CHAPTER II

.

REVIEW OF LITERATURE

2.1 Capillary electrophoresis as an appropriate equipment for analysis and about the three insecticides

Capillary electrophoresis (CE) developed as a technique in the late 1980s and has grown steadily in popularity since then, reaching a stage where it is likely that over 1000 published papers making particular reference to the method will appear in 1997. In its simplest and most commonly used form, it is a throwback to the original Tiselius method, but its popularity is based not only on the inherent simplicity but on the additional advantages of speed, versatility and low running costs. Essentially an analytical method, it has found application in the separation of biopolymers such as peptides, proteins, oligonucleotides, metal ions and inorganic ions as well as in the analysis of pharmaceuticals and the monitoring of water quality . Although the basic methodology involves the separation of molecules based on their charge to mass ratio, there are straightforward modifications to the procedure, borrowed from existing well-established techniques, which allow separations based on size or isoelectric point, or which permit the separation of non-charged molecules, while the degree of resolution can result in the separation of optical isomers. There have been recent reviews on the application of CE techniques to the separation of proteins, DNA fragments, carbohydrates, drug metabolism and pesticide residues in agricultures (2).

In Thailand CE was used for analysis of carbofuran and parathion methyl as residues in strawberry fruits for the first time in 2004. The report showed a good success in using CE to analyse both insecticides. The conditions suitable for analysis were tried and came out the same for the two insecticides: Column; id 75 μm, length 50 cm, length to detection point 40.2 cm, controlled temperaturen25 ^{o}C, UV detectin 205 nm, buffer; tetraborate+SDS at pH 8; electricity voltage 20 kV. However, the weak point of using CE method in that research was found that the CE could not detec the residues with small amounts while GC amd HPLC could.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) which is the most commonly sold under the trade name Furidan, an anticholinesterase carbamate, is commonly used as an insecticide, nematicide, and acaricide in agricultural practice around the world. Carbofuran is preferred over many other insecticides because it has a low persistence in most soil types (typically <60 days), breaks down in neutral or slightly alkaline water (half-life of 1 to 8 weeks depending upon water temperature), does not bind to sediments or suspended particles, and does not bioaccumulate. Carbofuran is highly toxic by inhalation and ingestion and moderately toxic by dermal absorption. As with other carbamate compounds, carbofuran's cholinesterase-inhibiting effect is short-term and reversible. Symptoms of carbofuran poisoning include: nausea, vomiting, abdominal cramps, sweating, diarrhea, excessive salivation, weakness, imbalance, blurring of vision, breathing difficulty, increased blood pressure, and incontinence. Death may result at high doses from respiratory system failure associated with carbofuran exposure. Complete recovery from an acute poisoning by carbofuran, with no long-term health effects, is possible if exposure ceases and the victim has time to regain their normal level of cholinesterase and to recover from symptoms. The oral LD_{50} is 2 mg/kg in mice, and 19 mg/kg in dogs. The dermal LD_{50} is >1000 mg/kg in rabbits. The LC_{50} (4-hour) for inhalation of carbofuran is 0.043 to 0.053 mg/L in guinea pigs (3).

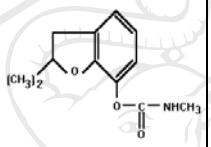
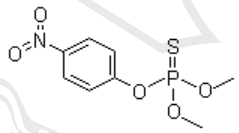
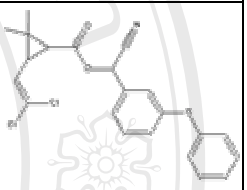
Parathion-methyl (O,O-Dimethyl O-(p-nitrophenyl) thionophosphate), sometimes called "cotton poison," is a chemical pesticide that should be used only in open fields to control insects. Most commonly, it is used on cotton, soybean and vegetable fields. Methyl parathion is not meant to be used inside buildings or homes. Such use is illegal. Methyl parathion is a brownish liquid that turns milky white when mixed with water. It can leave a yellow stain on areas where it has been sprayed and smells like rotten eggs. Parathion methyl is highly toxic via the oral route, with reported oral LD_{50} values of 6 to 50 mg/kg in rats, 14.5 to 19.5 mg/kg in mice, 420 mg/kg in rabbits, 1270 mg/kg in guinea pigs and 90 mg/kg in dogs . It is highly toxic via the dermal route as well, with reported dermal LD_{50} values of 67 mg/kg in rats, 1200 mg/kg in mice, and 300 mg/kg in rabbits . Effects associated with acute exposure to methyl parathion are similar to those associated with

exposure to other organophosphate pesticides. Symptoms of acute exposure to organophosphate or cholinesterase-inhibiting compounds may include the following: numbness, tingling sensations, in coordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, and convulsions or fatality. Persons with respiratory ailments, recent exposure to cholinesterase inhibitors, cholinesterase impairment, or liver malfunction are at increased risk from exposure to methyl parathion (4).

Cypermethrin [Cyano-3-phenoxybenzenyl (+Cis,trans) 3-(2,2-dichloro viniyl-2,2-dimethyl cycle propane carboxylate). Cypermethrin is a synthetic pyrethroid insecticide used to control many pests, including moth pests of cotton, fruit, and vegetable crops. It is also used for crack, crevice, and spot treatment to control insect pests in stores, warehouses, industrial buildings, houses, apartment buildings, greenhouses, laboratories, and on ships, railcars, buses, trucks, and aircraft. It may also be used in non-food areas in schools, nursing homes, hospitals, restaurants, hotels, in food processing plants, and as a barrier treatment insect repellent for horses. Technical cypermethrin is a mixture of eight different isomers, each of which may have its own chemical and biological properties. Cypermethrin is light stable. It is available as an emulsifiable concentrate or wettable powder. Cypermethrin is a moderately toxic material by dermal absorption or ingestion . Symptoms of high dermal exposure include numbness, tingling, itching, burning sensation, loss of bladder control, in coordination, seizures, and possible death. Pyrethroids like cypermethrin may adversely affect the central nervous system. Symptoms of high-dose ingestion include nausea, prolonged vomiting, stomach pains, and diarrhea which progresses to convulsions, unconsciousness, and coma. Cypermethrin is a slight skin or eye irritant, and may cause allergic skin reactions . The LD_{50} for cypermethrin in rats is 4123 mg/kg (in water). EPA reports an oral LD_{50} of 187 to 326 mg/kg in male rats and 150 to 500 mg/kg in female rats. The oral LD_{50} varies from 367 to 2000 mg/kg in female rats, and from 82 to 779 mg/kg in mice, depending on the ratio of cis/trans- isomers present. This wide variation in toxicity

may reflect different mixtures of isomers in the materials tested. The dermal LD_{50} in rats is 1600 mg/kg and in rabbits is greater than 2000 mg/kg (5).

Table 2.1. Physical and Chemical Properties of Carbofuran, Parathion- Methyl and Cypermethrin

Physical and Chemical Properties	**Carbofuran**	**Parathion- Methyl**	**Cypermethrin**
Structure			
Molecular Formula	$C_{12}H_{15}NO_3$	$C_8H_{10}NO_5PS$	$C_{22}H_{19}ClNO_3$
Molecular Weight	221.25	263.21	416.30
Appearance	crystalline solid	crystalline solid	colorless crystals
Solubility in Other Solvents	acetone, acetonitrile, benzene, cyclohexone	dichloromethane, 2-propanol, toluene, n-hexane	methanol, acetone, xylene
Melting Point	153-154 C	35-36 C	60-80 C
LC_{50} in rats	5 to 13 mg/kg	0.24 mg/L	250 mg/kg

Presently there are different ways to analyze the pesticide residue in agriculture products. It could be simple method to analyze by chemical reactions, colorimetry, UV-spectrophotometry, Thin Layer Chromatography (TLC) including apply Hi-End equipments for example Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). The Hi-End equipments could tell us the type of pesticide residue as well its volume. However, the difficulty of extraction that requires different specific solvent would make high consumption and producing waste which hazard to

environmental. Therefore, the Capillary Electrophoresis (CE) has become an important approach in the analytical laboratory. Initially introduced as a technique for separation of biological macromolecules , CE has since attracted much interest in other application areas, including pesticide-residue determination. The prospects for CE in pesticide analysis are very promising because of its advantages, such as higher separation efficiency, shorter analysis times, and very small consumption of expensive reagents and toxic solvents.

2.2 Relevant research

Molina *et al.* (6) analyzed herbicide in water reservoir by using the Capillary Electrophoresis method and found that this method able to separate 4-dimetylamino-3-methyl phenol, 2-isoproxyphenol, propoxur, 2,3-dihydro-2,2-dimethyl-7-benzofuranol, carbofuran, aminocarb, 1-naphthol, carbaryl, 4-methylthio-3,5-xylenol and methiocarb by using buffer: 20mM borate, 25 mM phosphate, 40mM SDS, pH8 and UV detector setting at 202 and 214 nanometers.

Henry *et al*. (7) analyzed pyrethrin ester by capillary electrophoresis and found that this method could separate six insecticidally active pyrethrin ester in typical extracts and commercial formulations by using acetonitrile–aqueous buffer–tetrahydrofuran (55:35:10) provided the elutropic solvent strength needed to resolve the six esters from an extract mixture in under 16 min. A 25 cm packed bed of Hypersil 3 μm C_{18} stationary phase was used with the ternary mobile phase at 25 °C and 30 kV voltage.

Garcia-Ruiz *et al.* (8) analyzed enantiomeric separation of organophosphorus pesticides by capillary electrophoresis and found that this method could separate enantiomers of a group of organophosphorus pesticides (OPs) by using of a 25 mM Tris buffer (pH 7.0), 20 mM in CM-β-CD together with an applied voltage of 24 kV with temperature at 25 ^{o}C.

Hernandez-Borges *et al*. (9) analyzed herbicide by capillary electrophoresis and found that this method could separate five triazolopyrimidine sulfonanilide pesticides (i.e., diclosulam, cloransulam-methyl, flumetsulam, metosulam and florasulam) in different types of water is investigated by using of 24 mM formic acid and 16 mM ammonium carbonate at pH 6.4.

Bermudo *et al*. (10). analyzed acrylamide in food samples by capillary zone electrophoresis found that this method could acrylamide (AA) after derivatisation with 2-mercaptobenzoic acid. The best separation was achieved using a 40mM phosphate buffer at pH 8.0, working at 25 kV in un-coated fused silica capillaries. Linear calibration curves over the range studied (0.3–100 μgmL−1), the limit of detection (0.07 μgmL−1).

Maeso *et al*. (11) analyzed caffeine and pyroglutamate in coffees by capillary electrophoresis and found that this method has been developed and validated for the simultaneous determination of caffeine and pyroglutamate in coffee by capillary electrophoresis. Separation conditions employed MECK conditions with 50 mM borate buffer at pH 9.5 with 130 mM SDS. The applied potential was 10 kV and detection was performed at 200 nm.

Yu *et al*. (12) analyzed active components in Chinese Medicine micellar electrokinetic capillary chromatography and found that this method could separating the active components contained in Chinese medicines. ‘SHUANGDAN’ granule, an important botanical drug in the treatment of cardiovascular diseases in China The optimized buffer system (25 mM borate, 29 mM phosphate and 50 mM SDS) provides the best separation with regard to resolution and analysis time.

2.3 Theory of Capillary electrophoresis

2.3.1.Electrophoresis theory

The theory that governs electrophoresis is directly applicable to CE and can be dealt with very briefly, with reference to few equations. As mentioned earlier, electrophoresis is the movement or migration of ions or solutes under the influence of an electric field. Therefore, separation by electrophoresis relies on differences in the speed of migration (migration velocity)of ions or solutes. Now, ion migration velocity can be expressed as:

$$\nu = \mu_e E \qquad (2.1)$$

where ν is ion migration velocity (ms^{-1} , μ_e is electrophoretic mobility ($m^2V^{-1}s^{-1}$ and E is electric fileld strength ($V\ m^{-1}$) .

The electric fileld strength is function of the applied voltage divided by the total capillary length. Electrophoretic mobility is factor that indicates how fast given ion or solute may move through given medium (such as buffer solution). It is an expression of the balance of forces acting on each individual ion; the electrical force acts in favour of motion and the frictional force acts against motion. Since these forces are in steady state during electrophoresis, electrophoretic mobility is constant (for given ion under given set of conditions).The equation describing electrophoretic mobility is:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad (2.2)$$

where q is the charge on the ion, is the solution viscosity and r is the ion radius. The charge on the ion (q) is foxed for fully dissociated ions, such as strong acids or small ions, but can be affected by pH changes in the case of weak acids or bases. The ion radius (r) can be affected by the counter-ion present or by any complexing agents used. From equation we can see that differences in electrophoretic mobility will be caused by differences in the

charge-to-size ratio of analyte ions. Higher charge and smaller size confer greater mobility, whereas lower charge and larger size confer lower mobility.

Electrophoretic mobility is probably the most important concept to understand in electrophoresis. This is because electrophoretic mobility is characteristic property for any given ion or solute and will always be constant. What is more, it is the defining factor that decides migration velocities. This is important, because different ions and solutes have different electrophoretic mobilities, so they also have different migration velocities at the same electric field strength. It follows that, because of differences in electrophoretic mobility, it is possible to separate mixtures of different ions and solutes by using electrophoresis(13).

A vitally important of capillary electrophoresis is the bulk flow of liquids through the capillary. This is called the electroosmotic flow and is caused as follows. An uncoated fused-silica capillary tube is typically used for capillary electrophoresis. The surface of the inside of the tube has ion sable silanol group, which are in contact with the buffer during CE. These silanol groups readily dissociate, giving the capillary wall a negative charge. Therefore, when the capillary is filled with buffer, the negatively charged capillary wall attracts positively charged ions from the buffer solution, creating an electric double layer and a potential difference (zeta potential).

2.3.2 Electroosmotic flow (EOF)

a) Introduction

In capillary electrophoresis, in addition to the solutes, the buffer solution usually also moves through the capillary under the influence of an electric field. This phenomenon is termed electroosmotic or electroendosmotic flow. In normal operation, the direction of the electroosmotic flow is toward the negatively charged cathode, which means the buffer flows from the source vial, through the capillary, through the detector, to the destination vial.

Charged solute molecules are separated due to differences in their electrophoretic mobilities, and will tend to migrate toward the electrode that has an opposite charge from that of the solute. Nagatively charged anions are attracted to the

positively charged anode and, with no electroosmotic flow, would simply migrate into the source vial without passing through the capillary and detector. The electroosmotic flow of the buffer is usually greater than the electrophoretic mobilities of negatively charged solutes so they are carried along with the buffer toward the detector. Electroosmotic flow can be strong enough to carry even small, triply charged anions toward the negative electrode. Since anionic solutes are pulled back toward the source vial by the positive charge of the anode, they move at a rate that is lower than the electroosmotic flow. Neutral solutes are not influenced by electrophoretic mobilities, and therefore move through the capillary at the same rate as the electroosmotic flow. Positively charged solutes migrate toward the negative electrode under the influence of both electrophoretic mobilities and electroosmotic flow, and so move faster than the electroosmotic flow. This is analogous to three boats in a river: one moving down stream, but under power (cation), so it moves faster than the river; a second that is floating with no power (neutral), so it moves at the same speed as the river; and a third heading upstream (anion), but at a rate that is slower than the river is moving downstream so it is still carried downstream.

The order in which solutes pass through the capillary is cations, neutrals, then anions, as shown in Fig. 2.1. charged solutes are separated from each other because of differences in their electrophoretic mobilities. Neutral solutes are separated from the charged solutes but not from each other. The solutes with the highest mobility, and the first to migrate through the capillary, are small, highly charged cations. Small, highly charged anions are the last to migrate through the capillary. A representation of the resulting electropherogram is shown in Fig. 1.2 Neutral solutes can be separated from each other using a different type of capillary electrophoresis, micellar electrokinetic capillary chromatography(14).

b) Benefits of electroosmotic flow

There are two major benefits of electroosmotic flow. First, since all solutes are carried through the capillary, anions and cations can be separated in a single run. Without electroosmotic flow, this would require two separate runs, one with the polarity of the voltage set to analyze cations, the other with the polarity switched for anions. Neutral solutes would not move through the capillary in either case. The second benefit is that even

ions with quite different charge – to – size ratios can be analyzed in a reasonable length of time.

c) What causes electroosmotic flow?

When a buffer is placed inside a capillary; the inner surface of the capillary acquires a charge. This may be due to ionization of the capillary surface of adsorption of ions from the buffer onto the capillary. Even Teflon capillaries exhibit electroosmotic flow, which is probably due to adsorption of the electrically charged ions in the buffer onto the capillary wall. In the case of fused silica, the surface silanol (Si – OH) groups are ionized to negatively charged silanoate (Si – O) groups at pH above about three. This ionization can be enhanced by first passing a basic solution through the capillary followed by the buffer. Often, a new capillary is conditioned by treatment with a KOH or NaOH solution. The negatively charged silanoate groups then attract positively charged cations from the buffer, which form an inner layer of cations at the capillary wall. These cations are not of sufficient density to neutralize all the negative charges, so a second, outer layer of cations forms. The inner layer is tightly held by the Si – O groups and is referred to as the fixed layer. The outer layer of cations is not tightly held because it is further away from the silanoate groups, and it is referred to as the mobile layer. These two layers make up the diffuse double layer of cations.

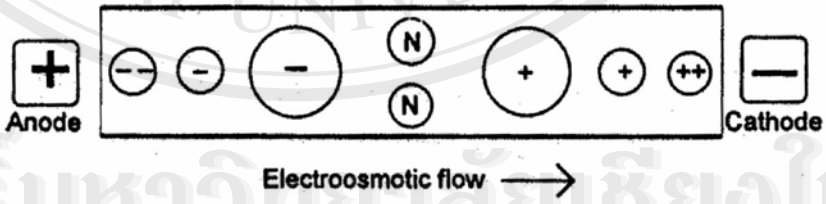


Figure 2.1 Respresentation of migration of neutral, anionic, and cationic solutes in a capillary, due to electroosmotic flow and electrophoretic mobility. Tn this example, the solutes migrate toward the negatively charged cathode. The circled +'s, N's, and –'s represent cationic, neutral, and anionic solutes, repectively. For clarity, buffer ions are not shown.

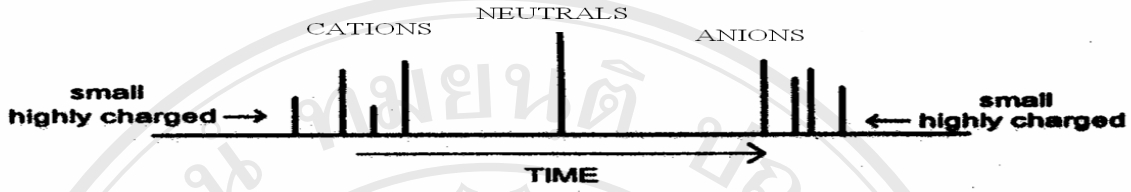


Figure 2.2 Drawing of an electropherogram showing the order of migration due to electroosmotic flow and electrophoretic mobility. Small, highly charge cations are the first to elute from the capillary. Neutral solutes are separated from each other. In this example, the outlet of the capillary is at the negatively charged cathode.

When an electric field is applied, the mobile, outer layer of cations. Pulled toward the negatively charged cathode. Since these cations are solvated, they drag the bulk buffer solution with them, thus causing electroosmotic flow, as represented in Fig. 2.3

Between the two layers is a "plane of shear", as shown in Fig. 2.3 and an electrical imbalance is created at that plane which is the potential difference across the layers; this is termed the zeta potential. Electroosmotic flow is proportional to the zeta potential, which is proportional to the thickness of the double layer. The zeta potential is given by

$$\zeta = 4\pi\delta e/\epsilon \qquad (2.3)$$

Where δ is the thickness of the diffuse double layer, e is the charge per unit surface area, and ϵ is the dielectric constant of the buffer.

The thickness of the diffuse double layer is inversely proportional to the concentration of the buffer, with a 10 mM concentration producing a layer approximately 1 nm thick.

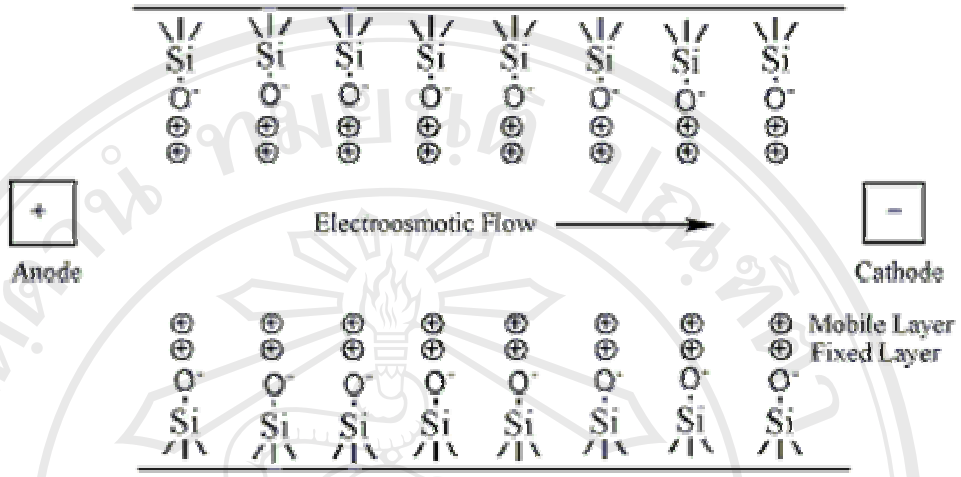


Figure 2.3 Respresentation of electroosmotic flow in a capillary. Electroosmotic flow is caused by the negatively charged Si-O^- groups on the inner wall of the capillary attracting the positively charged cations, represented by the circled by the circled +'s, forming the fixed layer. The mobile layer of cations is pulled toward the cathode, dragging the bulk buffer solution with it. The anions and the salvation of the cations are not shown.

Electroosmotic flow has a relatively flat profile, Fig. 2.4, compared to pumped or laminar flow, as in HPLC. Due to frictional drag, the electroosmotic flow at the wall is slower than the flow through the rest of the tube. This reduction in flow subtracts very little from the overall flat profile since the area near the wall is quite small. The advantage of the flat flow profile is that all of the solute molecules experience the same velocity component caused by electroosmotic flow regardless of their cross sectional position in the capillary, and they elute as narrow bands giving narrow peaks of high efficiency.

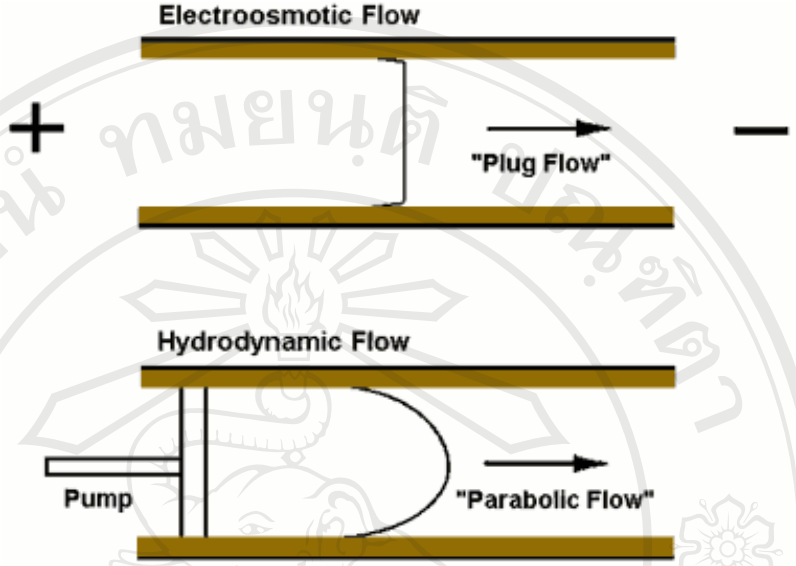


Figure 2.4 Electroosmotic and pumped flow profiles (15)

This is in contrast to solutes moving through a tube under the influence of pumped flow, where the solutes in the center of the tube move faster than those nearer the wall. The result of this uneven flow profile is relatively broad peaks.

It is important that electroosmotic flow be constant. If the flow varies, the migration times of the solutes will change, which may cause peaks to be identified incorrectly or erros in quantitation many electronic integrators look for peaks within a certain time window, and if the flow changes, the peaks may not be within that window and would be missed by the integrator and not reported. Variations in flow also cause changes in peak areas when concentration measuring detectors, such as UV/vis, are used. If the flow rate is reduced by a factor of two, a solute will reside in the detector cell twice as long and its peak area will be twice as large. If quantitation is done by peak area, there will be significant errors. If migration times are not reproducible, peak area/migration time should be used in the calibration plots instead of peak area. When concentration measuring detectors are used, peak heights will not change as much as peak areas with variations in flow.

d) Electroosmotic flow velocity and mobility

The velocity of the electroosmotic flow, is given by

$$\nu_{EOF} = \epsilon\zeta E/4\pi\eta \qquad (2.4)$$

Where $\in$ is the dielectric constant of the buffer, ζ is the zeta potential, E is the applied electric field in volts/cm, and η is the viscosity of the buffer.

The electroosmotic mobility, of the buffer is given by

$$\nu_{EOF} = \epsilon\zeta/4\pi\eta \qquad (2.5)$$

Note that electroosmotic mobility is dependent solely on buffer characteristics, that is, dielectric constant, viscosity, pH, and concentration (which influence the zeta potential) and is independent of the applied electric field.

Anything that causes changes in the right side of Eq. (2.4) will cause variations in the electroosmotic flow.

e) Applied voltage

Since the electric field, E, is voltage / length, changing the voltage is an easy way to modify the electroosmotic flow because it causes a variation in the electric field. An increase in voltage increases electroosmotic flow and reduces migration times. Also, raising the voltage gives higher efficiencies. This suggests that one should use the highest voltage available on the instrument. However, higher voltages lead to higher currents and increased joule heating.

The heat produced is proportional to power, P in watts, which is given by

$$P = VI \qquad (2.6)$$

where V is voltage and I is current.

Since higher voltages give shorter analysis times and narrower peaks, it is best to use the maximum voltage. But, the voltage should not be so high that the heat cannot be effectively dissipated. The maximum voltage that should be used for a given set of conditions can be determined from a graph of observed current versus applied voltage. Fig. 2.6. This is sometimes called an Ohm's law plot, and is graphical representation of Ohm's law:

$$E = IR \qquad (2.7)$$

where R is the resistance and I the current.

In Fig. 2.5, voltage, V, can be plotted instead of electric field, E, which is in units of volts / cm, since the capillary length is constant. This graph should be linear with a zero intercept and a slope of 1/R. When excessive heat is produced, the resistance goes down, causing an increase in current which is indicated by a deviation from linearity and an increase in the slope of the plot. The maximum voltage that should be used is indicated by the point at which nonlinearity occurs. The maximum voltage depends on buffer composition, pH, and concentration.

The maximum voltage also depends on capillary length and inner diameter. Assume that the voltage is kept the same, but the capillary length is cut in half. As a result of this, the electric field, in volts/cm, will double. Since the resistance has been reduced by cutting the capillary length in half, the current will increase and, as seen by Eq. (2.6), more heat will be generated. So, for the same applied voltage, a shorter capillary will have a lower maximum voltage. A smaller inner diameter capillary will have a higher maximum voltage compared to a larger diameter capillary of the same length. This is because for a given voltage and buffer, resistance increases and the current decreases for the smaller diameter capillary.

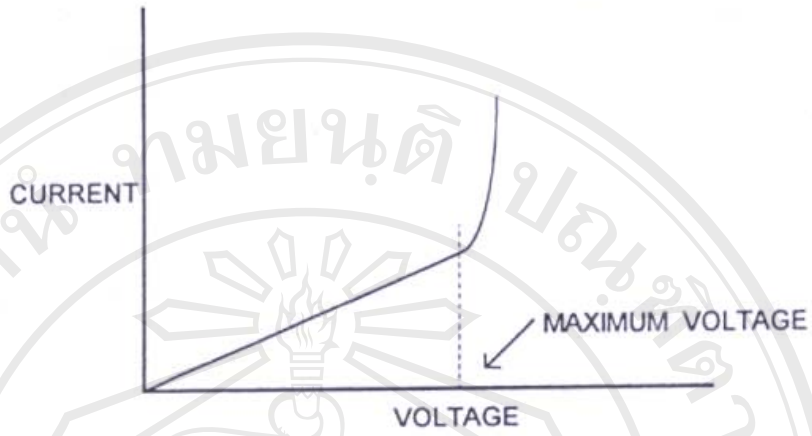


Figure 2.5 Ohm's law plot. The voltage indicated by the dashed line is the maximum voltage that shouls be used.

f) pH of the buffer

Buffer pH has a significant effect on electroosmotic flow because it changes the zeta potential. Fig. 2.6 shows electroosmotic mobility as a funtion of pH for Pyrex, silica, and Teflon capillaries. As pH increases, electroosmotic flow increases, primarily because at higher pH, there is more dissociation of Si – OH to Si – O on the inner capillary wall, The zeta potential is proportional to the surface charge on the capillary wall, Eq. (2.3). At higher pH, there are more charged Si – O groups and, consequently, a greater zeta potential, and as seen from Eq 2.7, an increase in electroosmotic velocity. At lower pH, there is less surface ionization and a lower zeta potential. At pH below about two, there is no electroosmotic flow in a fused silica capillary because most of the silanol groups are protonated. The plot of mobility versus pH for silica shown in Fig. 2.6 follows a similar profile to that for the dissociation of silica versus pH.

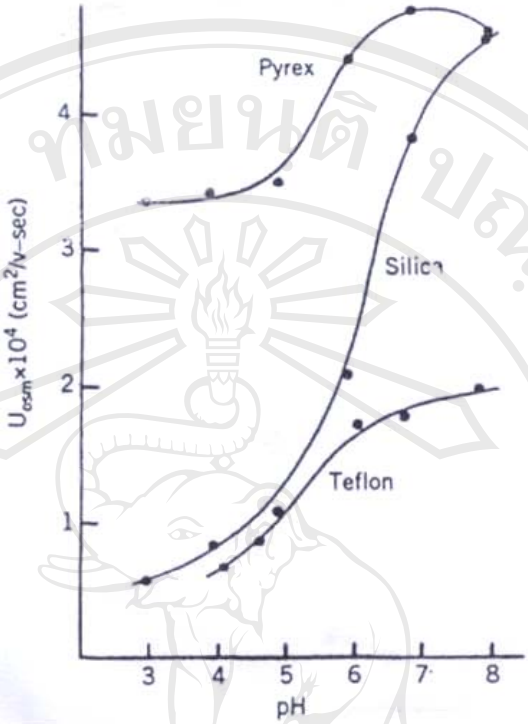


Figure 2.6 Effect of buffer, at constant ionic strength, on electroosmotic flow in 75 μm i.d. Pyrex, 75 μm i.d. fused silica, and 120 μm i.d. Teflon capillaries, all 50 cm long. Electroosmotic flow was measured by using phenol as a neutral marker with detection at 280 nm.

The pH of the buffer will also influence the degree of ionization of the solutes and, hence, their electrophoretic mobilities. Usually, the buffer is chosen to give the best separation and not necessarily the optimum electroosmotic velocity.

g) Buffer concentration or ionic strength

When the capillary temperature is controlled, increasing the buffer ionic strength or concentration will lower the eletroosmotic flow. If the temperature is not controlled, increasing ionic strength or concentration may cause an increase in electroosmotic flow because it increases the current and, consequently, the temperature, which lowers the viscosity.

h) Temperature

A rise in temperature causes an increase in electroosmotic flow because it decreases the viscosity of the buffer. A temperature increase o f 1^0 C, from 20 to 21 ^{0}C, reduces the viscosity of water by 2.4%, so similar increase in the magnitude of the electroosmotic flow is expected.

An increase in temperature also causes a decrease in the dielectric constant which, according to Eq. (2.4), suggests a reduction in electroosmotic flow. This is the opposite from the effect due to reduction in viscosity, mentioned above, and suggests that the two conflicting factors would cancel each other. The decrease in dielectric constant for water is only about 0.5% ^{0}C, which is less than the change in viscosity, so the overall result of a temperature rise is an increase in electroosmotic flow.

Because elevated temperature may cause zone spreading, sample decomposition, or boiling buffer, it is always advisable to control the temperature.

i) Organic solvent

It is hard to predict the effect of addition of an organic solvent to the buffer because it affects several variables, including viscosity, dielectric constant, and zeta potential. In addition, the effect of adding an organic solvent to the buffer depends on which and how much solvent is added. Adding methanol to water increases the viscosity of the solution until the percentage of methanol exceeds about 50% (v/v), then viscosity decreases up to 100% methanol. In contrast, adding acetonitrile to water decreases the viscosity of the mixture from 0 to 100% acetonitrile.

j) Chemical modification of the capillary wall

Electroosmotic flow can be greatly reduced or eliminated by coating or covalently bonding a chemical moiety to the capillary wall. These chemicals can reduce or eliminate electroosmotic flow by blocking the charges at the wall which, in turn, reduces the zeta potential. The chemicals can either be chemically bonded to the capillary wall or simply dissolved in the buffer (dynamic coating).

When a chemical is bonded to the capillary wall, it decreases electroosmotic flow due to shielding of the charges on the capillary wall. It may also decrease the flow due to increased viscous drag on the buffer at the capillary wall. The thicker the film, the more viscous drag, and the slower the electroosmotic flow. It is desirable to have enough electroosmotic flow to assure that all solutes elute in a reasonable time.

Dynamic coating of the capillary is relatively simple; the modifier is simply dissolved in the buffer. Surfactants and hydrophilic polymers can be used to cover the wall charge on the capillary and reduce electroosmotic flow. A wide variety of surfactants (detergents) can be used, including cationic surfactants, such as acetyltrimethyl ammonium bromide (CTAB), nonionic surfactants, such as polyoxyethylenesorbitan (TWEEN), and zwitterionic surfactants, such as tris (hydroxymethyl) aminomethane (TRIS).

k) Application of radial voltage to the capillary wall

Voltage applied through a conductive sheath wrapped around an outer portion of the capillary or a tangential steel plate applied to the outside of the capillary, Fig. 2.7, changes the zeta potential, and consequently, the electroosmotic flow. Hayes et al. observed an approximate doubling of electroosmotic flow by applying – 10 kV, radially, over 4% of the capillary length. This technique of changing electroosmotic flow is relatively easy, once the capillary has been prepared with the sheath or plate.

l) Summary of factors impacts that change electroosmotic flow

Increasing voltage increases electroosmotic flow.

Increasing buffer pH increases electroosmotic flow.

Increasing the concentration or ionic strength of the buffer decreases electroosmotic flow.

Increasing temperature increases electroosmotic flow.

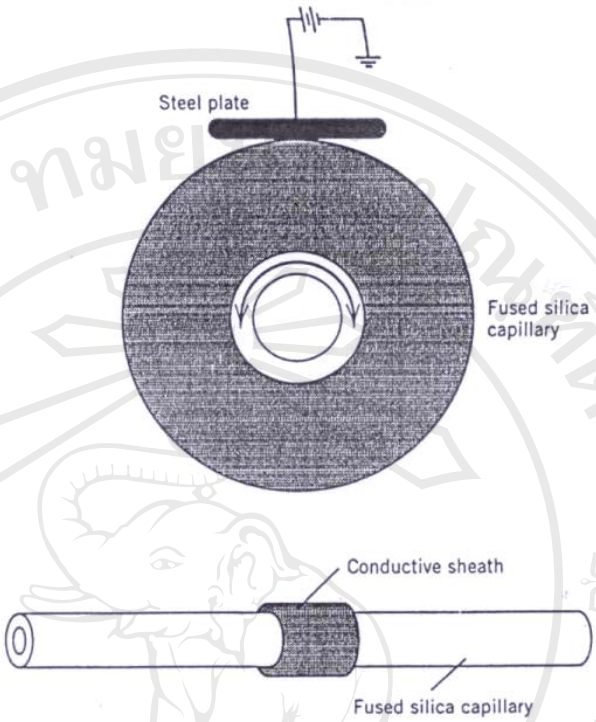


Figure 2.7 Drawing of capillaries with a tangential steel plate and conductive sheath of silver paint to modify electroosmotic flow.

Adding an organic solvent may increase or decrease electroosmotic flow, depending on how it affects the viscosity, dielectric constant, and zeta potential.

Modifying the capillary wall can reduce, eliminate, or even reverse the electroosmotic flow.

m) How to measure electroosmotic flow

The simplest way to measure electroosmotic flow is to inject an uncharged compound, a neutral marker. It can be added to the sample or injected by itself. Then, measure its migration time and, knowing the length of the capillary from the inlet to the detector, calculate the electroosmotic velocity, in distance/time (cm/s) from

$$\nu_{EOF} = l/t_{nm} \qquad (2.8)$$

where is the effective length of the capillary, in cm, from its inlet to the detector, and is the migration time, in seconds, of a neutral marker. the equation for electroosmotic mobility, is given by

$$\mu_{EOF} = \nu_{EOF}/E \qquad (2.9)$$

The electroosmotic mobility in $cm^2/V.\ s$, can be calculated by dividing the electroosmotic velocity, in cm/sm by the electric field, E, in volts/cm, Eq. (2.9). Typical magnitudes of are $10^{-5} - 10^{-4}\ cm^2\ /V\ s$.

When electroosmotic flow parameters are measured using a neutral marker, it is important that one is chosen which moves through the capillary under the influence of only electroosmotic flow. The main criteria in choosing a neutral marker are that it be uncharged at the pH of the buffer, detectable by whatever type of detector is used, pure, have no interaction with the capillary walls, and be soluble in the buffer. A variety of neutral markers have been used, including benzene or pyridine, phenol, methanol, mesityl oxide, and formamide.

Electroosmotic flow can also be measured gravimetrically by weighing the buffer coming out of the capillary for a given period of time and, from the capillary dimensions, buffer density, and electric field, calculate electroosmotic velocity and mobility. This is experimentally more cumbersome than using a neutral marker and would best be done using a recording balance.

n) Reversing electroosmotic flow

Not only can the magnitude of electroosmotic flow be changed, but also its direction. In “normal” capillary electrophoresis, the detector side of the capillary, the cathode, has a negative charge and attracts the positively charged cations in the double layer, As a result, electroosmotic now is toward the cathode, and the order of solute elution is cations, neutrals, then anions. The direction of electroosmotic flow can be reversed such that the order of elution is anions, neutrals, then cations.

o) How to reverse electroosmotic flow

A simple method of changing the direction of electroosmotic flow is to add a quaternary amine, as an alkyl ammonium salt, a flow modifier, to the buffer. As shown in Fig. 2.8 the positively charged hydrophilic ends of the quaternary amines attach to the capillary wall through ionic interacitions with the silanol groups on the capillary wall. The hydrophobic, hydrocarbon ends of the attached quaternary amines associate with the hydrocarbon ends of the quaternary amines in solution through hydrophobic inter actions. The hydrophilic, positively charged ends of the associated amines then attract anions from the buffer. These anions are attracted to the posivtive electrode, and their movement pulls the buffer toward the positive electrode, resulting in reversal in direction of electroosmotic flow.

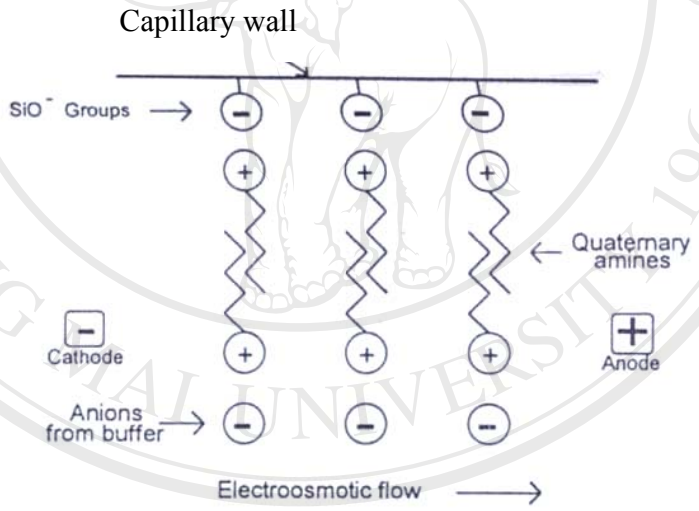


Figure 2.8 Drawing of the inside of a capillary representing reversal of electroosmotic flow by the addition of a quaternary amine to the buffer. The negatively charged Si-O$^-$ group at the capillary wall attract the positively cgarged quaternary amines. The hydrophobic ends of the attached quaternary amines associate with other quaternary amines in solution, and their exposed positive charges atteact negatively charged anions from the buffer. The solvated anions migrate toward the anode, and they grag the bulk solution with them, reversing the direction of electroosmotic flow. Solvation of the ions is not shown. A variety of alkyl ammonium salts have been used as flow modifiers, including cetyltrimethyl

ammoniumbromide (CTAB) and chloride (CTAC), tetradecyltrimethyl ammonium bromide (TTAB), amines, such as diethylenetriamine (DETA), and a diquaternary ammonium salt, 1,6 – bis (trimethylammonium) hexane bromide (the common name of this compound is hexamethonium bromide).

p) Effect of electroosmotic flow on resolution

The previous discussions may have sugested that it is desirable to adjust the operating conditions such that the electroosmotic flow is always very high. After all, increasing the electroosmotic flow decreases the separation time and gives higher efficiencies. While short separation times and high efficiencies are desirable, the most important thing in an electrophoretic separation is resolution, that is, separation of the solutes of interest. The sepped of the separation and the number of theoretical plates become unimportant if the components in the sample are not resolved. If the solutes are swept through the capillary too fast, there may not be sufficient time for the zones to be separated, even though they are very narrow.

Resolution takes into account how narrow and how far apart adjacent peaks are. Resolution, R, can be measured from

$$R = \Delta t / W_{AVE} \qquad (2.10)$$

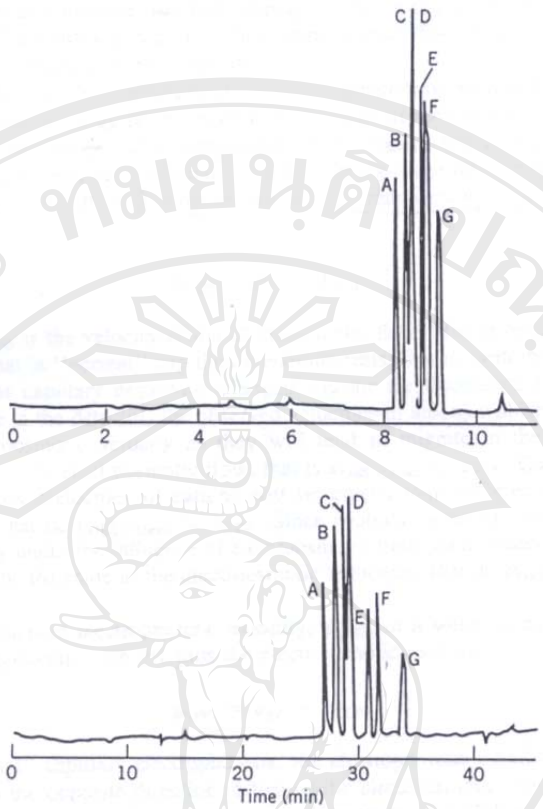


Figure 2.9 Electropherograms showing the effect of electroosmotic flow on resolution and separation time. Top : untreated capillary treated with trimethylchlorosilane to reduce electroosmotic flow. Resolution is improved with less electroosmotic flow, but the separation time is increased. Buffer : 0.05 M phosphate at pH 7. Capillary : borosilicate glass, 75 μm i.d., 100 cm long, treated with trimethylchlorosilane to obtain the bottom electropherogram. Injection : electrokinetic. Voltage : 30 kv. Peak : dansylated derivatives of amino acids, A = asparagines, B = isoleucine, C = threonine, D = methionine, E = serine, F = alanine, and G = glycine.

where Δt is the difference in migration times and is the average peak width, measured at the base, of two adjacent peaks.

An equation to calculate resolution, R, from electrophoretic prameters has been derived by Jorgenson and Lukacs

$$R=0.177(\mu_1-\mu_2)[V/(\mu_{AVE}+\mu_{EOF})D]]^{1/2} \quad (2.11)$$

Where μ_1 and μ_2 are the electrophoretic mobilities of the adjacent solutes, V is the applied voltage, is the average mobility of the two solutes, is the electroosmotic mobility, and D is the solute's diffusion coefficient. From Eq. (2.11), it can be seen that the highest resolution is attained when electroosmotic flow is equal, but in the opposite direction, to the average electrophoretic mobility of the solutes, that is when if this condition were met, resolution would be extremely high; however, separation times would be infinite since the sample ions would e moving at the same velocity as the electroosmotic flow, but in the oposite direction; and so, effectively, would not move at all. This is seen in Fig. 2.16 where the resolution between the peaks is improved when a lower electroosmotic flow is used, but the analysis time is more than tripled.

There is no best electroosmotic flow that can be used for all analyses so, in order to attain the best balance of resolution and separation time, a compromise must be made between electroosmotic flow and separation time.

2.3.3 Electrophoretic mobility

a) Introduction

Under the influence of an electric field, and electrically carged solute will migrate through a buffer with an electrophoretic velocity, in cm/s, given by

$$\upsilon_{EP} = \mu_{EP} E \quad (2.12)$$

where is the electrophoretic mobility and E the applied electric field. Separation is achieved because solutes migrate through the capillary at different velocities. Electrophoretic mobility is given by

$$\mu_{EP} = q/6\pi\eta r \quad (2.13)$$

where q is the charge of the ionized solute, η the buffer viscosity and r the solute radius. Electrophoretic mobility is analogous to electroosmotic mobility and has the sme units,

$cm^2/V.s$. Electrophoretic mobility is deoebdebt ti sine extent on the shape of the ions, but for this discussion, it is assumed that the ions are spherical.

From Eq. (2.13), it can be seen that the greater the charge to size ratio (q/r) the higher the electrophoretic mobility and, for a given applied electric field and buffer, the higher the velocity; see Eq. (2.12). Small, highly charged molecules move through the capillary the fastest, and large molecules with a lower charge move slower. Neutral molecules have an electrophoretic mobility of zero because, for them, q is zero. Just as an increase in viscosity of the buffer causes a reduction in electroosmotic flow, it also causes a reduction in electrophoretic mobility.

Note that the electrophoretic velocity is dependent on both mobility and electric field, whereas electrophoretic mobility is dependent only on solute and buffer properties and is independent of the applied electric field.

A solute's velocity is influenced both by its electroosmotic mobility and the electroosmotic flow. The observed electrophoretic velocity, is given by

$$\upsilon_{OBS} = \upsilon_{EP}+\upsilon_{EOF} \qquad (2.14)$$

where is the velocity of the electroosmotic flow, also in cm/s.

Note that in "normal" capillary electrophoresis, that is, with the detector side of the capillary negatively charged and the electroosmotic flow from the source to the detector, the observed velocities of anions will be less than the electroosmotic velocity as they will tend to migrate in the opposite direction of the electroosmotic flow, that is conversely, the observed velocities of cations will be greater than the electroosmotic velocity, that is Since neutrals move through the capillary only under the influence of electroosmotic flow, their observed velocities will be the same as the electroosmotic velocities, that is $V_{OBS,\ Neutrals} = V_{EOF}$

The observed electrophoretic mobility, of a solute is due to both its electrophoretic mobility plus the electroosmotic mobility:

$$\mu_{OBS} = \mu_{EP} + \mu_{EOF} \quad (2.15)$$

In "normal" capillary electrophoresis, the electrophoretic mobilities of anions is in the opposite direction to that of the electroosmotic flow, so their observed mobilities will be less than the electroosmotic mobility. And , conversely, the observed mobilities of cations are greater than the electroosmotic mobility since they move in the same direction as the electroosmotic flow. The observed mobilities of neutrals will be the same as the electroophoretic mobility because they move at the same velocity as the electrosmotic flow. That means may be greater than equal to, or less than the true, not observed, electrophoretic mobilities of most anions are usually less than the electroosmotic mobility, that is $\mu_{EOF} > \mu_{EP}$.

b) Measuring electrophoretic velocity and mobility

Migration time is the time it takes a solute to migrate from injection to detector. The observed electrophoretic velocity, in cm/s, can be calculated from

$$\upsilon_{OBS} = l/t_m \quad (2.16)$$

Where is the effective capillary length, from inlet to detector. The electroosmotic velocity, can be determined using Eq. (2.8) by measuring the migration time of a neutral marker Rearranging Eq. (2.14) and substituting Eqs. (2.8) and (2.16) the electrophoretic velocity is then given by

$$\upsilon_{EP} = l/t_m - l/t_{nm} \quad (2.17)$$

Electrophoretic mobility can then be determined by rearranging Eq. (2.12) and substituting Eq. (2.16) and E = V/L, where L is the total capillary length and V the voltage:

$$\mu_{EP} = \upsilon_{EP}/E \quad (2.18)$$

$$\mu_{EP} = \upsilon_{EP} L/V \quad (2.19)$$

$$\mu_{EP} = (1/t_m - 1/t_{nm})(L/V) \quad (2.20)$$

Eq. (2.20) can be used to calculate a solute's electrophoretic mobility from experimental parameters.

c) Influence electrophoretic mobility

From Eq. (2.13), it can be seen that anything that affects the charge or size of a solute or the viscosity of the buffer will cause changes in the electrophoretic mobility. Some parameters affect both electrophoretic mobility and electroosmotic flow. For instance, changing the pH of the buffer may change the degree of ionization of the solute, and hence, its charge, while also changing the electroosmotic flow; In addition anything that causes a change in the viscosity of the buffer such as a change in temperature or addition of an organic solvent, will also affect both electrophoretic mobility, Eq. (2.13) and electroosmotic flow, Eq. (2.5)

d) Solute charge

The pH of the buffer is very important in determining the charge of an ionized solute. The mobilities of anionic solutes increase as the pH is raised because they exist in the predominantly negatively charged form at higher pH. As pH is lowered, more of the negative charge is neutralized and their mobilities decrease. This is shown in Fig. 2.11 where the mobilities of negatively charged anti – inflammatories increase as the pH is raised, as seen by their shorter migration times. In this case, a coated capillary was used which eliminates electroosmotic flow, so the observed changes in migration times are due only to changes in solute mobilities.

In the case of cationic solutes, their mobilities increase as pH is lowered.

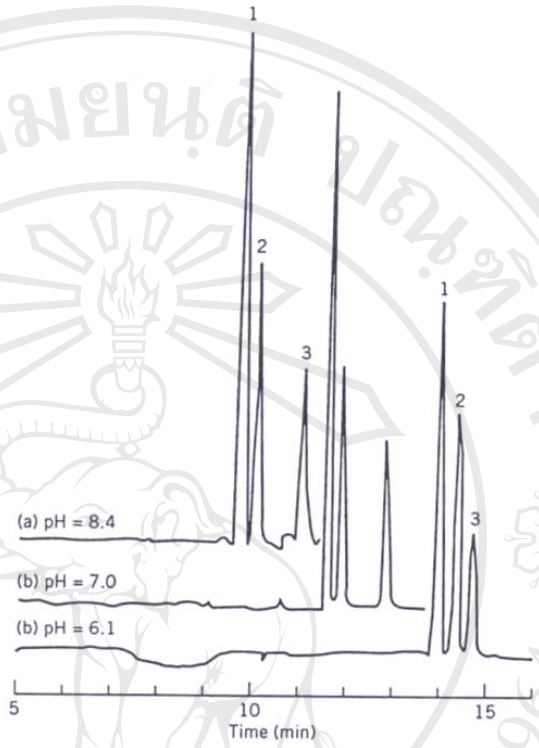


Figure 2.10 Electropherograms illustrating the effect of pH on mobilities.The inner wall of the capillary was coated with linear polyacrylamide to eliminate electroosmotic flow. Buffer : pH 8.4, 20 mM borate 100 mM boric acid. pH 7.0, 30 mM phosphate 9 mM borate; pH 6.1, 80 mM MES 30 mM tris.Capillary : fused silica, 75 μm i.d., 375 μm o.d., 40 cm long to the detector. Detection :UV, 215 nm. Injection : electromigration. Voltage : 275 V/cm. Peaks : 1 = naproxen, 2 = ibuprofen, and 3 = tolmetin

e) Solute size

There is not much that can be done to change the size of a given solute except perhaps, to change its degree of hydration and hence, its size. Fig. 2.11 shows that the addition of methanol to the buffer enhances the separation of iodide and chloride, and azide and perchlorate anions.

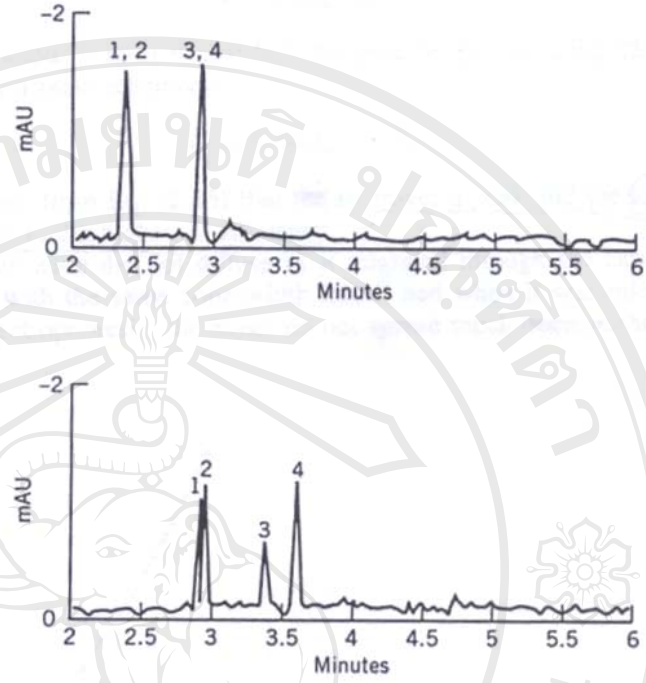


Figure 2.11 Electropherograms showing the effect of addition of an organic solvent to the buffer on resolution and migration times. Top : without organic solvent, bottom : with 15 % methanol added to the buffer. Buffer : 2.5 mM pyromrllitic acid, 6.5 mM NaOH. 0.75 mM hexamethonium hydroxide, 1.6 mM triethanolamine, pH 7.7, 15 % methanol was added to the buffer to obtain the bottom electropherogram. Capillary : fused silica, 50 μm i.d., 50 long to the detector. Detection :indirect UV at 250 nm. Injection : gravity, 100 mm for s. Temperature : ambient. Voltage : 30 kv. Peak : 1 =iodide, 2 = chloride, 3 = perchlorate and 4 = azide.

With no methanol in the buffer, these anion pairs are not separated. It is proposed that the addition of methanol to the buffer causes a change in the degree of hydration of iodide relative to chloride. The enthalpies of hydration, are – 331 kcal/mol for iodide and – 348 kcalk/mol for chloride, so the waters of hydration are easier to displace from iodide. Therefore, the addition of methanol should cause an increase in the mobility of iodide because of a reduction in its size. This is seen in Fig. 1.18 where the migration time of

iodide is shorter than that of chloride when methanol is present in the buffer. Note that the migration times of all solutes increase with the addition of methanol due to a reduction in electroosmotic flow; It is also proposed that the separation of azide and perchlorate is partially due to changes in their degrees of hydration with the addition of methanol.

2.3.4. Capillary diameter and joule heating

The production of heat in CE is the inevitable result of the application of high field strengths. Two major problems arise from heat production. Temperature gradients across the capillary and temperature changes with time due to ineffective heat dissipation.

The rate of heat generation in a capillary can be approximated as follows

$$\frac{dH}{dt} = \frac{iV}{LA} \qquad (2.21)$$

Where L is the capillary length and A, the cross-sectional area. Since $I = V/R$ and $R = L / kA$ where k is the conductivity, then

$$\frac{dH}{dt} = \frac{kV^2}{L^2} \qquad (2.22)$$

The amount of heat generated is proportional to the square of the field strength. Either decreasing the voltage or increasing the length of the capillary has a dramatic effect on the heat generation. The use of low conductivity buffers is also helpful in this regard although sample loading is adversely affected.

Temperature gradients across the capillary are a consequence of heat dissipation. Since heat is dissipated by diffusion, it follows that the temperature at the center of the capillary should be greater than at the capillary walls.

Since viscosity is lower at higher temperature, it follows that both the EOF and electrophoretic mobility (EPM) will increase as well. Mobility for most ions

increases by 2% per degree Kelvin, The result is a flow profile that resembles hydrodynamic flow, and bandbroadening occurs. Operating with narrow - diameter capillaries improves the situation for two reasons : the current passed through the capillary is reduced by the square of the capillary radius, and the heat is more readily dissipated across the narrower radial path. The resulting thermal gradient is proportional to the square of the diameter of the capillary, which can be approximated from the following equation:

$$\Delta T = \frac{0.24\ Er^2}{4K} \qquad (2.23)$$

Where W is the power, r is the capillary radius, and K, the thermal conductivity.

The second problem is ineffective heat dissipation. If heat is not removed at a rate equal to its production, a gradual but progressive temperature rise will occur until equilibrium is reached. Depending on the specific experimental conditions, imprecision in migration time will result due to variance in both EOF and electrophoretic velocity. Narrow – diameter capillaries help heat dissipation, but effective cooling systems are required to ensure heat removal. Liquid cooling is the most effective means of heat removal and capillary temperature control.

Capillary inner diameters range from 20-200 μm From the standpoint of resolution, the smaller the capillary i.d., the better the separation. However, smaller bore capillaries yield poorer limits of detection due to reduced detector parh length and sample loadability. Narrow capillaries are also more prone to clogging. As long as buffers are filtered through <0.5-μm filters, clogging is seldom a problem in the above mentioned size range. Since it may be impractical to filter samples, high speed centrifugation is usually sufficient to settle suspended particles.

2.3.5. Separation 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.

2.3.5.1. Capillary gel electrophoresis (CGE) [see appendix A]

2.3.5.2. Capillary zone electrophoresis (CZE) [see appendix A]

2.3.5.3. Capillary isotachophoresis (CITP) [see appendix A]

2.3.5.4. Capillary isoelectric focusing (CIEF) [see appendix A]

2.3.5.5. Capillary electro chromatography(CEC) [see appendix A]

2.3.5.6. Micellar electrokinetic capillary chromatography (MECC or MECK)

MEKC can be performed by dissolving an ionic surfactant in the CE running solution at a concentration higher than the critical micelle concentration (cmc) with no instrumental modifications. In general, neutral or alkaline buffer solutions are used to create conditions for a strong electroosmotic flow (EOF) that moves the entire liquid stream in the capillary toward the cathode (Figure 2.12).

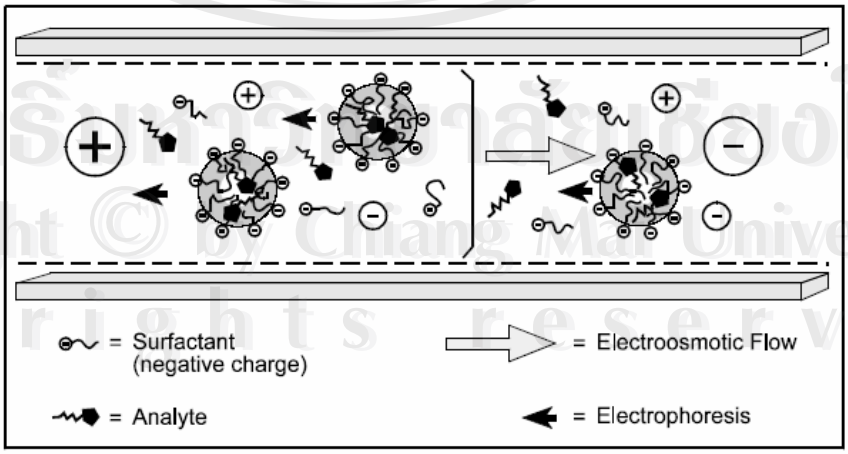


Figure 2.12 Illustration of the components the MECC

Therefore, even anionic micelles, such as sodium dodecyl sulfate (SDS), mi-grate toward the cathode. The neutral analyte that is not at all solubilized by oris free from the micelle migrates at the same velocity as that of EOF; the analyte that is totally incorporated into the micelle migrates at the same velocity as that of the micelle. Other neutral analytes are detected between t_0 and t_{mc} , which are the migration time of the EOF marker and the micelle, respectively. The interval between t_0 and t_{mc} is called the migration time window. The wider the window, the larger the peak capacity, which is the number of peaks that can be separated during a run. Migration time can be measured by using markers such as methanol for EOF and Sudan III for the micelle. Parameters similar to those in chromatography can be used to describe the migration behavior of the analyte. The retention factor k can be defined as

$$k = n_{mc} / n_{aq} \qquad (2.24)$$

in which n_{mc} and n_{aq} are the numbers of moles of the analyte in the micelle and surrounding aqueous phase, respectively; *k* can be measured by

$$k = \frac{t_R - t_0}{t_0 (1 - t_R / t_{mc})} \qquad (2.25)$$

in which t_R is the migration time of the analyte (1). The difference between this equation and the conventional one used in chromatography is the limited migration time window in MEKC. Although the micelle is not fixed inside the capillary, it plays the same role as the stationary phase in chromatography and is there-fore called the pseudo stationary phase.

a) Controlling selectivity and resolution

In CE, the separation principle is simple and so is the strategy for optimizing separation conditions. Resolution is based on the difference in electrophoretic mobility and separation selectivity and is manipulated mainly by optimizing pH and, if necessary, by using additives to modify the electrophoretic mobility. Other issues are often more important, such as band broadening caused by the adsorption of analytes onto the capillary wall and low sensitivity due to a short optical path length in the photometric detector.

These issues also occur in MEKC, but because MEKC separation is based on chromatographic separation, the optimization strategies are more versatile.

The MEKC resolution *R* S equation is

$$R_s = \frac{\sqrt{N}}{4}\left[\frac{\alpha-1}{\alpha}\right]\left[\frac{k_2}{1+k_2}\right]\left[\frac{1-t_0/t_{mc}}{1+(t_o/t_{mc}).k_1}\right] \qquad (2.26)$$

in which *N* is the plate number and is the selectivity factor equal to *k* 2 */k* 1 (3). Equation 3 is similar to the one used in conventional chromatography, except for the addition of the last term on the right-hand side. This variable comes from the migration of the micelle or pseudo stationary phase inside the capillary; that is, the migration of the pseudo stationary phase causes reduction of the column length (4). If the micelle migration is completely suppressed or *t* mc is infinity, the resolution equation is the same as the conventional one.

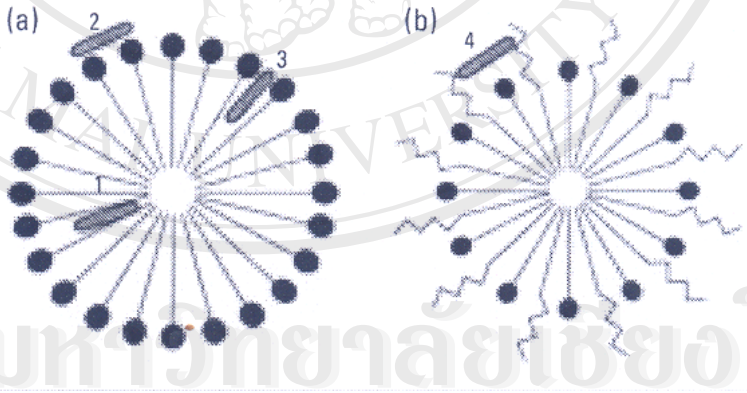


Figure 2.13 Micellar solubilization

(a) Ionic micelle and (b) mixed micelle of ionic and nonionic surfactants interacting (1) with the core (2) on the surface (3) as a cosurfactant and (4) with a nonionic surface

The plate number is not proportional to the capillary length as in other separation modes in CE under a constant applied voltage. Under conventional conditions, *N* is >100,000; if *N* is significantly lower than that, the experimental conditions must be reconsidered. The most probable cause of low *N* is adsorption of the analyte onto the capillary wall; if this happens, the capillary must be rinsed thoroughly. To manipulate the separation, must be changed (5). In re-versed-phase LC, usually the separation is not manipulated by changing the stationary phase because C 18 is the most widely accepted phase and different products vary little. In MEKC, however, pseudo stationary phases are micelles, and several different surfactants can be used to form micelles. Some typical surfactants with their (cmc) and aggregation numbers are listed in Table 2.2

Table 2.2 Aggregation number (AN) and cmc of selected surfactants.

Surfactant	**cmc (at 25 °C; 10 –3 M)**	**AN**
Anionic		
SDS	8.1	62
Sodium tetradecyl sulfate	2.1 (50 °C)	138
Sodium decanesulfonate	40	40
Sodium N-lauroyl-N-methyltaurate	8.7	NA
Sodium N-dodecanoyl-L-valinate	5.7 (40 °C)	NA
Sodium cholate	13–15	2–4
Sodium deoxycholate	4-6	4-10
Cationic		
Tetradecyltrimethylammonium bromide	3.5	75
Dodecyltrimethylammonium bromide	15	56
Cetyltrimethylammonium bromide	0.92	61
Cetyltrimethylammonium chloride	1.3	NA
Nonionic		
Polyoxyethylene(23) sorbitan monolaurate (Tween 20)	0.059	NA

Using mixed micelles, especially combinations of ionic and nonionic or ionic and zwitterionic, creates other possible choices to change selectivity. The chemical structure of polar groups of surfactant molecules affects selectivity more than the hydrophobic core of the micelle or lipophilic groups of surfactant molecules, because most analytes interact with the micelle at the surface. Mixed micelles with surfaces covered by polyoxyethylene groups will have different surface characteristics and hence selectivity from that of the SDS micelle.

According to the linear salvation energy relationship, hydrophobicity of the analyte is the major factor that deter-mines selectivity. The second factor in tuning selectivity is the hydrogen-bond basicity of the analyte or hydrogen-bond acidity of the surfactant. Unlike the mobile phase in reversed-phase LC, the aqueous phase is not very important. In most cases, only the aqueous buffer is used, but if the analytes are extremely hydrophobic, up to 30% miscible organic solvents, such as methanol or acetonitrile, can be added to the micellar solution to increase solubility into the aqueous phase. Analyzing extremely hydrophobic analytes is a challenge in MEKC.

The retention factor term (third term) in the right-hand side of Equation 3 is not independent of other variables because the last term includes k. The optimum *k* values to maximize resolution are easily determined by

$$k_{opt} = \sqrt{t_{mc}/t_0} \qquad (2.27)$$

which differentiates the product of the last two terms in Equation . Under neutral or acidic conditions, *t* mc /*t* 0 is 3–4 and *k* opt is 1.7–2.0. To adjust *k* in MEKC, the concentration of the surfactant can be increased or decreased because *k* can be expressed as

$$k = KV_{mc} \cong V\ Kv\ (\overline{C}_{sf} - cmc) \qquad (2.28)$$

in which *K* is the distribution coefficient of the analyte between the micelle and the aqueous phase; V_{mc} and V_{aq} are the volume of the micelle and the aqueous phase, respectively; v is the partial specific volume of the micelle; and C_{sf} is the surfactant concentration. As shown

by Equation 5, *k* is linearly proportional to the surfactant concentration; this is an advantage of MEKC, because the C_{sf} needed to obtain a given *k* can be calculated, provided the cmc and *k* at a certain C_{sf} are known. In Table 1, the cmc values are in pure water, but cmc in buffer solution is much lower.

MEKC rarely separates extremely hydrophobic analytes with high k. However, several strategies are possible. Adding an organic solvent significantly reduces *k* and gives better resolution of extremely hydrophobic analytes. The organic aqueous solution has higher viscosity, and the migration times will be long. Adding too much organic solvent may destroy the micellar structure and/or decrease the migration time window because the electrophoretic mobility of the micelle is reduced, probably as a result of the reduced charge on the micelle or the increased size of the micelle due to swelling caused by the organic solvent.

Adding β- or ϒ-cyclo-dextrin (CD) to the micellar solution is very effective. Although CD itself is electrically neutral and behaves like the aqueous phase, it can bind analytes in its cavity by hydrophobic interaction, depending on the size of the analyte and the cavity. In addition, CDs are chiral compounds and useful additives for separating enantiomers, particularly neutral ones, in MEKC with achiral surfactants. Adding a high concentration of urea increases the solubility of hydrophobic compounds in the aqueous solution and decreases k. Urea is very soluble in water up to 7 M, but the solution is UV-transparent and the viscosity is not very high (16).

2.4. Instrument of capillary electrophoresis

2.4.1. System overview

The instrumentation needed to perform capillary electrophoresis is relatively simple. A basic schematic of a capillary electrophoresis system is shown in Figure 2.14 The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high-voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial

containing the sample and then returned to the source vial (sample is introduced into the capillary via capillary action, pressure, or siphoning). The migration of the analytes is then initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. It is important to note that all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow, as will be explained. The analytes separate as they migrate due to their electrophoretic mobility, as will be explained, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different retention times in an electropherogram (17).

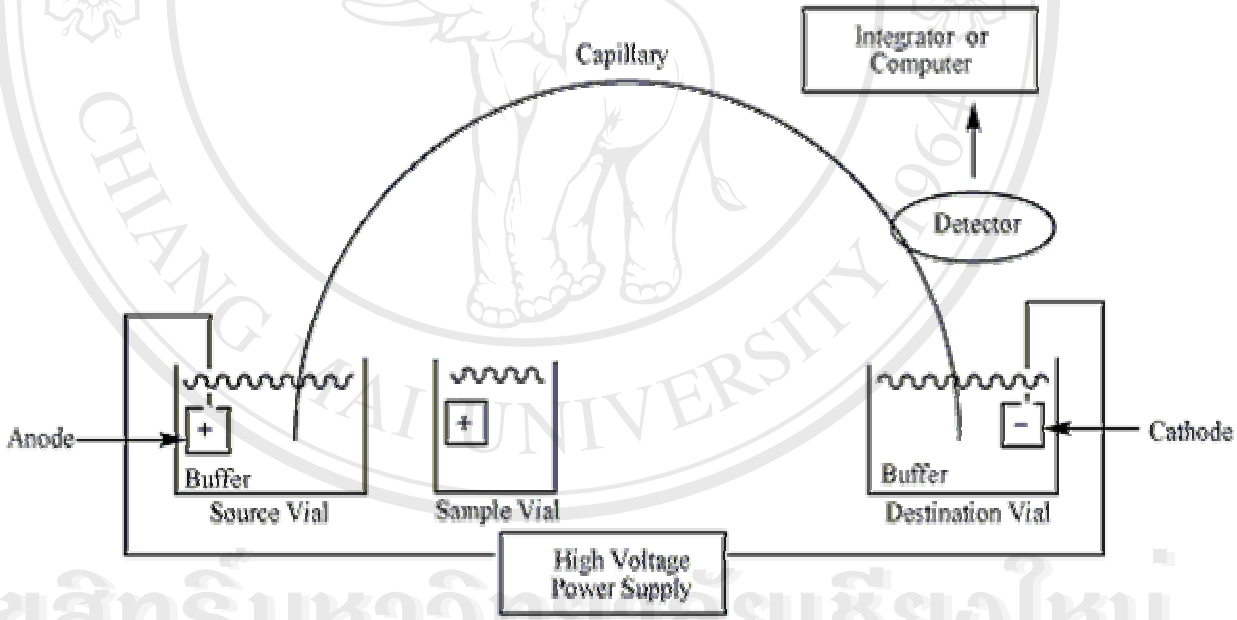


Figure 2.14 Diagram of capillary electrophoresis system

Electropherograms are different from chromatograms when a concentration-dependent detector, such as an ultraviolet/visible(UV/vis) absorbance detector, is used. Assuming equal solute concentrations and detector responses, peak eights and widths are different in HPLC. In isocratic HPLC, the longer the retention times, the broader and shorter the peak. This is because solutes are diluted more as they spend more time inside the column. Area of

all the peak are approximately the same since all solute move through the same rate, but later eluting peak are more dilute and therefore broader with smaller peak heights.

In capillary electrophoresis, peak heights remain constant as retention gets longer because the solutes move through the detector in zones of approximately the same length and, therefore, the same concentration. The more slowly, and consequently, reside in the detector cell longer. For equal concentrations and detector response, peak area increase with time. After the separated components pass through the detector, the individual components can be collected for identification or further analysis.

2.4.2. Sample injection

Sample injection refers to the means of getting the sample into the capillary strictly speaking, it is not an injection of sample into the capillary; rather, it is introduction of a sample. The term, sample injection „ has been taken from chromatography where the sample is, in some cases, injected directly into a column or flowing mobile phase. While sample introduction is the more appropriate term, sample injection will be used here to comply with common usage.

Samples are loaded into sample vials which usually have volumes of a few micro liters to a few milliliters. Only a small volume of sample is required since injection is performed by placing the inlet of the capillary, that is, the side of the capillary at the positive electrode into the samples are injected into the capillary by different techniques, the most common being hydrodynamic and electrokinetic injections Hydronamic injection can be done by pressure or siphoning. Siphon injection, also called gravity injection, is done by raising the sample vial causing the sample to siphon into the capillary. Pressure injection is performed by either pressurizing the sample vial or by applied a vacuum to the destination vial. In Electrokinetic injection, an electric field is applied to the sample vial, causing the sample components to migrate into the capillary. Hydrodynamic injections do not work for gel-filled capillaries, so electrokinetic injection is usually required in this case. A sample vial and applying a pressure to the vial, as show in Fig 2.14 The volume of sample injected, V_i, can be calculated from the Poiseulle equation

$$V_i = \Delta P r^4 \pi t / 8 \eta L \quad (2.29)$$

Where ΔP is the pressure across the capillary, d is the capillary inner diameter, t is the time the pressure is applied, η is the viscosity of the sample solution, and L is the total capillary length. The length of the sample plug.

L_p can be calculated from

$$Lp = Vi/\pi r2 = \Delta Pr2t/8\eta L \quad (2.30)$$

Where r is the capillary inner radius. For example, assume that a pressure of 25 mbar is applied for 2 s to an aqueous sample at 20 ^{o}C, which a capillary that is 75 cm long and 50 μm i.d., The injected volume is 1 nL, and the sample plug length is 0.5 mm

Pressure injections canals be made by applying a vacuum to the destination vial while the capillary inlet is in the sample vial, as show in fig 2.23 the injection volume and sample plug length in this case can be calculated using Eg 2.28 and Eq 2.29

Where ρ is the density of the buffer in the capillary (0.9972 g.mL^{-1}, for water at 20 ^{o}C), g is the gravitational constant(980 cm .s^{-2}), and ΔH is the difference I heights of the liquids in the sample and destination vials. A more practical equation for injection volume, V_i, in nL is

$$Vi = 2.84 \times 10^{-8} Htd^4/L \quad (2.31)$$

Where H is the height the sample is raised in mm, t is the sample is raised in seconds, d is the capillary inner diameter in μm, and L is the total capillary length in cm. For example, if the sample is raised 50 mm for 10 s, and the capillary is 50 cm long and 50 μm i.d., the injection volume is 1.78 nL. Eg 2.50 is valid when the sample has the same viscosity as water and its temperature is 20-22 ^{o}c Eg 2.31 does not apply when the sampling time, t, is

very short because the time it takes for the sample vial to be raised is not taken into account.

2.4.3. Source and destination vials

The source vial sometimes referred to as the inlet vial or inlet reservoir, and the destination vial is sometimes referred to as the exit vial or exit reservoir. Ordinarily, the source vial, capillary, and destination vial are filled with the same buffer.

The composition of the buffer is one of the most important variables in capillary electrophoresis, and small changes in buffer pH or concentration may cause significant changes in rate of solute migration and, consequently, change in migration times. Therefore, it is important that the source vial, destination vial, and capillary be thoroughly rinsed when the buffer is changed.

During the course of repeated analyses with the same buffer, the composition of buffer in the vial and the capillary may change due to electrolytic formation of hydrogen ions at the anode and hydroxide ions at the cathode. After repeated analyses, the buffer I the source vial may have a different composition from that in the destination vial, which can lead to nonreproducible migration times. Also, after the solutes migrate through the capillary, they exit into the destination vial, and as they accumulate there, will cause a change in the source vial and capillary even when the same buffer is used for each analysis. Most instruments have the ability to automatically rinse and fill the vial and capillary between injections, there by providing a constant buffer composition for each analysis.

Source and destination vials should be kept at the same level to prevent siphoning of buffer from one vial to the other, which may cause change in migration times.

Income instruments, certain sample vials in an auto sampler can be designated as source and destination vials.

2.4.4. Capillaries

Although Teflon and Pyrex capillaries have been used, fused silica capillaries are used the most. Fused silica is easily broken, own outer layer of polyimide is placed on the capillary to make it strong. An optical window can be made by removing a short section (approximately 1 cm) of the polyimide coating. This can be done by scraping it off or burning it off with a low-temperature flame from a match or lighter. The window

should be cleaned with a solvent such as methanol or acetone and dried. The window section with the coating removed is very fragile and easily broken, so one must be careful in handing that portion of the capillary. The position of the window is dictated by the positions of the detector and the destination vial, but when possible, it should be close to the outlet end of a the capillary.

A representation of the crosssection of the typically 50 or 75 μm i.d., although capillaries of 25 μm i.d., 12.7 μm i.d., and 5 and 2 μm i.d., have also been used. Outer diameter that are 10-30 μm thick. The larger the inner diameter of the capillary, the more heat is generated and the greater the temperature difference between the center and inner wall of the capillary, the temperature difference is proportional to the square of the inner radius of the capillary. If the internal diameter is doubled, temperature difference increases by a factor of four. This would suggest that it is best to use a capillary with the smallest inner diameter. This is not always the case though when UV/vis absorbance or fluorescence detector are used. Reducing the capillary diameter reduces the absorbance of light and peak height proportionally. With effective heat dissipation, capillaries of 50-100 μm i.d. can be used with little degradation in efficiency.

If it is necessary to accurately know the inner diameter of a capillary of the capillary, it is best determined gravimetrically by drying and weighing the capillary, filling it with water, and weighing it again. From the weight of the water, length of the capillary, and the density of water, the inner diameter of the capillary, i.d. in cm, can be calculated from

$$\text{i.d.} = 2\,[\text{wt}/\text{dL}\pi]^{1/2} \qquad (2.32)$$

where wt is the weight of water in g, d is the density of water in g/cm^3, and L is the capillary length in cm

The outer diameter of the capillary is important in terms of heat dissipation. A thick capillary wall provides better heat dissipation when no additional cooling is used. When a thermostated cooling system is used, a thinner wall is better as it allows faster heat removal.

2.4.5. Detection

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In these systems, a section of the capillary itself is used as the detection cell. The use of on-tube detection enables detection of separated analytes with no loss of resolution. In general, capillaries used in capillary electrophoresis are coated with a polymer for increased stability. The portion of the capillary used for UV detection, however, must be optically transparent. Bare capillaries can break relatively easily and, as a result, capillaries with transparent coatings are available to increase the stability of the cell window. The path length of the detection cell in capillary electrophoresis (~ 50 micrometer) is far less than that of a traditional UV cell (~ 1 cm). According to the Beer-Lambert law, the sensitivity of the detector is proportional to the path length of the cell. To improve the sensitivity, the path length can be increased, though this results in a loss of resolution. The capillary tube itself can be expanded at the detection point, creating a "bubble cell" with a longer path length or additional tubing can be added at the detection point as shown in figure 2.15 Both of these methods, however, will decrease the resolution of the separation.

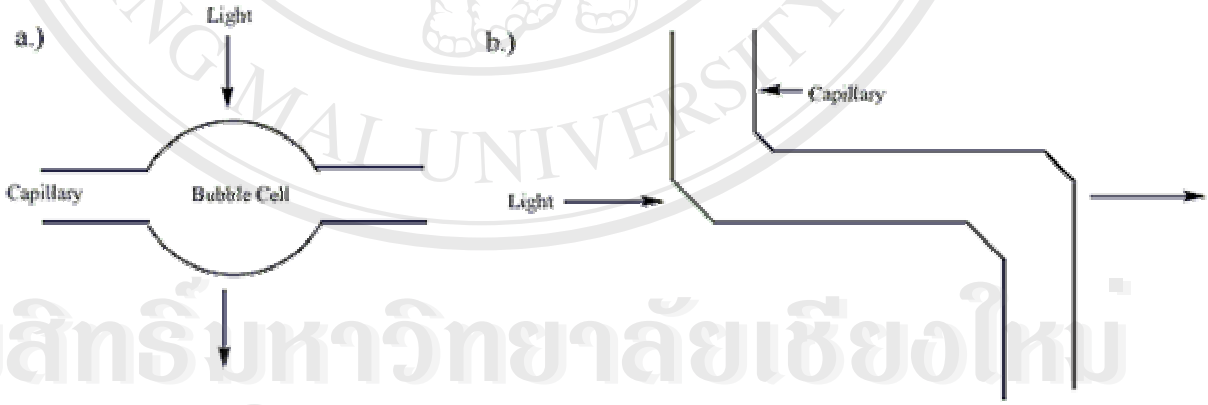


Figure 2.15 Techniques for increasing the pathlength of the capillary: a.) a bubble cell and b.) a z-cell (additional tubing).

Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be

utilized for samples that do not fluoresce. The set-up for fluorescence detection in a capillary electrophoresis system can be complicated. The method requires that the light beam be focused on the capillary, which can be difficult for many light sources.[1] Laser-induced fluorescence has been used in CE systems with detection limits as low as 10^{-18} to 10^{-21} mol. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary.

2.4.6. Power Supply

The purpose of the power supply is to provide and electric field across the capillary. Most can be operated in either the constant voltage, constant current, or constant power mode and have the ability to reverse the polarity. Voltages up to 30 kv, currents up to 300 μA, and power up to 6w are used. The constant voltage mode is most widely used. It is necessary to have a stable voltage as any variations in voltage will cause change in migration times.

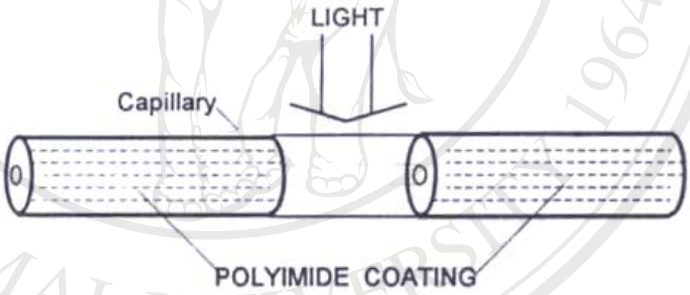


Figure 2.16 Drawing of apiece of a fused silica capillary with a section of the polymide coating removed with serves as the cell in a UV/vis or fluorescence detector. This drawing is not to scale.

In most electrophoresis separations, the molecules migration from the anode to the cathode. There are some cases where the direction of migration is reversed. In these cases, the polarity of the applied electric field is also reversed such that the negative electrode is at the capillary inlet and the positive electrode at the exit. It is necessary, the solute would simply migration of migration is reversed, the solutes would simply migrate into the source vial and not go through the capillary and detector.