CHAPTER II

THEORY

2.1 Sample Preparation

Sample preparation is a technique used to clean-up a sample before analyzing it and/or to concentrate a sample to improve its detection. The need for sample preparation steps from three majors concerns:

Sample Concentration

Frequently, the component of interest is present in level too low a for detection. Sample preparation can make the component to become concentrated to an adequate level for measurement.

Contaminations

The presence of interfering matrix elements can mask the analysis of the component of interest. Sample preparation can remove excess contaminants to yield clean, informative chromatograms.

In Solution

For most analyses (HPLC, GC, Spectrophotometry, etc.), the sample must be properly prepared in solution for subsequent analysis.

Classification of extraction methods depend on the type of samples (solids, liquids and gases)

2.1.1 Volatile Samples

There are many sampling techniques for volatile samples, including gas-solid adsorption, headspace (HS) analysis, purge and trap. There are many sampling and sample preparation methods for gases.

2.1.1.1 Solid-Phase Trapping

A gaseous sample passed through a tube packed with adsorbent (such as silica gel or activated carbon); trapped analytes are eluted with strong solvent. This method is used for semi volatile organic compounds in air. Control of gas flow rate is critical for trapping efficiency. Watch for aerosol formation, adsorbent overloading, and irreversible adsorption of reactive analytes. Popular sorbents include silica gel, alumina, porous polymers (Tenax, polyurethane foams), or carbon.

2.1.1.2 Headspace Sampling

Sample (solid or liquid) is placed in a closed, thermostated glass vial until equilibrium is established. At equilibrium, analytes partition themselves between a gas phase and the solid (or liquid) phase at a constant ratio; the gas phase is sampled and injected into GC for analysis. Instument of classical headspace sampling as shown in Figure 2.1. This method is used primarily for determination of trace concentrations of volatile substances in samples difficult to handle by conventional GC techniques. Sensitivity can be increased by heating (<100 °C), salting out, adjusting pH, and other means to shift equilibrium. Sometimes water or solvent is added to aid in sample dispersion or to free organics from the matrix, especially for soils and sediments.

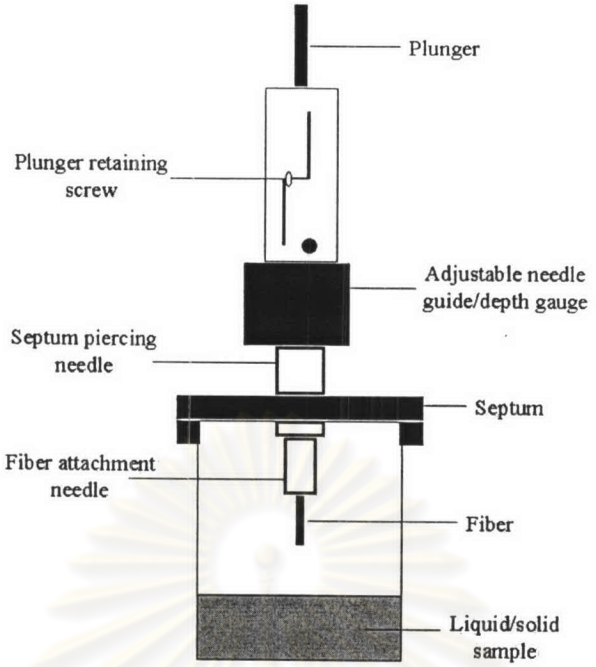


Figure 2.1 Instrument of calssical headspace sampling

2.1.1.3 Purge and Trap (Dynamic HS)

Sample (solid or liquid) is placed in closed, thermostated container and the HS vapors are continually removed by means of inert gas flow with subsequent trapping of sample components by solid phase extraction or cold trapping, and then thermally desorbed into GC injection port. This should be used when analytes are too low in concentration or have unfavorable partition coefficients in static HS sampling; sometimes called gas phase stripping. It can provide more sensitivity than static HS by accumulating the volatiles until concentration is sufficiently built up for thermal desorption and GC analysis. It can be manual or automated. Instrment of purge and trap as shown in Figure 2.2.

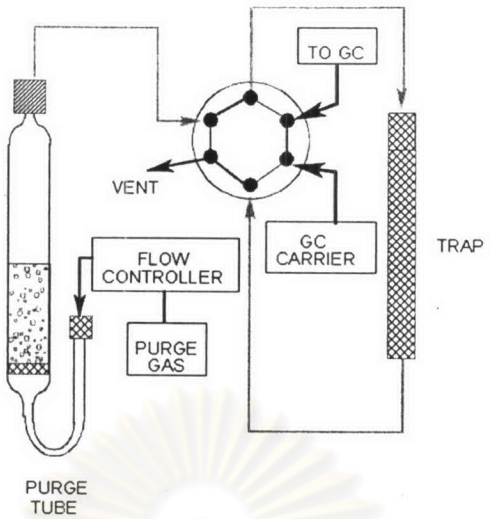


Figure 2.2 Instrument of purge and trap

2.1.1.4 Thermal Extraction

A form of dynamic HS but the sample is heated (controlled) much higher in temperatures, up to 350 °C. The system must be constructed by fused quartz or fused silica so that extracted analytes do not react with hot metal surfaces; system cold spots should be avoided. This should be used for semi volatile compounds.

2.1.2 Liquid Samples

Liquid samples are much easier to prepare for analytical measurement compared to volatile compounds or solids, because dissolution or an extraction step may not be involved. Often, dilution in a compatible solvent is all that is required. The major considerations for liquid samples are the matrix interferences, the concentration of analytes, and compatibility with the analytical technique.

2.1.2.1 Liquid- Liquid Extraction (LLE)

Sample is partitioned between two immiscible phases chosen to maximize differences in solubility; separator funnel is used for small sample volumes. Beware of formation of emulsions; break them with heat, addition of salt, filtration through a filter paper; change Kp by different solvent or chemical equilibria affecting additives (such as buffers for pH adjustment, salts for ionic strength, complexing agents, and ion pairing agents); many published methods are available; continuous extractions are used for low Kp or large volumes.

2.1.2.2 Dilution

Sample is diluted with solvent that is compatible with analytical measurement technique to avoid chromatographic column overload or to be in linear range of detector or spectrophotometer. Solvent should be compatible with analytical measurement technique; solvent should not be too strong for HPLC mobile phase conditions so that injection causes unacceptable band broadening.

2.1.2.3 Evaporation

Liquid is removed by gentle heating at atmospheric pressure with flowing air or inert gas or under vacuum. Do not evaporate too quickly; bumping can lose sample; watch for sample loss on wall of container; do not overheat to dryness. Best under inert gas such as nitrogen; rotary evaporator works best; automated systems are available. The instrument of evaporator as shown in Figure 2.3.

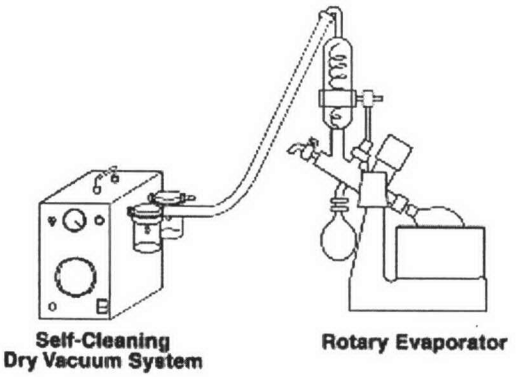


Figure 2.3 Instrument of evaporator

2.1.2.4 Distillation

Sample is heated to boiling point of solvent and volatile analytes are concentrated in vapor phase, condensed, and collected; steam distillation involves boiling with water or purging with steam and collecting distillate. Mainly for samples that can be volatilized; sample can decompose if heated too high; vacuum distillation can be used for nonvolatile compounds. The instrument of distillation as shown in Figure 2.4.

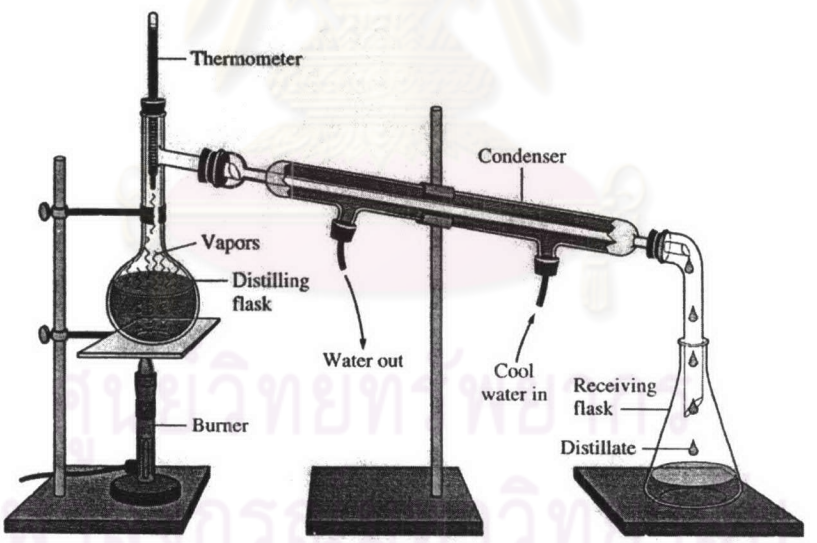


Figure 2.4 Instrument of distillation

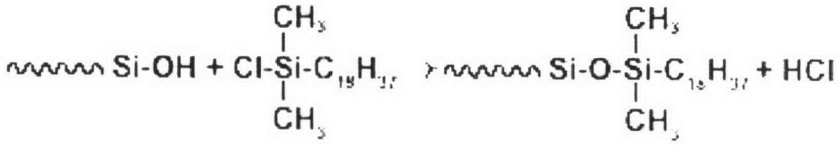
2.1.2.5 Centrifugation

Sample is placed in tapered centrifuge tube and spun at high force, liquid is decanted. Quantitatively removing solid sample from tube sometimes presents practical problem; ultracentrifuge normally not used for simple particulate removal.

2.1.2.6 Solid Phase Extraction (SPE)

Solid Phase Extraction is an extraction technique based on the selective partitioning of one or more components between two phases, one of which is a solid sorbent. The second phase typically is a liquid, but it may also be an emulsion , a gas or a supercritical fluid. The coponents of interest may either preferentially adsorb to the solid, or they may remain in the second, non-solid phase. Once equilibrium has been reached, the two phases are physically separated by decanting, filtration, centrifugation or a similar process. If the desired analytes are adsorbed on the solid phase, they can then be selectively desorbed by washing with an appropriate solvent. If the components of interest remain in a liquid phase, they can be recovered via concentration, evaporation, chromatographic separation, and/or recrystallization.

Reversed Phase SPE: Reversed phase separations involve a polar (usually aqueous) or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The analyte of interest is typically mid - to nonpolar. Several SPE materials, such as the alkyl- or aryl-bonded silicas (C-18, , C-8, C-4, and -Ph) are in the reversed phase category. Here, the hydrophilic silanol groups at the surface of the raw silica packing have been chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes.



Retention of organic analytes from polar solutions (e.g. water) onto these SPE materials is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. These nonpolar-nonpolar attractive forces are commonly called vander Waals forces, or dispersion forces. To elute an adsorbed compound from a reversed phase SPE tube or disk, use a nonpolar solvent to disrupt the forces that bind the compound to the packing C-18 and C-8 are standard, monomerically bonded silicas. Polymerically bonded materials, result in a more complete coverage of the silica surface and higher carbon loading. Polymeric bonding is more resistant to pH extremes, and thus is more suitable for environmental applications for trapping organic compounds from acidified aqueous samples. All silica-based bonded phases have some percentage of residual unreacted silanols that act as secondary interaction sites. These secondary interactions may be useful in the extraction or retention of highly polar analytes or contaminants, but may also irreversibly bind analytes of interest. The following materials also are used under reversed phase conditions: carbon-based, polymer- based), and polymer-coated and bonded silica.

Carbonaceous adsorption media, such as the materials, consist of graphitic, nonporous carbon that has a high attraction for organic polar and nonpolar compounds from both polar and nonpolar matrices. The carbon surface is comprised of atoms in hexagonal ring structures, interconnected and layered in graphitic sheets. The hexagonal ring structure demonstrates a strong selectivity for planar aromatic or hexagonal ring-shaped molecules and hydrocarbon chains with potential for multiple surface contact points. Retention of analytes is based primarily on the analyte's structure (size and shape), rather than on interactions of functional groups on the analyte with the sorbent surface. Elution is performed with mid- to nonpolar solvents. Styrene/divinylbenzene material is used for retaining hydro-phobic compounds which contain some hydrophilic functionality, especially aromatics. Phenols are sometimes difficult to retain on C-18-modified silica under reversed phase conditions, mainly due to their greater solubility in water than in

organic matrices. Styrene/divinylbenzene material has been shown to retain phenols well under reversed phase conditions. Elution steps can be done with mid- to nonpolar solvents, because the polymeric packing is stable in almost all matrices. Hydrophobic (C-18-like) bonded silica that is coated with a hydrophilic polymer and is typically used under reversed phase conditions. The porous polymer coating prevents the adsorption of large, unwanted molecules onto the silica surface. The pores in the polymer allow small, hydrophobic organic compounds of interest (such as drugs) to reach the bonded silica surface, while large interfering compounds (such as proteins) are shielded from the bonded silica by the polymer and are flushed through the SPE tube.

Normal Phase SPE: Normal phase SPE procedures typically involve a polar analyte, a mid- to nonpolar matrix (e.g. acetone, chlorinated solvents, and hexane), and a polar stationary phase. Polar-functionalized bonded silicas (e.g. CN, NH_2, and Diol), and polar adsorption media (Si, Florisil, and Alumina), typically are used under normal phase conditions. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. These include hydrogen bonding, π - π interactions, dipole-dipole interactions, and dipole-induced dipole interactions, among others. A compound adsorb by these mechanisms is eluted by passing a solvent that disrupts the binding mechanism - usually a solvent that is more polar than the sample's original matrix.

The bonded silica, -CN, -NH_2, and -Diol - have short alkyl chains with polar functional groups bonded to the surface. These silicas, because of their polar functional groups, are much more hydrophilic relative to the bonded reversed phase silicas. As with typical normal phase silicas, these packings can be used to adsorb polar compounds from nonpolar matrices. Such SPE tubes have been used to adsorb and selectively elute compounds of very similar structure (e.g. isomers), or complex mixtures or classes of compounds such as drugs and lipids. These materials also can be used under reversed phase conditions (with aqueous samples), to exploit the hydrophobic properties of the small alkyl chains in the bonded functional groups. The Si material is underivatized silica commonly used as the backbone of all of the bonded phases. This silica is extremely

hydrophilic, and must be kept dry. All samples used with this material must be relatively water-free. The functional groups that are involved in the adsorption of compounds from nonpolar matrices are the free hydroxyl groups on the surface of the silica particles. Si may be used to adsorb polar compounds from nonpolar matrices with subsequent elution of the compounds in an organic solvent that is more polar than the original sample matrix. In most cases, LC-Si is used as an adsorption media, where an organic extract is applied to the silica bed, the analyte of interest passes through unretained, and the unwanted com-pounds adsorb onto the silica and are discarded. This procedure is usually called sample clean-up. Florisil SPE tubes are packed with a magnesium silicate that is used typically for sample clean-up of organic extracts. This highly polar material strongly adsorbs polar compounds from nonpolar matrices. Alumina SPE tubes are also used in adsorption/sample cleanup-type procedures. The luminum oxide materials can either be of acidic (Alumina-A, pH ~5), basic (Alumina-B, pH ~8.5), or neutral (Alumina-N, pH ~6.5) pH, and are classified as having Brockmann Activities of I. The activity level of the alumina may be altered from grade I through grade IV with the controlled addition of water, prior to or after packing this material into tubes.

Ion Exchange SPE: Ion exchange SPE can be used for compounds that are charged when in a solution (usually aqueous, but sometimes organic). Anionic (negatively charged) compounds can be isolated on strong anionic exchanger (SAX) or NH_2 bonded silica cartridges. Cationic (positively charged) compounds are isolated by using strong cationic exchanger (SCX) or weak cationic exchanger (WCX) bonded silica cartridges. The primary retention mechanism of the compound is based mainly on the electrostatic attraction of the charged functional group on the compound to the charged group that is bonded to the silica surface. In order for a compound to retain by ion exchange from an aqueous solution, the pH of the sample matrix must be one at which both the compound of interest and the functional group on the bonded silica are charged. Also, there should be few, if any, other species of the same charge as the compound in the matrix that may interfere with the adsorption of the compound of interest. A solution having a pH that neutralizes either the compound's functional group or the functional group on the sorbent surface is used to elute the compound of interest. When one of these functional groups is

neutralized, the electrostatic force that binds the two together is disrupted and the compound is eluted. Alternatively, a solution that has a high ionic strength, or that contains an ionic species that displaces the adsorbed compound, is used to elute the compound.

Anion Exchange SPE: The SAX material is comprised of an aliphatic quaternary amine group that is bonded to the silica surface. A quaternary amine is a strong base and exists as a positively charged cation that exchange or attracts anionic species in the contacting solution - thus the term strong anion exchanger (SAX). The pKa of a quaternary amine is very high (greater than 14), which makes the bonded functional group charged at all pH when in an aqueous solution. As a result, SAX is used to isolate strong anionic (very low pKa, <1) or weak anionic (moderately low pKa, >2) compounds, as long as the pH of the sample is one at which the compound of interest is charged. For an anionic (acidic) compound of interest, the pH of the matrix must be 2 pH units above its pKa for it to be charged. In most cases, the compounds of interest are strong or weak acids. Because it binds so strongly, SAX is used to extract strong anions only when recovery or elution of the strong anion is not desired (the compound is isolated and discarded). Weak anions can be isolated and eluted from SAX because they can be either displaced by an alternative anion or eluted with an acidic solution at a pH that neutralizes the weak anion (2 pH units below its pKa). If recovery of a strongly anionic species is desired, use NH_2. The NH_2 SPE material that is used for normal phase separations is also considered to be a weak anion exchanger (WAX) when used with aqueous solutions. The NH_2 material has an aliphatic aminopropyl group bonded to the silica surface. The pKa of this primary amine functional group is around 9.8. For it to be used as an anion exchanger, the sample must be applied at pH at least 2 units below 9.8. The pH must also be at a value where the anionic compound of interest is also charged (2 pH units above its own pKa). NH_2 is used to isolate and recover both strong and weak anions because the amine functional group on the silica surface can be neutralized (2 pH units above its pKa) in order to elute the strong or weak anion. Weak anions also can be eluted from LC-NH_2 with a solution that neutralizes the adsorbed anion (2 pH units below its pKa), or by adding a different anion that displaces the analyze.

Cation Exchange SPE: The SCX material contains silica with aliphatic sulfonic acid groups that are bonded to the surface. The sulfonic acid group is strongly acidic (pKa <1), and attracts or exchanges cationic species in a contacting solution - thus the term strong cation exchanger (SCX). The bonded functional group is charged over the whole pH range, and therefore can be used to isolate strong cationic (very high pKa, >14) or weak cationic (moderately high pKa, <12) compounds, as long as the pH of the solution is one at which the compound of interest is charged. For a cationic (basic) compound of interest, the pH of the matrix must be 2 pH units below its pKa for it to be charged. In most cases, the compounds of interest are strong or weak bases. SCX SPE tubes should be used to isolate strong cations only when their recovery or elution is not desired. Weak cations can be isolated and eluted from SCX; elution is done with a solution at 2 pH units above the cation's pKa (neutralizing the analyte), or by adding a different cation that displaces the analyte. If recovery of a strongly cationic species is desired, use WCX.

The WCX SPE material contains an aliphatic carboxylic acid group that is bonded to the silica surface. The carboxylic acid group is a weak anion, and is thus considered a weak cation exchanger (WCX). The carboxylic acid functional group in WCX has a pKa of about 4.8, will be negatively charged in solutions of at least 2 pH units above this value, and will isolate cations if the pH is one at which they are both charged. WCX can be used to isolate and recover both strong and weak cations because the carboxylic acid functional group on the silica surface can be neutralized (2 pH units below its pKa) in order to elute the strong or weak cation. Weak cations also can be eluted from WCX with a solution that neutralizes the adsorbed cation (2 pH units above its pKa), or by adding a different cation that displaces the analyze.

In many cases, the analyte in ion exchange SPE is eluted in an aqueous solution. If you must use an acidic or basic solution to elute an analyte from an SPE tube, but the extracted sample must be analyzed in an organic solvent that is not miscible with water, try to elute the compound with acidic methanol (98% methanol / 2% concentrated HCl) or basic methanol (98% methanol / 2% NH_4OH). The methanol can be evaporated

quickly, and the sample may be reconstituted in a different solvent. If you need a stronger (more nonpolar) solvent to elute the analyte from the SPE tube, add methylene chloride, hexane, or ethyl acetate to the acidic or basic methanol.

Secondary Interactions

The primary retention mechanisms for compounds on the SPE materials are described above. For the bonded silicas, it is possible that secondary interactions will occur. For reversed phase bonded silicas, the primary retention mechanism involves nonpolar interactions. However, because of the silica particle backbone, some polar secondary interactions with residual silanols: such as those described for normal phase SPE could occur. If a nonpolar solvent does not efficiently elute a compound from reversed phase SPE packing, the addition of a more polar solvent (e.g. methanol) may be necessary to disrupt any polar interactions that retain the compound. In these cases, methanol can hydrogen bond with the hydroxyl groups on the silica surface, thus reaking up any hydrogen bonding that the analyte may be incurring.

The silanol group at the surface of the silica, Si-OH, can also be acidic, and may exist as Si-O group above pH 4. As a result, the silica backbone may also have cation exchange secondary interactions, attracting cationic or basic analytes of interest. In this case, a pH adjustment of the elution solvent may be necessary to disrupt these interactions for elution (acidic to neutralize the silanol group, or basic to neutralize the basic analyte). This can be done by using acidic methanol (98% MeOH: 2% concentrated HCl) or basic methanol (98% MeOH: 2% concentrated NH_4OH), or by mixtures of these with a more nonpolar, methanol-miscible solvent. Normal phase bonded silicas will exhibit primary polar retention mechanisms via the bonded functional group, but also can have some secondary nonpolar interactions of the analyte with the small alkyl chain that supports the functional group. In this case, a more nonpolar solvent, or a mix of polar and nonpolar solvents, may be needed for elution. As with the reversed phase silicas, secondary polar or cation exchange interactions of the adsorbed compound may occur with the silica backbone. Ion exchange bonded silicas can provide secondary nonpolar interactions of analytes with the nonpolar portions of their functional groups, as well as

polar and cation exchange interactions of the analyte with the silica backbone. A delicate balance of pH, ionic strength, and organic content may be necessary for elution of the analyte of interest from these packings.

Solid phase extraction is used to separate compounds of interest from impurities in three ways. Choose the most appropriate scheme for your sample:

Selective Extraction: Select an SPE sorbent that will bind selected components of the sample - either the compounds of interest or the sample impurities. The selected components are retained when the sample passes through the SPE tube or disk (the effluent will contain the sample minus the adsorbed components). Then, either collect the adsorbed compounds of interest through elution, or discard the tube containing the extracted impurities.

Selective Elution: The adsorbed compounds of interest are eluted in a solvent that leaves the strongly retained impurities behind.

SPE is a five-step process: The SPE process provides samples that are in solution, free of interfering matrix components, and concentrated enough for detection. This is done in five steps:

1. Select the Proper SPE Tube or Disk
2. Condition the SPE Tube or Disk
3. Add the Sample
4. Wash the Packing
5. Elute the Compounds of Interest

1 Selecting SPE Tube

1.1 Selecting an SPE Tube: Bed Weight

Reversed Phase, Normal Phase, and Adsorption-Type Procedures: The mass of the compounds to be extracted should not be more than 5% of the mass of the packing in the tube. In other words, if you are using a 100 mg/1mL SPE tube, do not load more than 5 mg of analytes.

1.2 Selecting an SPE Tube: Sorbent Type

Concentrate analyte by evaporation. Evaporate to dryness and reconstitute with another solvent. Use SPE. Is the organic solvent polar and water-miscible (e.g. methanol or acetonitrile) or mid- to nonpolar and not water-miscible (e.g. dichloromethane or hexane). If polar: dilute with water to <10% organic and follow the matrix scheme for aqueous analytes. If mid- to non-polar: use normal phase, or evaporate to dryness, reconstitute with water or a water-miscible solvent, then dilute with water as above and use the matrix scheme for aqueous analytes.

2. Condition the SPE Tube or Disk

Reversed phase: type silicas and nonpolar adsorption media usually are conditioned with a water-miscible organic solvent such as methanol, followed by water or an aqueous buffer. Methanol wets the surface of the sorbent and penetrates bonded alkyl phases, allowing water to wet the silica surface efficiently. Sometimes a pre-conditioning solvent is used before the methanol step. This solvent is usually the same as the elution solvent, and is used to remove any impurities on the SPE tube that could interfere with the analysis, and may be soluble only in a strong elution solvent.

Normal phase: type SPE silicas and polar adsorption media usually are conditioned in the organic solvent in which the sample exists.

Ion exchange: packings that will be used for samples in nonpolar, organic solvents should be conditioned with the sample solvent. For samples in polar solvents, use a water-miscible organic solvent, then an aqueous solution with the proper pH, organic solvent content, and salt concentration. To ensure that the SPE packing does not dry between conditioning and sample addition, allow about 1mm of the last conditioning solvent to remain above the top tube frit or above the surface of the disk. If the sample is to be introduced from a reservoir or filtration tube, add an additional 0.5 mL of the final conditioning solution to a 1 mL SPE tube, 2 mL to a 3 mL tube, 4 mL to a 6 mL tube, and so on. This prevents the tube from drying out before the sample actually reaches the tube. If the packing dries before the sample is added, repeat the conditioning procedure. Flush buffer salts from the tube with water before reintroducing organic solvents. If appropriate, attach the sample reservoir at this time using a tube adapter.

3. Add the Sample

Accurately transfer the sample to the tube or reservoir, using a volumetric pipette or micropipette. The sample must be in a form that is compatible with SPE. Total sample volume can range from microliters to liters. When excessive volumes of aqueous solutions are extracted, reversed phase silica packings gradually lose the solvent layer acquired through the conditioning process. This reduces extraction efficiency and sample recovery. For samples >250 mL, add small amounts of water-miscible solvents (up to 10%) to maintain proper wetting of reversed phase packings. Maximum sample capacity is specific to each application and the conditions used. If recoveries are low or irreproducible, test for analyte break-through using the following technique: Attach two conditioned SPE tubes of the same packing together using an adapter. Pass the sample through both tubes. When finished, detach each tube and elute it separately. If the analyte is found in the extract of the bottom tube, the sample volume is too great or bed weight is too small, resulting in analyte breakthrough. To enhance retention of appropriate compounds on the packing, and elution or recipitation of unwanted compounds, adjust the pH, salt concentration, and/or organic solvent content of the sample solution. To

avoid clogging SPE tube frits or the SPE disk, pre-filter or centrifuge samples prior to extraction if possible. Slowly pass the sample solution through the extraction device, using either vacuum or positive pressure. The flow rate can affect the retention of certain compounds. Generally, the flow rate should not exceed 2 mL/min for ion exchange SPE tubes, 5 mL/min for other SPE tubes, and may be up to 50 mL/min for disks. Dropwise flow is best, when time is not a factor.

4. Wash the Packing

If compounds of interest are retained on the packing, wash off unwanted, unretained materials using the same solution in which the sample was dissolved, or another solution that will not remove the desired compounds. Usually no more than a tube volume of wash solution is needed, or 5-10 mL for SPE disks. To remove unwanted, weakly retained materials, wash the packing with solutions that are stronger than the sample matrix, but weaker than needed to remove compounds of interest. A typical solution may contain less organic or inorganic salt than the final eluant. It also may be adjusted to a different pH. Pure solvents or mixtures of solvents differing sufficiently in polarity from the final eluant may be useful wash solutions. If you are using a procedure by which compounds of interest are not retained on the packing, use about one tube volume of the sample solvent to remove any residual, desired components from the tube, or 5-10 mL to remove the material from a disk. This rinse serves as the elution step to complete the extraction process in this case.

5. Elute the Compounds of Interest

Rinse the packing with a small volume (typically 200 µL to 2 mL depending on the tube size, or 5-10 mL depending on the disk size) of a solution that removes compounds of interest, but leaves behind any impurities not removed in the wash step. Collect the eluate and further prepare as appropriate. Two small aliquots generally elute compounds of interest more efficiently than one larger aliquot. Recovery of analytes is best when each

aliquot remains in contact with the tube packing or disk for 20 seconds to 1 minute. Slow or drop-wise flow rates in this step are beneficial.

Sample Pretreatment

In addition to ensuring proper pH of the sample, you should consider other sample pretreatment needs. The following section describes how some difficult sample matrices should be pretreated before being applied to the SPE device:

Liquids;

Serum, plasma, and whole blood: Serum and plasma samples may not need to be pretreated for SPE. In many cases, however, analytes such as drugs may be protein-bound, which reduces SPE recoveries. To disrupt protein binding in these biological fluids, use one of the following methods for reversed phase or ion exchange SPE procedures: Shift pH of the sample to extremes (pH<3 or pH>9) with acids or bases in the concentration range of 0.1M or greater. Use the resulting supernatant as the sample for SPE. Precipitate the proteins using a polar solvent such as acetonitrile, methanol, or acetone (two parts solvent per one part biological fluid is typical). After mixing and centrifugation, remove the supernatant and dilute with water or an aqueous buffer for the SPE procedure. To precipitate proteins, treat the biological fluid with acids or inorganic salts, such as formic acid, perchloric acid, trichloroacetic acid, ammonium sulfate, sodium sulfate, or zinc sulfate. The pH of the resulting supernatant may be adjusted prior to use for the SPE procedure. Sonicate the biological fluid for 15 minutes, add water or buffer, centrifuge, and use the supernatant for the SPE procedure.

Urine: Urine samples may not require pre-treatment for reversed phase or ion exchange SPE, but often is diluted with water or a buffer of the appropriate pH prior to sample addition. In some cases, acid hydrolysis (for basic compounds) or base hydrolysis (for acidic compounds) is used to ensure that the compounds of interest are freely solvated in the urine sample. Usually a strong acid (e.g. concentrated HCl) or base (e.g. 10M KOH) is added to the urine. The urine is heated for 15- 20 minutes, then cooled and diluted with

a buffer, and the pH adjusted appropriately for the SPE procedure. Enzymatic hydrolysis that frees bound compounds or drugs also may be used.

Milk: Milk generally is processed under reversed phase or ion exchange SPE conditions. The sample may be diluted with water, or with mixtures of water and a polar solvent such as methanol (up to 50%). Some procedures may require precipitation of proteins by treatment with acid (typically HCl, H_2SO_4 , or trichloroacetic acid). After precipitation, the sample is centrifuged and the supernatant is used for SPE.

Water Samples Drinking water, groundwater, and waste-water samples may be extracted directly by SPE, as long as they are not heavily laden with solid particles. Groundwater and wastewater samples might need to be filtered prior to the SPE procedure. Filtering may reduce recoveries if compounds of interest are bound to the removed particles. If possible, do not filter the sample. Pass the unfiltered sample directly through the SPE device and, during elution, allow the solvent to pass through the particles on the adsorbent bed. This will improve recoveries, since particle-bound com-pounds of interest will be recovered using this process. In most cases, water samples are used with reversed phase or ion exchange SPE procedures.

Wine, Beer, and Aqueous Beverages Aqueous and alcoholic beverages may be processed for SPE without pretreatment under reversed phase or ion exchange conditions. For reversed phase procedures, if alcohol content is high, dilution with water or buffer to <10% alcohol may be required. If necessary, solids in the sample can be re-moved by centrifugation or filtration prior to SPE.

Fruit Juices Fruit juices typically are processed without pretreatment or are centrifuged for reversed phase or ion exchange SPE. If centrifuged, the resulting supernatant is used for the SPE procedure. Viscous juices may need to be diluted with water or buffer at the proper pH.

Liquid Pharmaceutical Preparations Because liquid pharmaceuticals are mainly aqueous, these samples generally are processed by reversed phase or ion exchange SPE. If the preparation is viscous, dilution with water or an appropriate buffer may be necessary. Organic extracts of the preparation may be processed using normal phase SPE. **Oils** Hydrocarbon or fatty oils are commonly processed under normal phase conditions, because they cannot be diluted with water. The diluent is usually a mid-polar to nonpolar solvent such as hexane or a chlorinated solvent. The diluted sample is passed through a normal phase bonded silica or adsorption medium, and the sample is collected as it passes through. The compound of interest should pass through unretained, while impurities remain in the adsorbent. If the compound of interest is retained on the packing, successive washes of the SPE packing with increasingly polar solvents, or with mixtures of the diluent with a polar solvent, are per-formed until the analyte is recovered in one of the fractions. For collecting oil in water samples, reversed phase SPE is used.

Solids;

Soil and Sediment: Soil and sediment samples typically are extracted with mid-polar to nonpolar solvents via Soxhlet extraction or sonication. The resulting extracts are then processed by normal phase SPE to remove interferences. The cleaned extracts then can be evaporated and reconstituted with another solvent for additional SPE (reversed phase, ion exchange, or normal phase) if necessary. If extraction efficiency of the compound of interest is pH-dependent, soil and sediment samples may need to be homogenized with water at the appropriate pH prior to extraction and SPE cleanup. In some cases, small amounts of soil or sediment can be homogenized with an appropriate solvent and then passed through the SPE device without pretreatment, as long as the particles do not clog the device. The analyte is then eluted with the appropriate solvent by passing it directly through any particles that rest on the SPE tube packing or disk.

Plant Tissues, Fruits, Vegetables, and Grains Plant tissues, fruits, vegetables, and commodities such as animal feeds and grains are homogenized either in water, in a polar organic solvent (e.g. methanol or acetonitrile), or in mixtures of water with these

solvents, for reversed phase or ion exchange cleanup procedures. After centrifugation or filtration to remove the precipitated proteins and solids, the pH of the sample may need to be adjusted. The analyte may adsorb onto the SPE packing or may simply pass through, free from interferences. The sample also may be homogenized with a mid-polar to nonpolar solvent for normal phase SPE procedures. Again, the sample may need to be centrifuged or filtered prior to SPE.

Meat, Fish, and Animal Tissues Meat, fish, and other tissues can be processed in the same manner as described above for solid fruits and vegetables. In addition to homogenization with water, sample preparation for reversed phase and ion ex-change SPE procedures may also involve hydrolysis or digestion of the meat or tissue with acid (typically HCl or richloroacetic acid) or saponification with base (e.g. NaOH). Enzymatic hydrolysis also may be used. The sample can then be centrifuged and the supernatant used for the SPE procedure. Tissue extracts obtained with mid-polar to nonpolar solvents can be processed using normal phase procedures.

Tablets and Other Solid Pharmaceutical Preparations Tablets and solid pharmaceutical preparations should be crushed into a fine powder, then extracted or homogenized with water or an appropriate buffer for reversed phase and ion exchange SPE procedures. A mid-polar to nonpolar solvent is used for normal phase cleanup procedures.

2.1.3 Solid Samples

When a sample is a solid, the sample pretreatment process can be more complex. There are two specific cases: the entire sample is of interest and must be solubilized, or only a part of the solid is of interest and the analytes must be selectively removed. If the solid sample is a soluble salt or drug tablet formulation, the only sample preparation that may be required is finding a suitable solvent that will totally dissolve the sample and the components of interest. If the sample matrix is insoluble in common solvents but the analytes of interest can be removed or leached out, then sample preparation can also be

rather straightforward. In these cases, techniques such as filtration, Soxhlet extraction, supercritical fluid extraction (SFE), ultrasonication, or solid-liquid extraction may be useful.

2.1.3.1 Solid – Liquid Extraction

Sample is placed in stoppered container and solvent is added that dissolves analyte of interest; solution is separated from solid by filtration (sometimes called shake/filter method). Solvent is usually boiled or refluxed to improve solubility; sample is in finely divided state to aid leaching process. Sample can be shaken manually or automatically; sample is filtered, decanted, or centrifuged to separate from insoluble solid.

2.1.3.2 Soxhlet Extraction

Sample is placed in disposable porous container (thimble); constantly refluxing solvent flows through the thimble and leaches out analytes, which are continuously collected in boiling pot. Extraction occurs in pure solvent; sample must be stable at boiling point of solvent. The process is slow but extraction is carried out unattended until complete; process is also inexpensive and excellent recoveries. Intrument as shown in Figure 2.5.

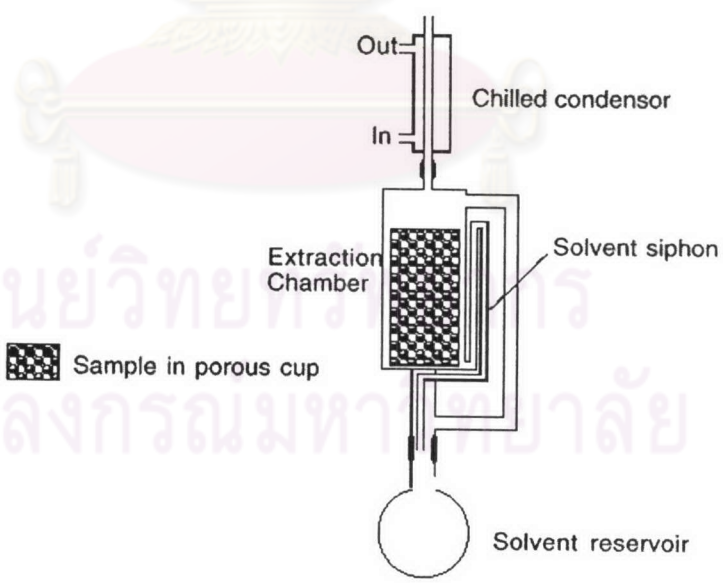


Figure 2.5 Instrument of soxhlet extraction

2.1.3.3 Homogenization

Sample is placed in a blender, solvent is added, and sample is homogenized to a finely divided state; solvent is removed for further workup. This is used for plant and animal tissue, food, environment samples. Organic or aqueous solvent can be used; dry ice or diatomaceous earth can be added to make sample flow more freely. Finely dispersed sample promotes more efficient extraction.

2.1.3.4 Sonication

Finely divided sample is immersed in ultrasonic bath with solvent and subjected to ultrasonic radiation. An ultrasonic probe or ultrasonic cell disrupter can also be used. Dissolution is aided by ultrasonic process; heat can be added for additional extraction. Process is safe and rapid; best for coarse, granular materials. Multiple samples can be done simultaneously; contact with solvent is efficient.

2.1.3.5 Dissolution

Sample is treated with solvent and taken directly into solution with or without chemical change. Inorganic solids may require acid or base to completely dissolve; organic samples can often be dissolved directly in solvent. Heat is required for many samples, especially organic polymers; consult solubility tables for common compounds and salts.

2.1.3.6 Supercritical Fluid Extraction (SFE)

Sample is placed in flow-through container and supercritical fluid (such as CO2) is passed through sample; after depressurization, extracted analyte is collected in solvent or trapped on adsorbent, followed by desorption by rinsing with solvent. Automated and manual versions are available; to affect "polarity" of supercritical fluid, density can be varied and solvent modifiers added. Collected sample is usually concentrated and pure

because CO_2 is removed after extraction; matrix has an effect on extraction process. The instrument of supercritical fluid extraction as shown in Figure 2.6.

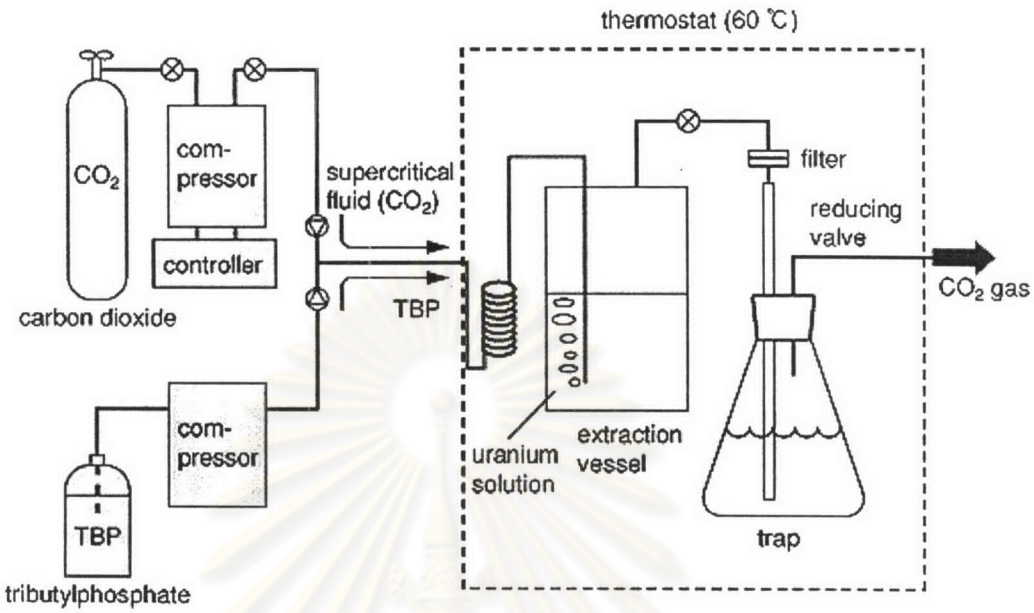


Figure 2.6 Instrument of supercritical fluid extraction

2.1.3.7 Accelerated (Enhanced) Solvent Extraction (ASE or ESE)

The sample is placed in a sealed container and heated to above its boiling point, causing pressure in vessel to rise; extracted sample is automatically removed and transferred to vial for further treatment. Greatly increases speed of liquid-solid extraction process and is automated. Vessel must withstand high pressure; extracted sample in diluted form requires further concentration; safety provisions are required. The instrument of accelerated solvent extraction as shown in Figure 2.7.

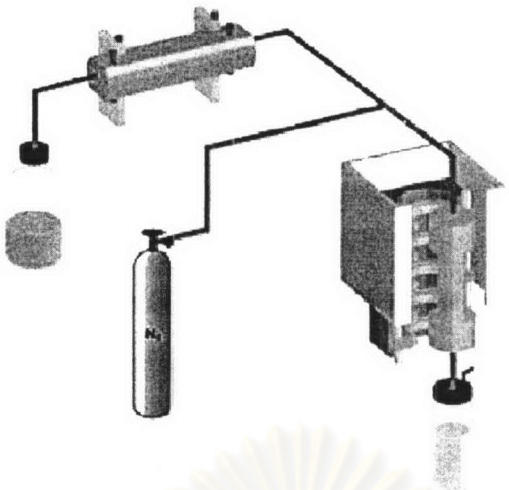


Figure 2.7 Instrument of accelerated (enhanced) solvent extraction

2.1.3.8 Automated Soxhlet Extraction

A combination of hot solvent leaching and Soxhlet extraction (Figure 2.8); sample in thimble is first immersed in boiling solvent, and then thimble is raised for Soxhlet extraction with solvent refluxing. Manual and automated versions are available; used less solvent than traditional Soxhlet, and solvent is recovered for possible reuse. Extraction time is decreased due to two-step process.

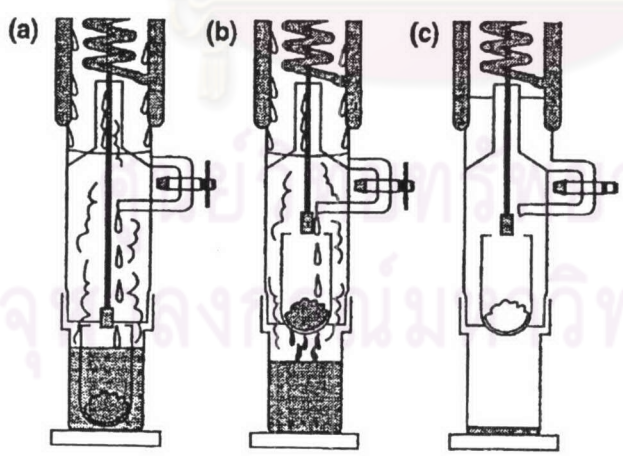


Figure 2.8 Instrument of automated Soxhlet extraction

2.1.3.9 Microwave-Assisted Extraction (MASE)

Sample is placed in an open or closed container and heated by microwave energy, causing extraction of analyte. Extraction solvent can range from microwave absorbing (MA) or non-microwave-absorbing (NMA); in MA case, sample is placed in high-pressure, non-microwave-absorbing container and heated well above its boiling point. Also in the MA case, the sample and solvent can be refluxed at atmospheric pressure, analogous to solid-liquid extraction; in NMA case, container can be open, with no pressure rise; safety provisions are required.

2.2 Pesticides

The class of agricultural chemicals commonly referred to as pesticides includes any compound intended to destroy, repel or otherwise control insects, weeds, nematodes, rodents and any other form of life declared to be a pest by the Environmental Protection Agency.

Post – World War II, chemical pesticides have become the most important consciously – applied form of pest management. The first "generation pesticides" were largely highly toxic compounds, such as arsenic and hydrogen cyanide. Their use were largely abandoned because they were either too ineffective or too toxic. The "second generation" pesticides largely included synthetic organic compounds. ('Synthetic' here means made by humans – not naturally occurring, while 'organic' means carbon containing, not to be confused with the popular use of "organic" as in organic farming).

The first important synthetic organic pesticide was a chlorinated hydrocarbon (organochlorine): dichlorodiphenyltrichloroethane or DDT. DDT was discovered in 1939 by a Swiss chemist Paul Mueller. It was hailed as a miracle for a number of reasons:

- It was toxic to wide range of insect pests yet appeared to have low toxicity to mammals.

- It was persistent (didn't break down rapidly in the environment) so that it didn't to be reapplied often.
- It was not water–soluble, so didn't get washed off by rains.
- It was inexpensive and easy to apply

It was so effective at killing pests and thus boosting crop yields and was so inexpensive to make that its use quickly spread over the globe. It was used for many non–agricultural applications as well, until the 1960's. It was used to control mosquitoes in residential areas of the US. Yields increased on treated crops, disease such as malaria were brought under control as never before (incidentally, malaria is now is on the increase again as the vectoring insects develop resistance to chemical pesticides). People could cheaply and easily control so many pests. The pesticide manufacturers said that the minute amounts found in the environment could not possibly be killing them. However, some experimental work demonstrated that even small amounts of some of the pesticides could affect the survival and reproduction of some species. More important, although concentrations were very low in the soil, atmosphere and water, concentrations were higher in plants, higher still in herbivores, and still higher as one moved up the food chain.

DDT was being transported for long distances in the atmosphere and then being washed from the atmosphere by rains. It also showed up in human breast milk at remark high concentrations. It is increasingly believed that, at high concentrations and for chronic, long–term exposure, it may be involved with liver cancers, reproductive abnormalities, and other human and mammalian effects. These concerns and the resulting public outcry prompted the US. Environmental Protection Agency (EPA) to cancel the registration of DDT in the US in 1972. Manufacture of DDT in the US did continue for export until the late 1970's. Currently these are no manufacturing of DDT in the US. DDT is still, however, widely used in less developed countries. When the last DDT manufacturing plant in the US was dismantled in 1983, it was sold to Indonesia, where it is currently manufacturing DDT. In December 2000, 122 nations (including the US) signed a treaty intended to phase out completely Persistent Organic Pollutants (POPs), including DDT.

These POPs are generally fat n-soluble (as in DDT), and are also very dispensable. The list of 12 POPs covered by the treaty includes nine pesticides (aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex and toxaphene) as well as dioxins.

The EPA wrote, in 1985, that dioxins are "the most potent carcinogen ever tested in laboratory animals". Many more POPs are likely to be list of globally–banned compounds. The chemical industry responded to the concern over DDT and its relatives with new classes of pesticides, which are less persistent than DDT, are often also acutely toxic. Today's pesticides are designed to persist for shorter periods in the environment and are supposedly less lethal than the early days of DDT.

2.2.1 Toxicity of Pesticides

The EPA banned use of chlordane, heptachlor and dieldrin in 1988 because they can remain in the environment for very long periods of time. The ability of these pesticides to accumulate in fat tissue means that very small amounts in air, water, soil and food can result in detectable levels in fat, blood and breast milk. The main toxic effect of these pesticides is on the liver and had only been demonstrated at very high doses, primarily in laboratory animals. Nervous system toxicity has been documented in humans receiving large doses of poisoning; include mild symptoms such as loss of balance, headache, dizziness and tumors as well as severe effects such as convulsion, seizures and death depending upon the dose. Some pesticides have the ability to cause cancer too.

2.2.2 Classification of Pesticides

Pesticides can be divided more than one class by many types such as type of pesticides to control (insecticide, acaricide, fungicide, bactericide, herbicide, nematicide, rodenticide and molluscicide), kind of pesticides to destroy (anti – feedant, anti – respirant, attractant, chemosterilant, defoliant, desiccant, repellent and growth regulator), source of pesticides (chemical from synthesis and plants), chemical compositions. For chemical composition pesticides can be divided into 3 types.

Inorganic compounds—they have an element that can be found in the environment to be the composition, but there is no carbon, stable, non–volatile, water soluble and highly toxic for humans and animals.

Organic compounds—synthetic compounds that have carbon, hydrogen and one or more elements such as chlorine, oxygen, sulfur, phosphorous or nitrogen.

Compound from plant extracts—extraction of plant that has pesticides such as pyrethrin.

Organic compounds can be classified to 11 minor groups.

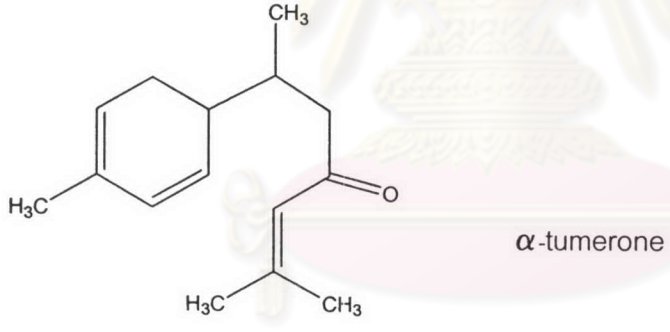
1. Organochlorine compounds or chlorinated hydrocarbons consist of carbon, hydrogen, chlorine and/or oxygen. This group is stable to decomposition and water–soluble such as DDT, endrin and heptachlor. Moreover, organochlorine compounds can be divided by structures too—diphenyl, aliphatic, benzene derivative and cyclodiene.
2. Organophosphorous compounds consist of phosphate, very severe toxic to humans and animals such as Malathion, parathion methyl and pirimiphos methyl.
3. Organosulfur compounds consist of sulfur such as tetradifon.
4. Carbamate like organophosphorous compounds such as carbaryl and carbofuran.
5. Formamedine—formetanate, amitraz.
6. Dinitrophenol—binapacryl, dinobuton.
7. Organotin compounds—cyhexatin.
8. Pyrethoids—deltamethrin, permethrin and cypermethrin.
9. Fumigant—methyl bromide, magnesium phosphide.
10. Petroleum oils
11. Antibiotic—penicillin.

2.3. Turmeric (*Curcuma Longa.*)

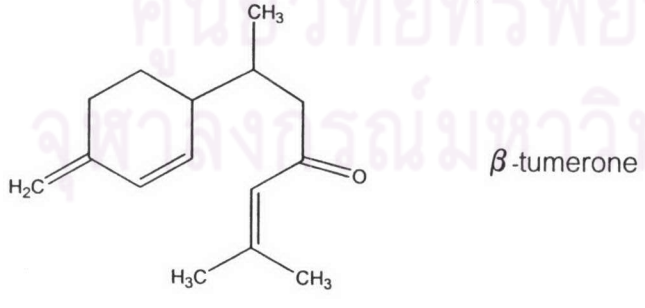
Figure 2.9 Picture of Turmeric (*Curcuma Longa.*)

Curcuma Longa, which belongs to the Zingiberaceae family, Curcuma Genus and Longa species. It has many common names, such as curcuma. It is a perennial herb that measures up to 1 m height with a shot stem, distributed throughout tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China [35]. As a powder, called turmeric, it has been in continuous use for its flavouring, as a spice in both vegetarian and non-vegetarian food preparations and it also has digestive properties.

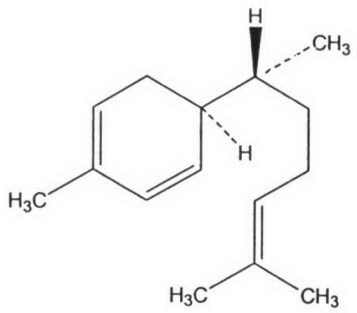
The coloring principle of turmeric was isolated in the 19th century and was named curcumin, which was extracted from the rhizomes of *Curcuma Longa.*, with yellow color and is the major component of plant. Pale yellow to orange-yellow volatile oil (6%) is composed of a number of monoterpenes and sesquiterpenes, including zingiberene, curcumene, α- and β- turmerone among others. The coloring principles are curcuminoids, 50–60% of which are a mixture of curcumin, monodesmethoxycurcumin and bisdesmethoxycurcumin. The structure of mixture of curcumin and volatile oil in *Curcuma Longa.* as shown in Figure 2.10.



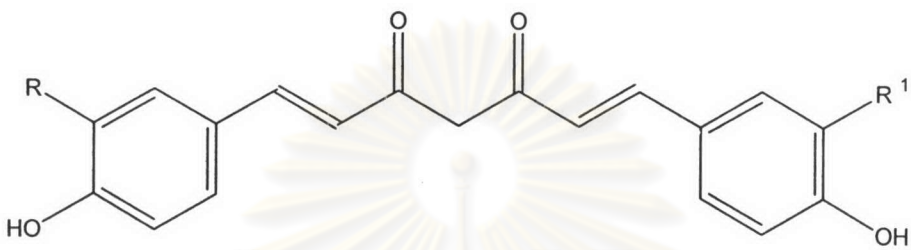
α-tumerone



β-tumerone



Zingiberene



curcumin	R = OCH_3, R' = OCH_3
desmethoxycurcumin	R = OCH_3, R' = H
bismethoxycurcumin	R = H, R' = H

Figure 2.10 Representative structures of curcuminoids

2.3.1 Activities of Turmeric (*Curcuma Longa.*)

2.3.1.1 Anti - Inflammatory Activity

There are a great number of papers on the literature relating to the activity of compounds extracted from *Curcuma Longa.* being potent inhibiters of inflammation. The volatile oil has exhibited anti-inflammatory activity in rats against adjuvant-induced arthritis, carrageemin-induced paw oedema. [36] Curcumin and its derivatives are the active anti-inflammatory constituents of the drug. [37] The anti-inflammatory activity of curcumin may be due to its ability to scavenge oxygen radicals, [38] which have been implicated in the inflammation process. [39]

2.3.1.2 Antioxidant Activity [40]

Unnikrishnan and Rao (1995) studied the anti-oxidative properties of curcumin and its there derivatives (desmethoxycurcumin, bisdesmethoxy curcumin and diacethyl curcumin). These substances provide a protection of hemoglobin from oxidation. curcumin is a good anti-oxidant and inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy & Lokesh 1994). The lipid peroxidation has a main role in the inflammation, in heart diseases, and in cancer. Pulla Reddy and Lokesh (1992) observed that curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals, which are important to the initiation of lipid peroxidation.

2.3.1.3 Anti - HIV Activity

Mazumber et al. (1995) demonstrated that curcumin has an anti-viral activity, being a HIV-I integrase inhibitor and suggested that curcumin analogs may be developed as anti-Aids drugs.

2.3.1.4 Anti - Tumor Activity

Ozaki et al. (2000), examining the action of curcumin on rabbit osteoclast apoptosis, demonstrated that curcumin drastically inhibits bone resorption in parallel with its stimulation of apoptosis in the cells. Since cancer and bone inflammation are diseases that increase bone resorption.

2.3.1.5 Other Activities

Curcumin can decrease high cholesterol levels and have anti-mutagenic activity. Moreover, curcumin could effectively inhibit diethylnitrosamin-induced liver inflammation in rats. Other interesting action was acute heptotoxicity and anti-bacterial.