APPENDIX A

MODE OF CAPILLARY ELECTROPHORESIS

As a technology, CE encompasses a family of related separation techniques that use narrow-bore fused silica capillaries to separate a complex variety of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Depending on the types of capillary and electrolytes used, CE can be segmented into several different separation techniques, as follows.

1. Capillary Gel electrophoresis (CGE)

CGE is a technique for separating charged molecules with different sizes. Two kinds of gels are commonly used: agarose and polyacrylamide. Agarose gels can be applied to a wider range of sizes than polyacrylamide gels. By using standard agarose electrophoresis, nuclei acids up to 50 kb may be separated. If Pulsed Field Gel Electrophoresis is used, the upper limit can be extended to 10 Mb. Polyacrylamide gels may separate nucleic acids that differ in length by only 1 nucleotide if their length is less than 500 bp.

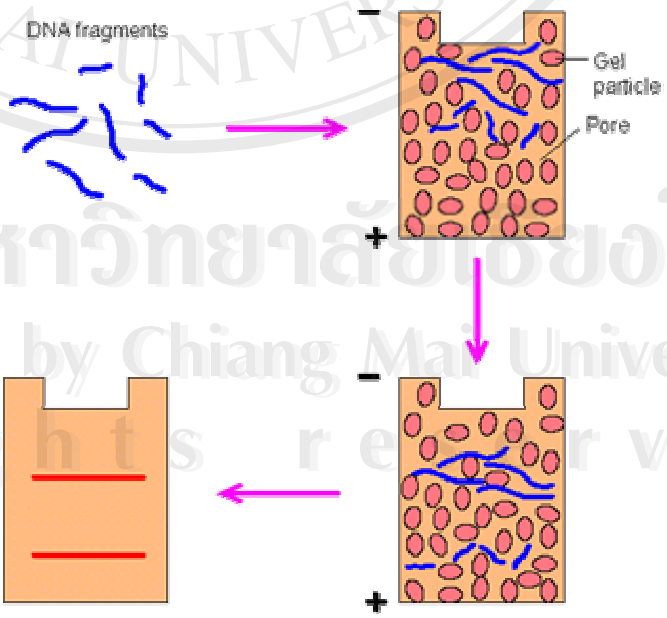


Figure A1 Gel eletrophoresis (20)

If a molecule of net charge, q, is placed in an electric field, a force, F, is exerted upon it:

E = potential difference
q = net charge
d = distance between the positive and negative electrodes
E/d = field strength

This force is opposed by friction:

$$F = \frac{E \times q}{d} \quad \text{(A1)}$$

$$E \times q = \frac{6 \pi r n v}{d} \quad \text{(A2)}$$

$$v = \frac{Eq}{do \, \pi \, rn} \quad \text{(A3)}$$

n = viscosity
v = velocity

However this equation is not adequate to explain electrophoresis in a gel, since it does not take into account the existence of gel pores. In reality, electrophoresis in a gel matrix can be thought of as a type of "gel filtration" where the driving force is the electric field. Penetration of the gel matrix by a mixture of nucleic acid molecules of different sizes results in the retardation of larger molecules. The electrophoretic mobility of nucleic acid molecules through a gel matrix is a function not only of molecular configuration (duplex or single stranded nucleic acid molecules), gel porosity, and ionic strength of the electrophoresis buffer. Pores in a normal fashion pile up at a short distance from the origin.

2. Capillary Zone Electrophoresis (CZE)

CZE is also referred to as free solution capillary electrophoresis (FSCE).While FSCE is the more descriptive term, CZE has come into common usage, and so will be used here. CZE is a widely used mode of electrophoresis because it is applicable to separations of anions and cations, although not neutrals, in the same run and is relatively simple. In CZE, the capillary is filled with a buffer of constant composition, and the source and destination vials are fills with the same buffer. This is in constrast to capillary isoelectric focusing where the capillary is fills with a solution they form a pH gradient, and isotachophoresis where leading and trailing buffers are placed in the capillary. Unlike CGE, where the capillary is filled with a gel, in CZE, the capillary is filled with a free solution, and therefore there are no mateix effects in CZE, although, in some instances, there may be interaction of the solute with the capillary wall.

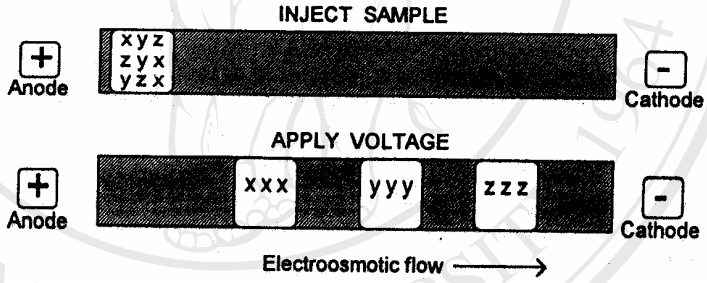


Figure A2 Separation by capillary zone electriphoresis with normal electroosmotic flow and polarity. The x's, y's, and z's represent ionic solutes with different charge to size ratios, with z having the highest ratio and x the lowest

Compounds are not separate in CZE, whereas ions are separated on the basis of their charge-to-size ratios. Electrophoretic mobility is dependent to some extent on the shape of the ions, but for this discussion, it is assumed that the ions are spherical. Since cations migrate through the capillary in the same direction as the electroosmotic flow. Cations elute in order of their charge-to-size ratios, with small, highly charged cations eluting first. Neutral molecules, which move through the capillary under the influence of only the electroosmotic flow and are not separated from each other, elute after the cations. Anions, which are attracted to the positive electrode and consequently tend to

migrate in the opposide direction to the electroosmotic flow, elite last. Usually, the electroosmotic flow is greater than the electrophoretic velocities of the anions, so most of them are also carries toward the cathode. Anions elute ineverse order to their charge-to-size ratios, with small, highly charged anions eluting last.

CZE can be used to separate almost any ionized compound that are soluble in a buffer. Amplest as diverse as small inorganic anions and large biomolecules have been separate by CZE. Even water insoluble compounds have been separate by CZE with nonaqueous buffers

3. Capillary isotachophoresis (CITP)

CITP is isotachophoresis performed in a capillary. In CITP, a sample is inserted between a leading electrolyte and a trailing (or terminating) electrolyte without electroosmotic flow. The leading electrolyte has a higher mobility and the trailing electrolyte has a lower mobility than ions in the sample zone. Separation in CITP relies on differences in the velocities, ν_i, of analyte ions within the sample zone.

$$\nu_i = \mu_{ep,i} E_{sz} \qquad \text{(A4)}$$

Here $\mu_{ep,\,i}$ is the electrophoretic mobility of species i and E_{sz} is the electric field strength of the sample zone before separation. During the transient separation process, all analyte ions are separated into consecutive bands under constant electric current and temperature. According to Ohm's law, the electric field strength increases as the mobility of ion band decreases (an ion band is defined as a homogeneous solution separated by moving or stationary boundaries). After separation, each of the analyte ions in different bands migrate at the same velocity, ν ; therefore, a steady-state stacking of bands is achieved.

$$\nu = \mu_{ep,i} E_i = \mu_{ep,j} E_j \qquad \text{(A5)}$$

Here the subscripts i and *j* represent different analyte ions. The analyte concentrations in each band are adjusted to the concentration of the leading electrolyte ion according to the Kohlraugh regulating function

$$C_i = \frac{C_l \mu_I (\mu_l + \mu_c)}{\mu_l (\mu_I + \mu_c)} \qquad (A6)$$

where C_i is the concentration of species in ion band , C_l is the concentration of the leading electrolyte, and μ_i, μ_l and μc are the mobilities of species , the leading electrolyte ion, and the counter ion in the steady state, respectively. CITP will concentrate those analyte ions whose concentration in the sample is lower than their steady-state concentration defined by

CITP cannot be used to separate cations and anions at the same time. Detection methods are based on conductivity, differential conductivity, or direct UV adsorption. In the latter case, spacers are placed between analyte bands. The spacers contain solutes that do not absorb in the UV and have mobilities between those of the two neighboring bands

4. Capillary Isoelectric Focusing (CIEF)

CIEF is an electrophoretic method for separating proteins based on their isoelectric point. The isoelectric point is the pH at which the net charge of the protein is zero. With the presence of a pH gradient in the IEF technique, the protein will migrate to the position in the gradient where its charge is zero. Proteins with a positive net charge will migrate toward the cathode until it meets its pI. Proteins with a negative net charge will migrate toward the anode until it meets its pI. If the protein diffuses away from its pI, it will regain its charge and migrate back. This focusing effect allows proteins to be separated based on very small charge differences. IEF is performed under high voltages (> 1000 V) until the proteins have reached their final position in the pH gradient. If IEF is performed under denaturing conditions very high resolution and cleanliness of sample can be obtained. can be described as electrophoresis in a pH gradient set up between a cathode and anode with the cathode at a higher pH than the anode. Because of the amino acids in proteins, they have amphoteric properties and will be positively charged at pH values below their IpH and negatively charged above. This means that proteins will migrate toward their IpH. Most proteins have a IpH in the range of 5 to 8.5. Under the influence of the electrical force the pH gradient will be

established by the carrier ampholytes, and the protein species migrate and focus (concentrate) at their isoelectric points. The focusing effect of the electrical force is counteracted by diffusion which is directly proportional to the protein concentration gradient in the zone. Eventually, a steady state is established where the electrokinetic transport of protein into the zone is exactly balanced by the diffusion out of the zone. From the factors that regulate the widths of the protein zones and distance between the zones, Sven son and Westerberg derived an equation for the resolution of two similar proteins, based on the following assumptions:

1. Straight and continuous pH gradients, dpH/dx.
2. Constant field strength, E
3. The two different proteins have the same diffusion coefficient, 'D'.
4. The electrophoretic mobility change with pH, dμ /dpH, is constant and the same for both proteins (This is not a good assumption).
5. Two closely spaced proteins are considered are considered separated when the position of their peak maxima differs by 3 standard deviation or more.

Equation 1: The minimum difference in IpH, for two proteins to be resolved is expressed

$$\Delta IpH = -3\frac{\sqrt{D(dpH/dx)}}{E(d\mu/dpH} \qquad (A7)$$

$$D = \frac{RT}{\eta f} \qquad (A8)$$

$$\mu = V/E = Q/(6\pi\eta r) \qquad (A9)$$

$$F = 6\pi\eta r \qquad (A10)$$

η = Viscosity

π = 3.14

μ = Mobility

R = radius of the molecule

F = Frictional coefficient

From equation A7 it can be seen that by reducing the diffusion, D, the resolution would increase. With a given separation, the only way to accomplish this is to increase the viscosity of the medium. Inert non-charged substances such as sucrose, glycerol etc. may be added or the experiment can be performed in a sieving medium such as a high concentration of polyacrylamide (PAA) gel. Increased viscosity will also affect the mobility (μ) the mobility of the proteins. This will make the isoelectric separation longer and decrease the resolution by decreasing the dμ /dpH in equation A7 Therefore, increasing the viscosity is not generally a successful way to improve the resolution although it may explain why there is a clear tendency for better resolution in sieving PAA gels than in more porous agarose gels. Since the diffusion coefficient is inversely related to molecular size it follows that larger proteins will tend to focus better than smaller ones, other things being equal (See equation A8). The shallower the gradient, dpH/dx (lower values of dpH/dx), the further apart will two proteins be and hence better separated. Note that the factor only applies as the square root. There are some drawbacks with use of extremely shallow gradients: Long focusing times since proteins must migrate a relatively long distance close to the IpH with very low charge: Only the limited number of proteins with IpH values within the narrow pH interval can be analyzed simultaneously; The carrier ampholine may not manage to maintain a completely smooth pH gradient. High field strength (E) will not only increase the resolution, the experimental time is also reduced. Too high field strength may give heat problems if the cooling is inefficient, especially when focusing in the very basic or acid pH region. The higher the pH dependence of the mobility, (dμ /dpH), the better the focusing. A high electrophoretic mobility close to the IpH will efficiently transfer diffused protein molecule back to IpH. This is essentially an intrinsic factor for the protein that cannot be manipulated. The effect of modifying mobility by affecting the viscosity will be counteracted by the effect of viscosity on diffusion. The overall effect is difficult to predict. A high value of dμ /dpH results from the presence of many groups with pKa values close to the IpH. Statistically this is more likely to be the case for a larger than for smaller proteins.

Both dμ /dpH and diffusion thus favor the focusing of large proteins and the influence of these factors explains the difficulties of focusing small proteins and peptides to sharp zones.

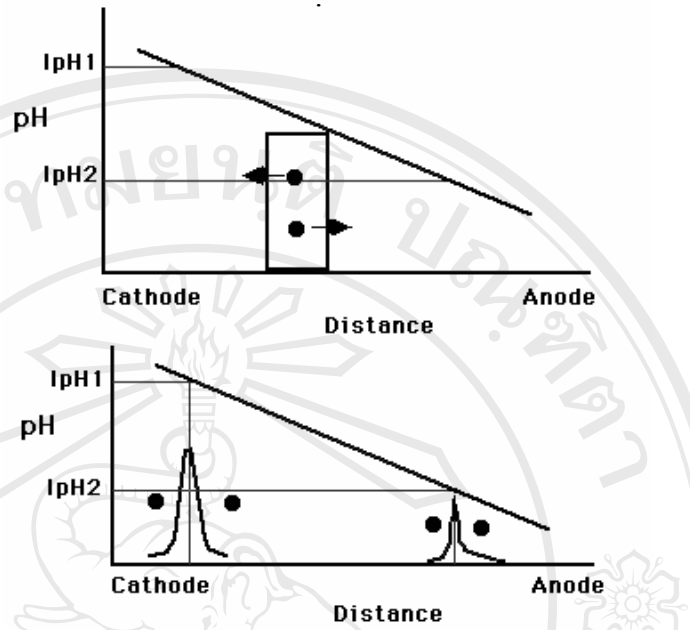


Figure A3 Principle of isoelectric focusing

Figure A3 is a schematic illustration of a sample with two proteins P1 and P2 place in the center of a pH gradient. P1 is positively charged and will migrate toward the cathode; P2 is negatively charged and will migrate toward the anode. As the proteins approach their IpH, they gradually become less and less charged. The proteins will thus concentrate at the position where pH = IpH. The proteins cannot concentrate in a indefinitely concentrated zone. widening by diffusion is inevitable. Any protein molecule diffusing away from the IpH will acquire a net charge and be transferred back to IpH again by electrophoresis. A balance will be set up between electrophoretic accumulation at IpH and diffusion. Formation of Natural pH gradients The formation of a pH gradient is schematically illustrated in Figure 3. Hydrogen ions form at the anode and hydroxyl ions at the cathode in the electrode reactions. This results in region of low and high pH near the anode and cathode respectively and steep pH gradients as one moves into the bulk solution. An amphoteric species with a IpH lower than the average pH in the system will concentrate in the steep gradient close to the anode.

A substance with good buffering capacity at its IpH will create a H plateau around its IpH. Given a sufficient number of such substances with evenly distribute IpH

values their corresponding plateau will overlap, resulting in a continuous pH gradient. The amphoteric substances that form and stabilize the pH gradient are collectively called carrier ampholytes. The most essential property for a good carrier ampholyte molecule is a good buffering capacity at its isoelectric point. This requires many pK values close to the buffering capacity at its isoelctric point for each molecular species, making most naturally occurring ampholytic substance, especially most naturally occurring amino acids, useless as carrier ampholytes. Svensson realized that the only way to produce suitable carrier ampholytes was to synthesize substances with the required properties. It was not until the first synthetic carrier ampholytes were successfully prepared that isoelectric focusing could be developed into the practically useful technique of today. The established pH gradient is maintained by hundreds or thousands of carrier ampholytes molecules lined up in order of IpH with partially overlapping distributions.

Since there are no other ionic species in the system, each carrier ampholyte must act as counter ion to other carrier ampholytes consequently each position in the pH gradient will have a unique chemical composition. Electrical conductance and buffer capacity will therefore vary over the pH gradient. Regions with low buffer capacity are more prone to distortion. In preparative experiments with protein loads, buffering capacity form the proteins may affect the pH gradient. Local heating will occur in the regions with the highest field strength (lowest conductance) and these regions will determine how high an overall voltage can be used. Consequently, other regions with lower field strength will no be focused at optimal conditions.

Optimal conditions over the whole pH gradient thus requires even field strength conductance and buffering capacity across the gradient. A large number of carrier ampholyte mixture are available giving different pH gradients. Many can also be obtained in precast gels ready to use. The optimal pH gradient will depend on the purpose of the experiment. For screening purposes, a broad range interval (pH 3-10 or similar) should be used. A narrow pH range interval is useful for careful IpH determinations or when analyzing proteins with very similar IpH points. Generally, one should not use a narrower gradient than necessary because the shallower gradient will lead to longer focusing times and more diffuse bands. When choosing pH gradient one should be aware that the interval stated by the manufacturer can only be an

approximation. The exact gradient obtained depends on many factors such as choice of electrolyte solutions, gradient medium (PAA or agarose), focusing time etc.

Despite the large number of pH intervals available, there may be occasions where none of them fits perfectly. In such cases one can either choose to work with Immobiline or use "pH gradient engineering" in any of the following variants:

1. Extend a given pH interval by adding carrier ampholytes covering the adjacent or a partly overlapping region. Extension into the extreme pH ends can be accomplished by adding acidic or basic compounds.
2. Expand a certain pH area by adding an amphoteric substance, "spacer", such as an amino acid. The spacer should be a "bad" ampholyte so that it does not focus too well.
3. Extend a certain pH range by manipulating the thickness of the gel. The gradient will be shallower in areas with thinner gel.
4. Manipulating the carrier ampholyte concentration will also affect the steepness of the final gradient. Areas with lower concentration will give shallower gradients. (21)

2.5.5 Capillary ElectroChromatography(CEC)

Electrochromatography is a term used to describe narrow bore packed column separations where the liquid mobile phase is driven not by hydraulic pressure as in HPLC but by electroosmosis. The advantages of using electroosmosis to propel liquids through a packed bed are the same as for in open capillaries i.e. reduced plate heights as a result of the plug flow profile and the ability to use smaller particles leading to higher peak efficiency than is possible in pressure driven systems (HPLC). The driving force in electro separation methods results from the electrical double layer that exists at the liquid - solid interface between, in the case of CZE, the bulk liquid and the capillary surface and in CEC, the packing material and mobile phase, and this is illustrated below.

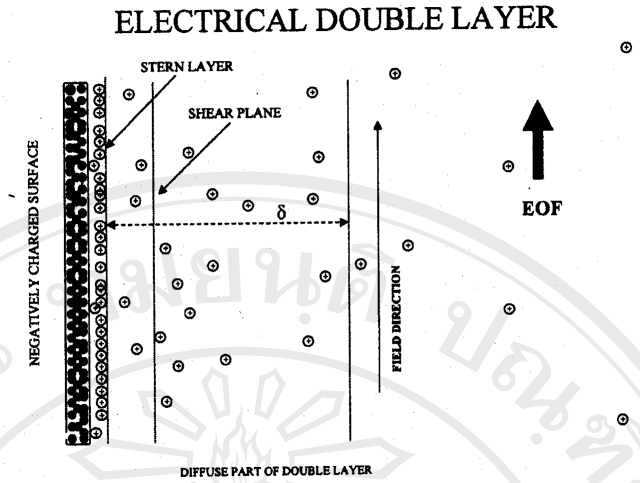


Figure A4 Electical Double Layer

Under alkaline conditions, the surface silanol groups of the fused silica will become ionised leading to a negatively charged surface. This surface will have a layer of positively charged ions in close proximity which are relatively immobilised. This layer of ions is called the Stern layer. The remainder of the excess charge, constituting the so called Goüy layer, is solvated and has the characteristics of a typical solvated ion. This layer extends into the bulk liquid and is the so called double layer. The concentration of ions in the double layer is relatively small compared to the total ionic concentration, and falls off exponentially from the capillary surface, as does the corresponding electric potential which is proportional to the charge density. The potential at the boundary between the Stern layer and the interface with the diffused double layer, is called the zeta potential, z, and values range from 0-100mV. As the charge density drops off with distance from the surface, so does the zeta potential, and the distance from the immobile layer to a point in the bulk liquid at which the potential is 0.37 times the potential at the interface with the Stern layer and the diffuse layer, is defined as the thickness of the double layer and is denoted by δ

The equation describing d was given by Knox as follows:

$$\delta = [\varepsilon_r \varepsilon_o RT / 2cF^2]^{1/2} \qquad (A11)$$

where e_r = dielectric constant or relative permitivity of medium

e_o = permitivity of a vacuum

R = universal gas constant

T = absolute temperature

c = molar concentration

F = Faraday constant

Using the above equation with water as the solvent (e r = 80), Knox calculated that for a 1:1 electrolyte such as NaCl, at a concentration of 0.001M in water, the thickness of the electrical double layer would be 10nm, and at a concentration of 0.1M it would be 1nm. Electroendosmotic flow within a capillary arises when an electric field is applied longitudinally along the length of a column. When this field is applied, ions in the diffuse (Goüy) layer which are not absorbed in the Stern layer, will migrate towards the cathode and shearing will occur within this region. Electroosmosis results because the core of liquid within this sheath will also be transported to the cathode. Since there is no charge imbalance within the sheath no shear takes place in this region. Because shearing only occurs within the diffuse layer, the resulting flow profile is plug-like and its velocity is in dependent of the capillary bore d c , provided that d c Å10 d. (usually d c > 20 d). If d_c approaches d then double layer overlap occurs and the EOF is considerably reduced and assumes a parabolic flow profile. In the case of packed capillaries, the open capillary diameter is replaced by the mean channel diameter and Knox calculated that using aqueous electrolytes between 0.001M and 0.01M there would be no double layer overlap as long as the partial diameter d_p is ⊕ 20 δ.

The relationship between the linear velocity u and the applied electric field is given by the Smoluchowski equation as follows:

$$u = \frac{\varepsilon_o \varepsilon_r \zeta E}{\eta} \qquad \text{(A12)}$$

where ε_o = the permittivity of a vacuum

ε_r = dielectric constant of the eluent

ζ = zeta potential

E = electric field strength

η = viscosity of the solvent

It can be seen from this equation that the velocity in the absence of electrical double layer overlap, is directly proportional to z. The zeta potential on the other hand is dependent on the charge density s as shown in the following equation:

$$\zeta \cong \frac{\delta\sigma}{\varepsilon_r \varepsilon_o} \qquad \text{(A13)}$$

When equations 1-3 are combined we obtain the expression

$$\upsilon = \frac{1}{\kappa}\frac{(E}{\eta} \qquad \text{(A14)}$$

where $\frac{1}{\kappa}$ = the thickness of the double layer.

It is now apparent that the electroosmotic flow depends upon the surface charge density, the field strength, the thickness of the electrical double layer, and the viscosity of the separation medium which in turn is dependent upon the temperature.

Silica gels used in the manufacture of stationary phases possess different properties depending on the method of preparation. As a result of this it is not surprising that such phases can have different charge densities depending on the surface area of the silica and the acidity of the surface silanols groups. Assuming a zeta potential of 100mV in an aqueous medium, electroosmotic flow velocities in open tubes of 0.8mm/s at 100V/cm and 3.2mm/s at 400V/cm are expected. Since HPLC columns packed with 3-5mm diameter reversed-phase particles require linear velocities in the region of 0.5 - 3.0mm/s for optimum separation, then it seems reasonable to assume that sufficient electroosmotic flow could be generated in capillaries packed with similar diameter particles to achieve efficient separation. Since silica based reversed-phase packing materials also contain silanol groups, it is possible to generate electroosmotic flow in capillaries packed with silica gel and/or chemically modified silica's as illustrated below:

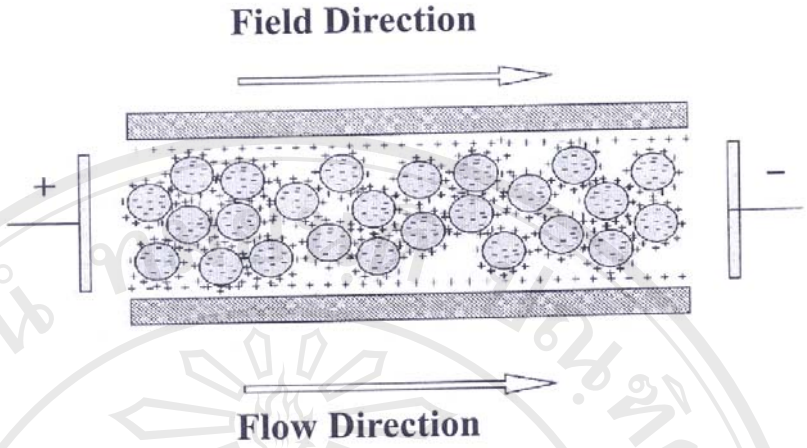


Figure A5 Electroosmotic flow in capillaries packed with silica gel

In fact, because the surface areas of micro particulate silica based packing materials are much greater than that of the capillary walls, most of the electro-osmotic flow is generated by the surface silanol groups of the stationary phase. Clearly this would not be expected to apply in the case of totally base deactivated materials, such as Hypersil BDS or Spherisorb ODS-B which have minimal levels of residual silanol groups.

The linear flow in a pressure driven system is given by the following eq.A15

$$\upsilon = \frac{\delta^2 \Delta\pi}{\phi\, \eta \Lambda} \qquad \text{(A15)}$$

where υ = linear velocity

d = particle diameter

ϕ = pressure resistance factor for packed columns

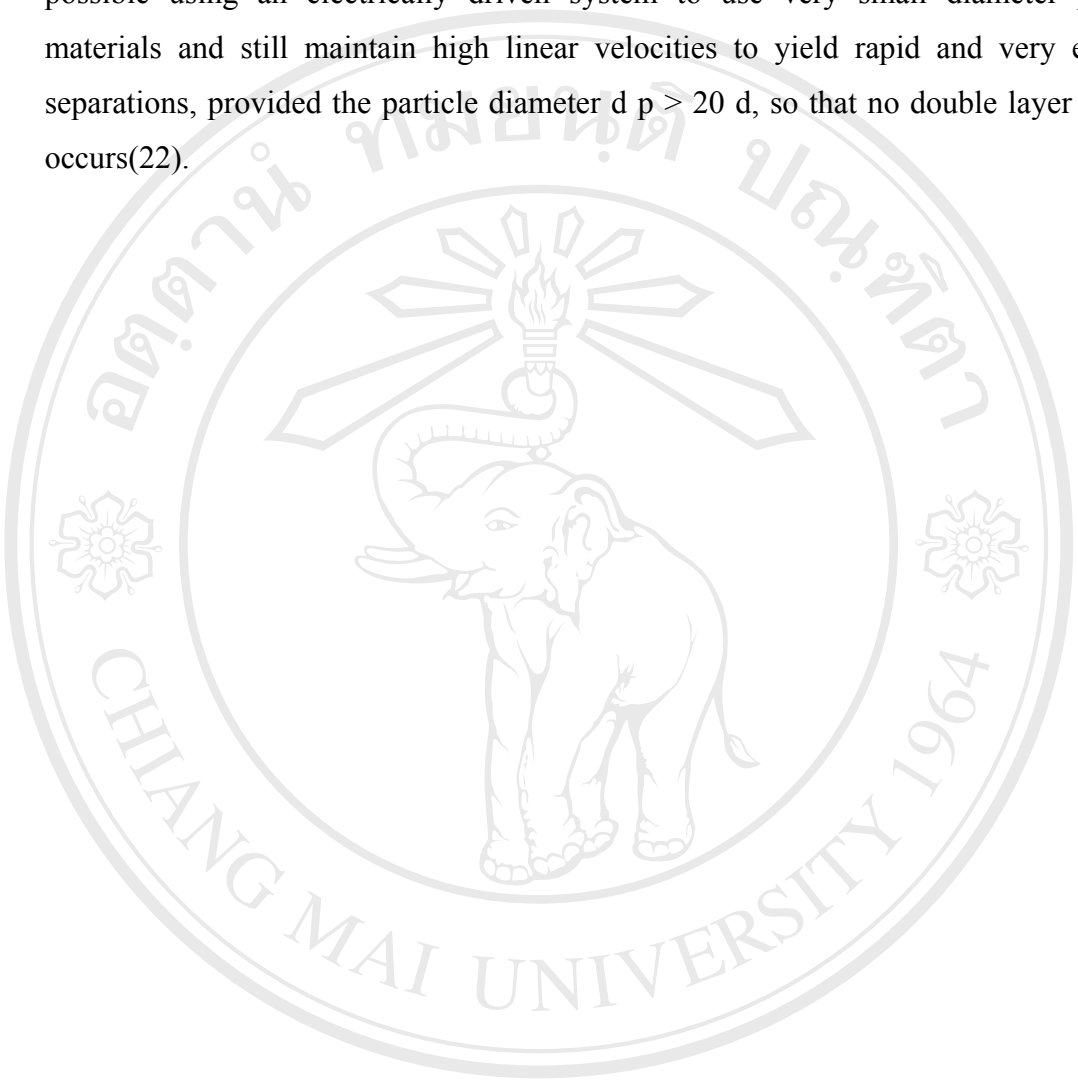
Δp = pressure drop across the column

L = column length

η = viscosity of the solvent

whereas the linear velocity through a packed capillary under an applied electric field is given by the Smoluchowski equation . What is noticeable when comparing equations and is that the linear velocity u is proportional to d 2 in a pressure driven system whilst

it is independent of the particle diameter in an electrically driven system. Since plate values are generally lowered as a result of using small diameter particles, it should be possible using an electrically driven system to use very small diameter packing materials and still maintain high linear velocities to yield rapid and very efficient separations, provided the particle diameter $d_p > 20\ d$, so that no double layer overlap occurs(22).



APPENDIX B

THEORY OF HPLC

High performance liquid chromatographs (HPLC) use a liquid mobile phase to separate the components of a mixture. The components are dissolved in a solvent and forced to flow through a chromatographic column under high pressure. In the column, the mixture is resolved into its components. The components flow through a detector and a chromatogram is generated. Most high performance liquid chromatographs (HPLC) use high-pressure pumps to force solvents through packed stationary beds. Isocratic pumps use constant mobile phase composition to elute compounds. By contrast, gradient pumps vary the strength of the organic solvent to elute different compounds. Pumps with flow control are well-suited for high performance liquid chromatographs (HPLC) that deliver gradients automatically. Specifications such as flow rate, maximum pump pressure, and pump accuracy vary among devices. In simple systems, the sample is introduced with an injection valve. More complex high performance liquid chromatographs (HPLC) incorporate an autosampler with a microprocessor

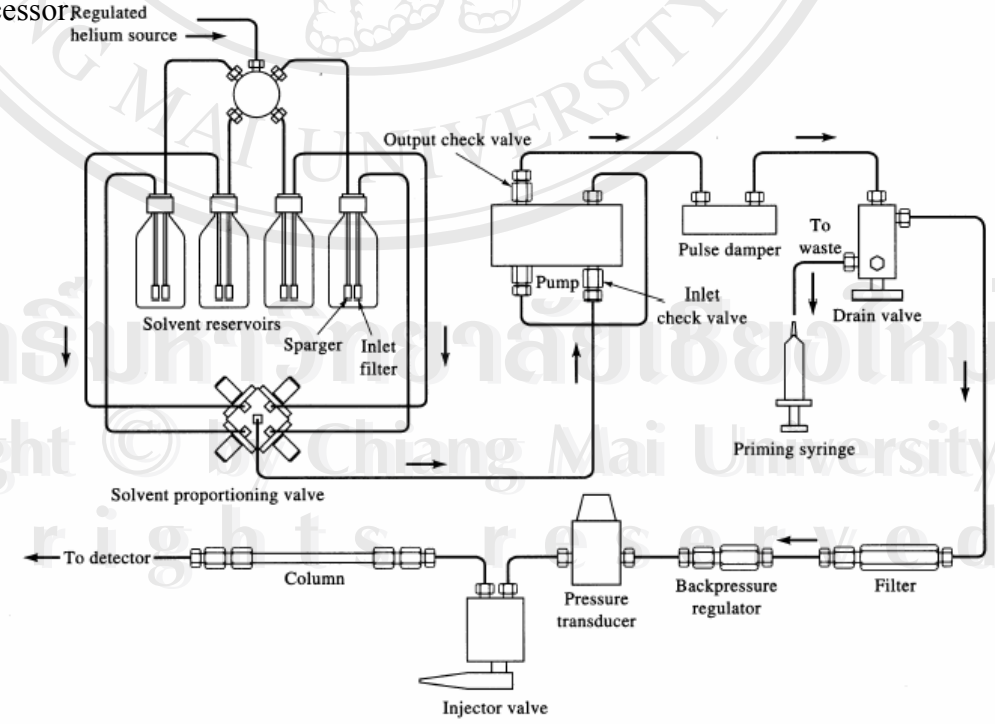


Figure B1 Diaram of high performance liquid chromatography (23)

1. The basic components of an HPLC system include:

1.1 Solvent Reservoir

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with the special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium was found not to be sufficient for degassing of aqueous solvents. It is useful to apply a vacuum for 5-10 min. And then keep the solvent under a helium atmosphere

1.2 Pump

High pressure pumps are need to force solvents through peaked stationary phase beds. However, many separation problems can be resolved with larger particle packings that require less pressure.

Flow rate stability is another important pump feature that distinguishes pumps. For most types of separation stable flow rate is not very important. However, for size exclusion chromatography the flow rate has to be extremely stable

External electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods.

Modern pumps have the following parameters :

- Flow rate range : 0.01 to 10 ml/min
- Flow rate stability: not more than 1 % (short term)
- For SEC flow rate stability should be less than 0.2 %
- Maximum pressure : up to 5000 psi (345 bar, 340 atm)

It is desirable to have an integrated degassing system, either helium purging, or better vacuum degassing

1.3 Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where sample introduction is done with the help of autosamplers and microprocessors.

In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoids detector interference, column/component interference, loss in efficiency or all of these. It is always best to remove particles from the sample by filtering, or centrifuging since continuous injection of particulate material will eventually cause blockage of injection devices or column

Sample size. Typical sample mass with 4.6 mm i.d. column range from the nanogram level up to about 2 mg deluted in 20 μl of solvent. In general, it will be noted that much less smple preparation is required in LC thanin GC since unwanted or interfering compounds, or both, may often be eatracted, or eliminated, by selective detection.

1.4 Column

Column is used for separating compounds. It has a made of stainless steel with silicon beads. Column contains the stationary phase. Interaction of stationary phase happens when it moves with the mobile phase. Difference types of column are available. Selection of the column can effect the performance of a High-Performance Liquid Chromatography

Guard Columns are placed in front of the separating column. This improves the life of the separation column. Guard columns need to be changed regularly to optimize their function. Capillary Columns are also called micro columns. The diameter of a capillary column is less than a millimeter. Open-tubular, tightly packed and partially packed are different types of capillary columns.

Micro-bore and small-bore are used for small and reasonable volumes assays.

Fast Columns are used to obtain better sample throughput. Fast columns are designed in such a way to reduce the time taken for chromatographic analysis. This type of column has shorter length compared to other columns. Some advantages of fast columns include decreased mobile phase usage and increased sensitivity.

1.5 Detector

Detector has an optical sensor which detects the changes in the solvent stream and sends an electronic signal to a computer data station or recorder. Ultraviolet light (UV), Refractive index (RI), Fluorescence (FD), Mass spec (MS) are different types of detectors available. The mobile phase carrying the sample componenets is passed through the low volumn cell in the detector.

1.6 Recorder or data system

The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operate attention

In routine analysis, where no auto automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

The advantages of intelligent process in chromatographs :

- additional automation options become easier to implement;
- complex data analysis becomes more feasible;
- software safeguard can be designed to reduce accidental misuse of the system.

For example, the controller can be set to limit the rate of solvent switching. This acts to extend column life by reducing thermal and chemical shocks. In general, these standalone, user programmable systems are becoming less expensive and increasingly practical.

Other more advanced features can also be applied to a chromatographic system. These features include computer controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors. These added features are not found are not found on many systems, but they do exist, and can save much time and effort for the chromatographer.

APPENDIX C

THEORY OF GC

Gas chromatography - specifically gas-liquid chromatography - involves a sample being vapourised and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. (24)

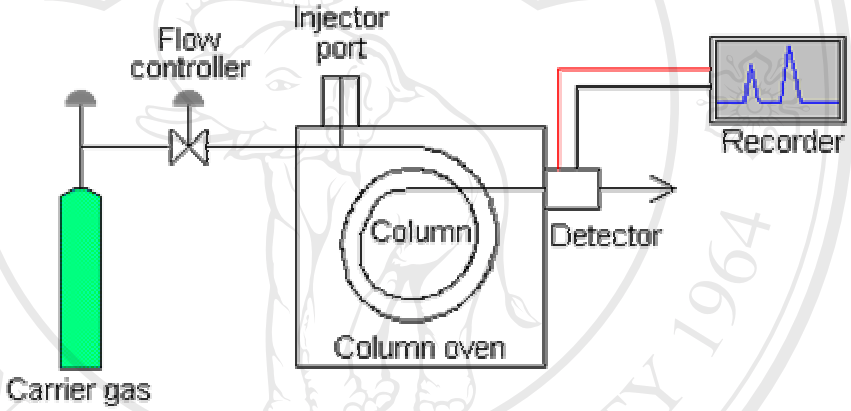


Figure C1 Diagram of gas chromatography

1. Instrumental components

1.1 Carrier gas

The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependant upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities.

1.2 Sample injection port

For optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapour - slow injection of large samples causes band broadening and loss of resolution. The most common injection method is where a microsyringe is used to inject sample through a rubber septum into a flash vapouriser port at the head of the column. The temperature of the sample port is

usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample, typically around 10^{-3} mL. For capillary GC, split/splitless injection is used. Have a look at this diagram of a split/splitless injector;

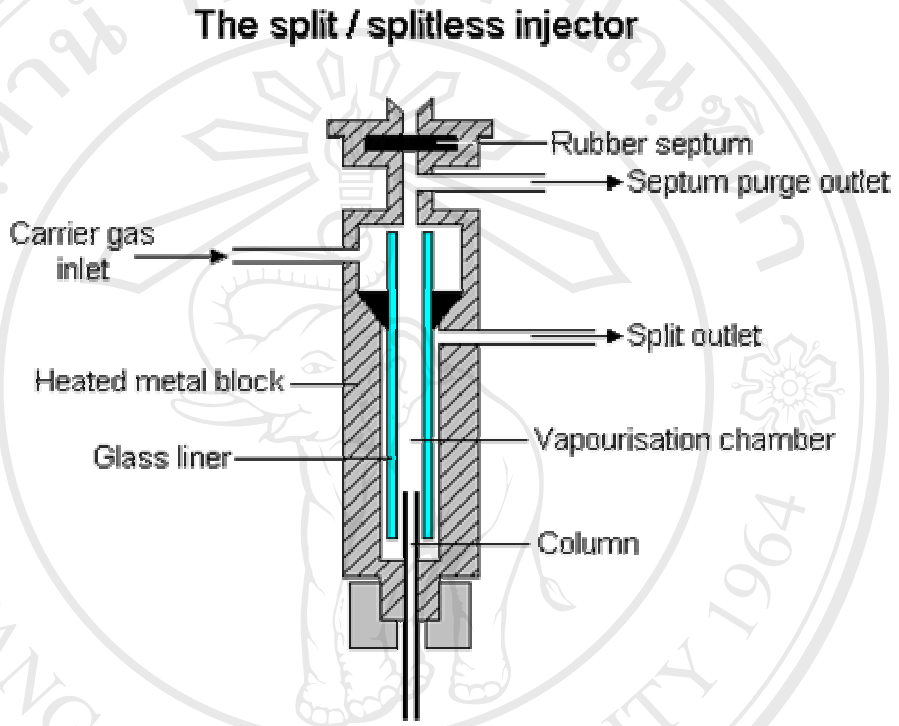


Figure C2 Split and splitless injector

The injector can be used in one of two modes; split or splitless. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vapourises to form a mixture of carrier gas, vapourised solvent and vapourised solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.

1.3 Columns

There are two general types of column, packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase. Most

packed columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm. Capillary columns have an internal diameter of a few tenths of a millimeter. They can be one of two types; wall-coated open tubular (WCOT) or support-coated open tubular (SCOT). Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase. In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the stationary phase has been adsorbed. SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns.

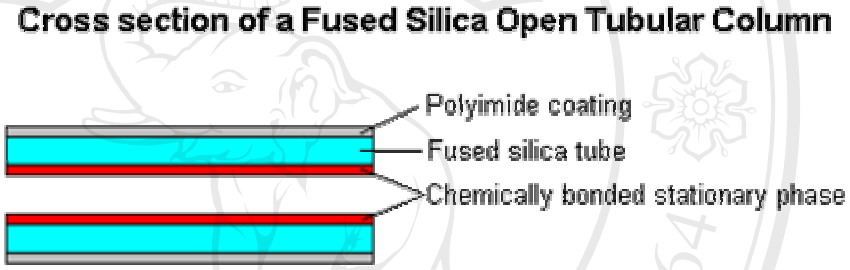


Figure C3 Cross section of a Fused Silica Open Tubular Column

These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating. These columns are flexible and can be wound into coils. They have the advantages of physical strength, flexibility and low reactivity.

1.4 Column temperature

For precise work, column temperature must be controlled to within tenths of a degree. The optimum column temperature is dependant upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes. Minimal temperatures give good resolution, but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful. The column temperature is increased (either continuously or in steps) as separation proceeds.

1.5 Detectors

There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property and a specific detector responds to a single chemical compound. Detectors can also be grouped into concentration dependant detectors and mass flow dependant detectors. The signal from a concentration dependant detector is related to the concentration of solute in the detector, and does not usually destroy the sample Dilution of with make-up gas will lower the detectors response. Mass flow dependant detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependant detector is unaffected by make-up gas

2. Specific GC detectors

2.1 Electron Capture Detectors (ECD)

The ECD uses a radioactive Beta emitter (electrons) to ionize some of the carrier gas and produce a current between a biased pair of electrodes. When organic molecules that contain electronegative functional groups, such as halogens, phosphorous, and nitro groups pass by the detector, they capture some of the electrons and reduce the current measured between the electrodes. The ECD is as sensitive as the FID but has a limited dynamic range and finds its greatest application in analysis of halogenated compounds.

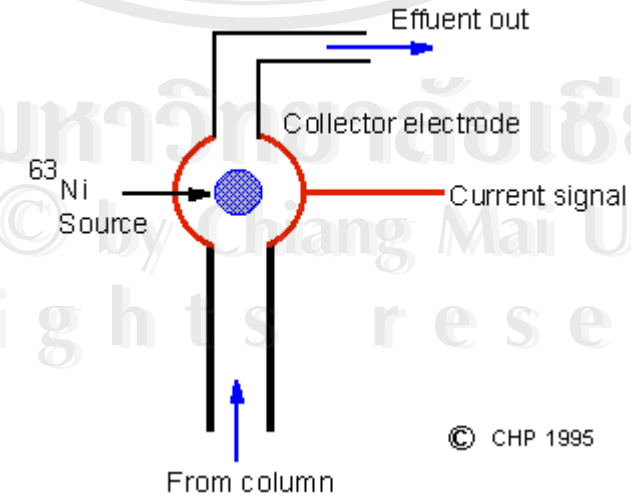


Figure C4 Schematic of a gas chromatographic Electron Capture Detectors

2.2 Flame Photometric Detector (FPD)

The reason to use more than one kind of detector for gas chromatography is to achieve selective and/or highly sensitive detection of specific compounds encountered in particular chromatographic analyses. The determination of sulfur or phosphorus containing compounds is the job of the flame photometric detector (FPD). This device uses the chemiluminescent reactions of these compounds in a hydrogen/air flame as a source of analytical information that is relatively specific for substances containing these two kinds of atoms. The emitting species for sulfur compounds is excited S_2. The lambda max for emission of excited S_2 is approximately 394 nm. The emitter for phosphorus compounds in the flame is excited HPO (lambda max = doublet 510-526 nm). In order to selectively detect one or the other family of compounds as it elutes from the GC column, an interference filter is used between the flame and the photomultiplier tube (PMT) to isolate the appropriate emission band. The drawback here being that the filter must be exchanged between chromatographic runs if the other family of compounds is to be detected.

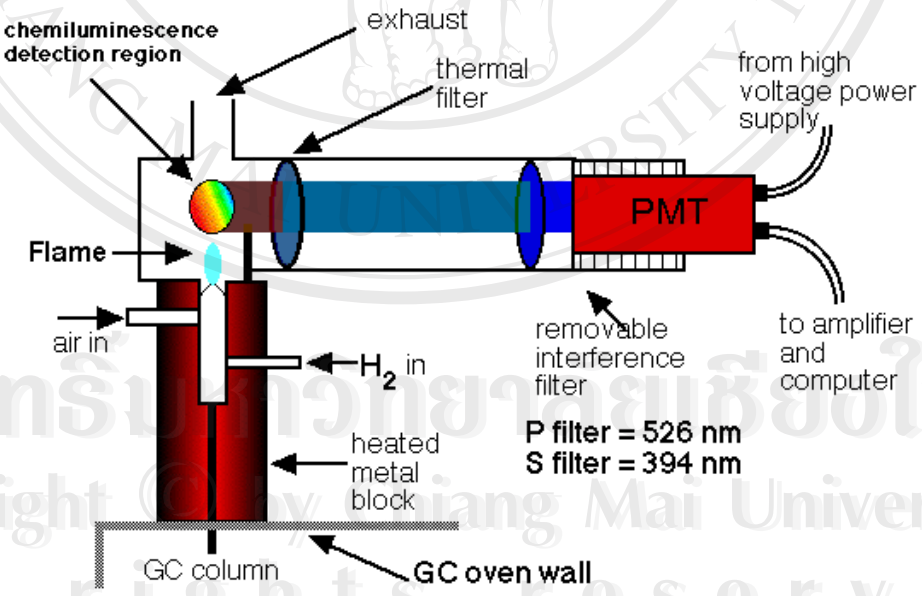


Figure C5 Schematic of a gas chromatographic flame photometric detector

2.3 Atomic-Emission Detector (AED)

As capillary column based gas chromatography takes its place as the major, highest resolution separation technique available for volatile, thermally stable compounds, the requirements for the sensitive and selective detection of these compounds increases. Since more and more complex mixtures can be successfully separated, subsequent chromatograms (output of a chromatographic separation) are increasingly more complex. Therefore, the need to differentiate between the sample components using the GC detector as a means of compounds discriminating is more and more common. In addition, each detector has its own characteristics (selectivity, sensitivity, linear range, stability, cost, etc.) that helps in a decision about which detector to use. One of the newest additions to the gas chromatographer's arsenal is the atomic emission detector (AED). This detector, while quite expensive compared to other commercially available GC detectors, is an extremely powerful alternative. FOR INSTANCE, Instead of measuring simple gas phase (carbon containing) ions created in a flame as with the flame ionization detector, or the change in background current because of electronegative element capture of thermal electrons as with the electron capture detector, the AED has a much wider applicability because it is based on the detection of atomic emissions.

The components of the AED include 1) an interface for the incoming capillary GC column to the microwave induced plasma chamber, 2) the microwave chamber itself, 3) a cooling system for that chamber, 4) a diffraction grating and associated optics to focus then disperse the spectral atomic lines, and 5) a position adjustable photodiode array interfaced to a computer. The microwave cavity cooling is required because much of the energy focused into the cavity is converted to heat.

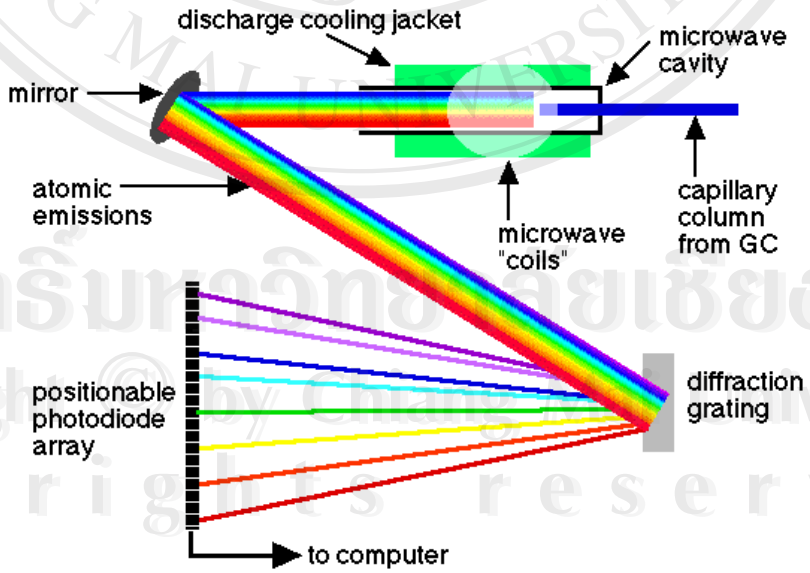


Figure C6 Schematic of a gas chromatographic atomic emission detector

2.4 Flame-Ionization Detectors (FID)

An FID consists of a hydrogen/air flame and a collector plate. The effluent from the GC column passes through the flame, which breaks down organic molecules and produces ions. The ions are collected on a biased electrode and produce an electrical signal. The FID is extremely sensitive with a large dynamic range, its only disadvantage is that it destroys the sample.

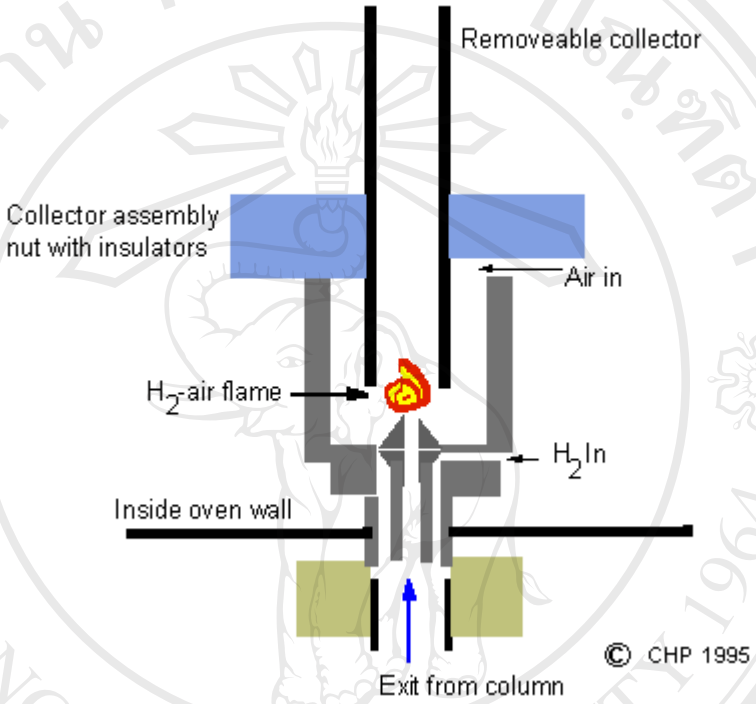


Figure C7 Schematic of a gas chromatographic flame-ionization detectors

2.5 Photoionization Detector (PID)

The reason to use more than one kind of detector for gas chromatography is to achieve selective and/or highly sensitive detection of specific compounds encountered in particular chromatographic analyses. The selective determination of aromatic hydrocarbons or organo-heteroatom species is the job of the photoionization detector (PID). This device uses ultraviolet light as a means of ionizing an analyte exiting from a GC column. The ions produced by this process are collected by electrodes. The current generated is therefore a measure of the analyte concentration.

Since only a small (but basically unknown) fraction of the analyte molecules are actually ionized in the PID chamber, this is considered to be a nondestructive GC detector. Therefore, the exhaust port of the PID can be connected to another detector in series with the PID. In this way data from two different detectors can be taken simultaneously, and selective detection of PID responsive compounds augmented by response from, say, an FID or ECD. The major challenge here is to make

the design of the ionization chamber and the downstream connections to the second detector as low volume as possible (read small diameter) so that peaks that have been separated by the GC column do not broaden out before detection.

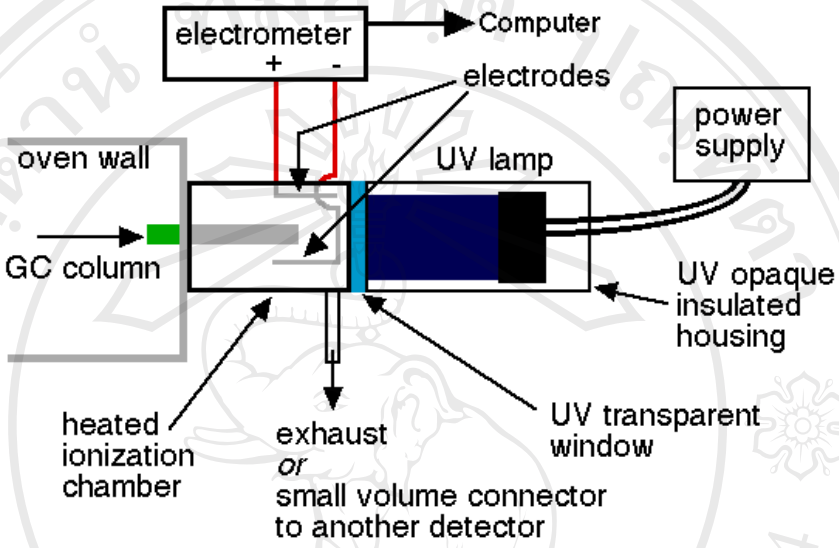


Figure C8 Schematic of a gas chromatographic photoionization detector

2.6 Thermal Conductivity Detectors (TCD)

A TCD detector consists of an electrically-heated wire or thermistor. The temperature of the sensing element depends on the thermal conductivity of the gas flowing around it. Changes in thermal conductivity, such as when organic molecules displace some of the carrier gas, cause a temperature rise in the element which is sensed as a change in resistance. The TCD is not as sensitive as other dectectors but it is non-specific and non-destructive.

Two pairs of TCD are used in gas chromatographs. One pair is placed in the column effluent to detect the separated components as they leave the column, and another pair is placed before the injector or in a separate reference column. The resistances of the two sets of pairs are then arranged in a bridge circuit.

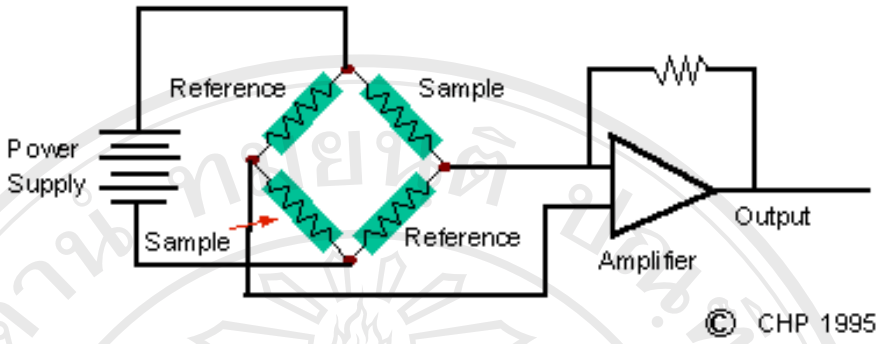


Figure C9 Schematic of a bridge circuit for TCD detection

The bridge circuit allows amplification of resistance changes due to analytes passing over the sample thermoconductors and does not amplify changes in resistance that both sets of detectors produce due to flow rate fluctuations, etc.

APPENDIX D

HPLC CHROMATOGRAM EXAMPLE

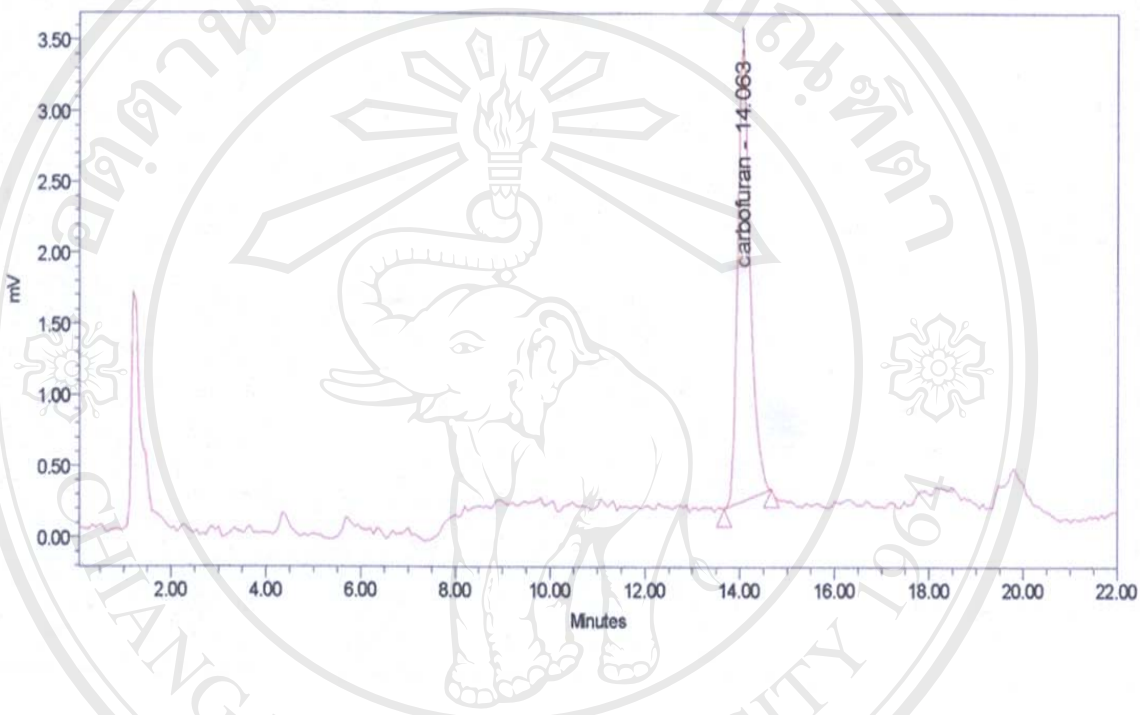


Figure D1 Chromatogram of carbofuran in sample

APPENDIX E

GC-FPD CHROMATOGRAM EXAMPLE

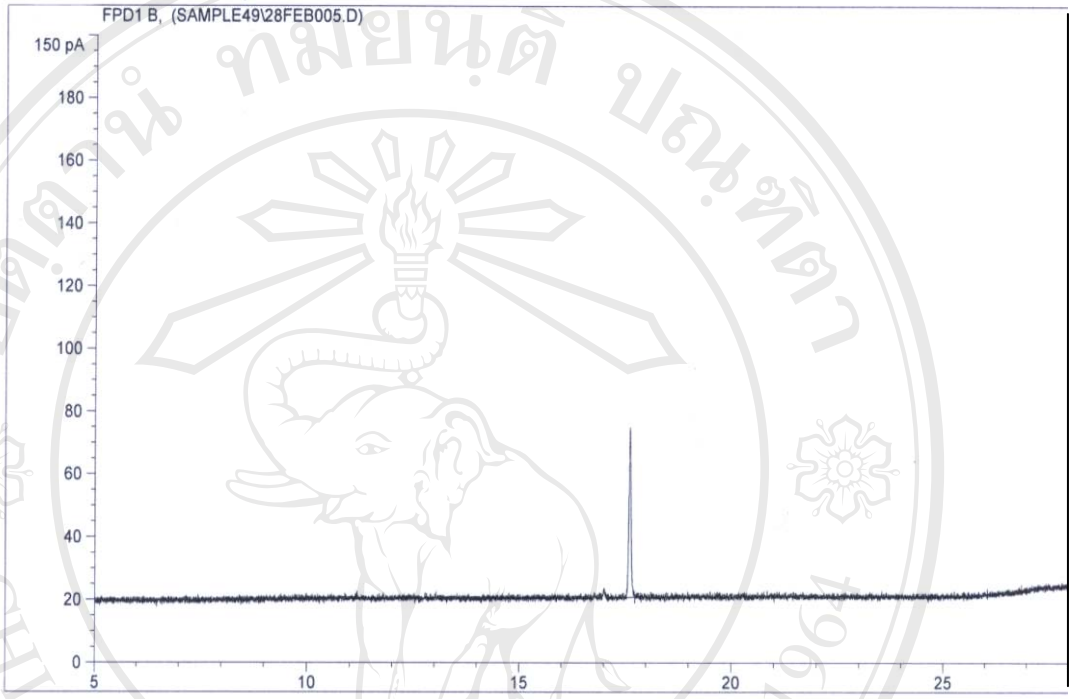


Figure E1 Chromatogram of parathion methyl in sample

APPENDIX F

GC-ECD CHROMATOGRAM EXAMPLE

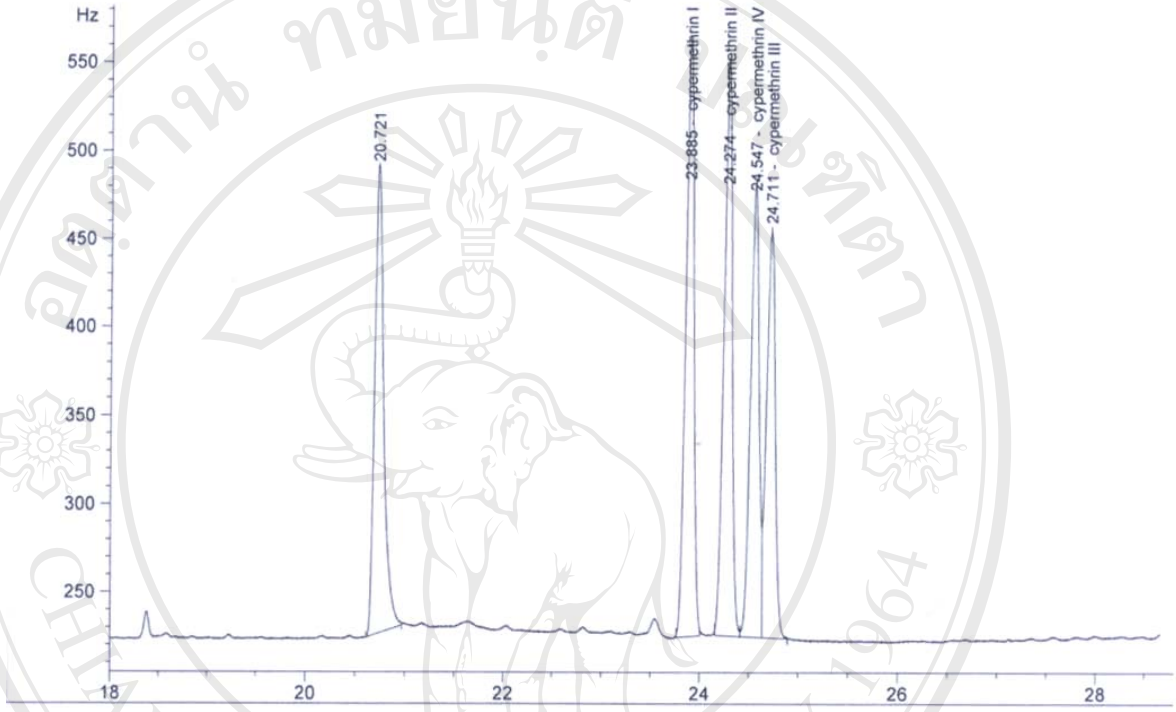


Figure F1 Chromatogram of cypermethrin in sample

APPENDIX G

INSECTICIDE GROUP

The insecticide can be classified by chemical structure as the following:

1) Carbamate Group

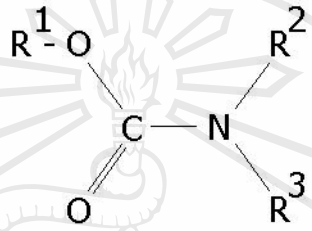


Figure G1 General Structure of Carbamate

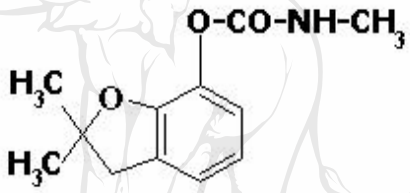


Figure G2 Carbofuran Structure

2) Organophosphate Group

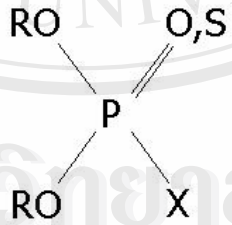


Figure g3 General Structure of Organophosphate

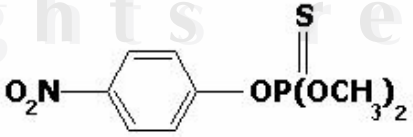


Figure G4 Parathion methyl Structure

3) Organochlorine Group

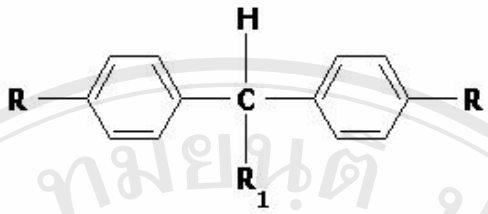


Figure G5 General Structure of Organochlorine

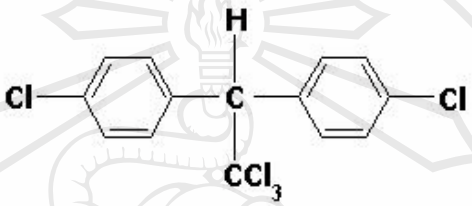


Figure G6 DDT Structure

4) Pyrethroid Group

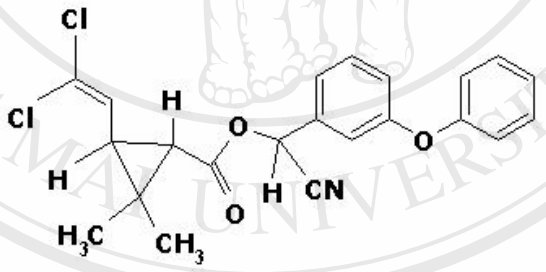


Figure G7 Cypermethrin Structure

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