadrupole (QqQ). The configuration of QqQ consists of three sets of quadrupole rods in a series (Figure 2.19). Both the first and third sets of quadrupoles are used for mass separation, while the second set acts as a collision cell. The selected precursor ions pass from first quadrupole, are then fragmented and focused in the second quadrupole before transmitted into third quadrupole, where the fragmented ions of analytes are separated and subsequently detected. With this mechanism, MS/MS separates components of same molecular masses but different product ions with high specificity.

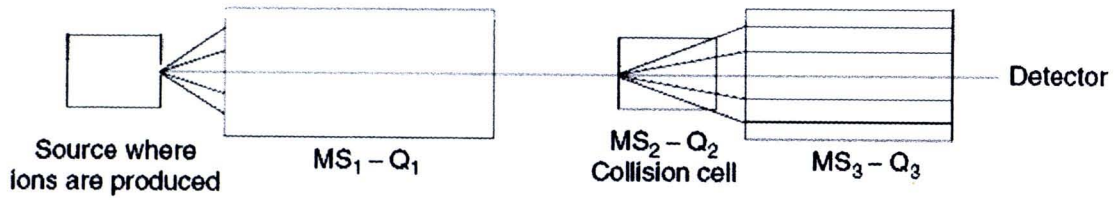

Figure 2.20 Triple quadrupole mass analyzer (QqQ). *(36)*