CHAPTER I

INTRODUCTION

1.1 Introduction to Capillary Electrophoresis

Capillary electrophoresis (CE) is electrophoretic separation of charged compounds in a small internal diameter capillary containing an electrolyte solution under the influence of an applied voltage. The separation mechanism is based on the difference in electrophoretic mobilities of charged analytes. CE is similar to chromatography in many ways, and most of the words used in chromatography are also found in CE. For example, resolution and efficiency are common to both techniques, and are defined in similar way. However, some terms are different. For example, in chromatography, a pump is used to drive the sample through the column; in CE, the sample migrates due to its electrophoretic mobility and electroosmotic flow under the electric field.

1.2 Basic Theories of CE

1.2.1 **Electrophoretic mobility** [Grossman and Colburn 1992, Foret *et al.* 1993]

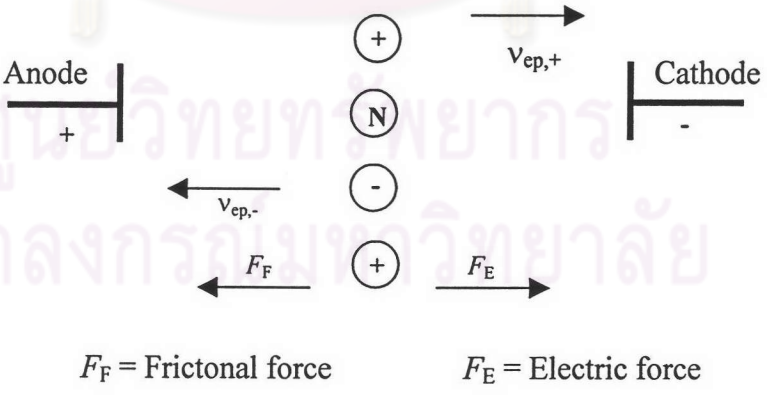


Figure 1.1 Migration behaviour of each species. Adapted from Foret *et al.* [1993].

Under the influence of electric field strength E ($E = V/L$, where V is the applied voltage across the capillary of length L) as shown in Figure 1.1, charged analytes migrate toward the electrode having opposite polarity with an electric force, F_E,

which is proportional to the electric field strength and the charge on the ion, q, (q = ze, where z is the charge of an ion, and e the electronic charge):

$$F_E = zeE \tag{1.1}$$

Meanwhile, the migration of the ion is resisted by the frictional force, F_F, due to the medium viscosity η (N s m^{-2})

$$F_F = 6\pi r_h \eta \nu_{ep} \tag{1.2}$$

where r_h is the hydrodynamic radius of an ion (m), and ν_{ep} the electrophoretic velocity (m s^{-1}). The acceleration of the ion will proceed until F_E is balanced by F_F, giving the equation

$$zeE = 6\pi r_h \eta \nu_{ep} \tag{1.3}$$

Rearrengement of Equation 1.3 gives

$$\nu_{ep} = \frac{ze}{6\pi\eta r_h} E = \mu E \tag{1.4}$$

The electrophoretic velocity of the ion in the electric field strength of 1 V m^{-1} is defined as **electrophoretic mobility**, μ (m^2 V^{-1} s^{-1}).

$$\mu = \frac{\nu_{ep}}{E} = \frac{ze}{6\pi\eta r_h} \tag{1.5}$$

From Equation 1.5, it can be seen that, under a particular medium, μ depends on the ratio of the charge and the size of an ion, z/r_h. The electrophoretic mobility is the property of charged analytes under given conditons, depending on charge density of the analyte, ionic strength and viscosity of the electrolyte, and temperature.

1.2.2 **Electroosmotic flow** [Grossman and Colburn 1992, Foret *et al.* 1993]

In CE, the migration of analytes in the presence of the electric field depends on not only their electrophoretic mobilities, but also electroosmotic flow (EOF). The EOF, as shown in Figure 1.2, is the movement of a medium toward the electrode when the voltage is applied. In the presence of the background electrolyte (BGE) at pH > 2, silanol groups at the surface of the fused-silica capillary ionise as the equation below, resulting in the negative charges of the capillary and excess positive ions in the solution.

$$\text{-SiOH}\ (s) \rightleftharpoons \text{-SiO}^{-}\ (s) + \text{H}^{+}\ (aq)$$

These positive ions arrange themselves into a double electric layer as illustrated in Figure 1.3. Some positive ions are attached at the negative surface of the capillary to form an imobilised layer, called *the compact or Stern layer*, held by electric forces. Some positive ions from *the diffusion layer*, and the rest of the excess positive ions is in the bulk solution.

The electric potential at the capillary-BGE interface, ψ_o, decreases linearly with increasing distance in the Stern layer and exponentially in the diffuse layer. With the electric potential ψ_d at the interface between the diffuse layer and Stern layer, the distance from the capillary surface where the potential is equal to $0.37\psi_d$ is defined as the thickness of double layer (δ). The electric potential at the plan of shear, located just outside the interface between the Stern and diffuse layer, is called zeta potential (ζ).

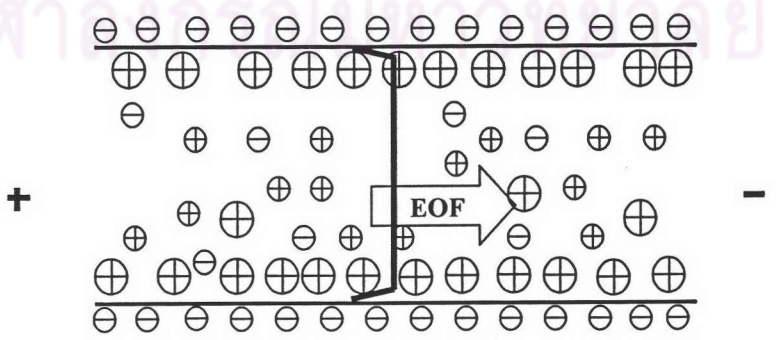


Figure 1.2 Electroosmotic flow (EOF). Adapted from Andrea *et al.* [1997].

When the electric field is applied across the capillary, excess solvated positive ions in the diffuse layer and bulk solution migrate toward the cathode. This results in a flow of water molecules in the same direction. This phenomena is called *electroosmosis*, and the movement of water or solvent is called *electroosmotic flow* (EOF).

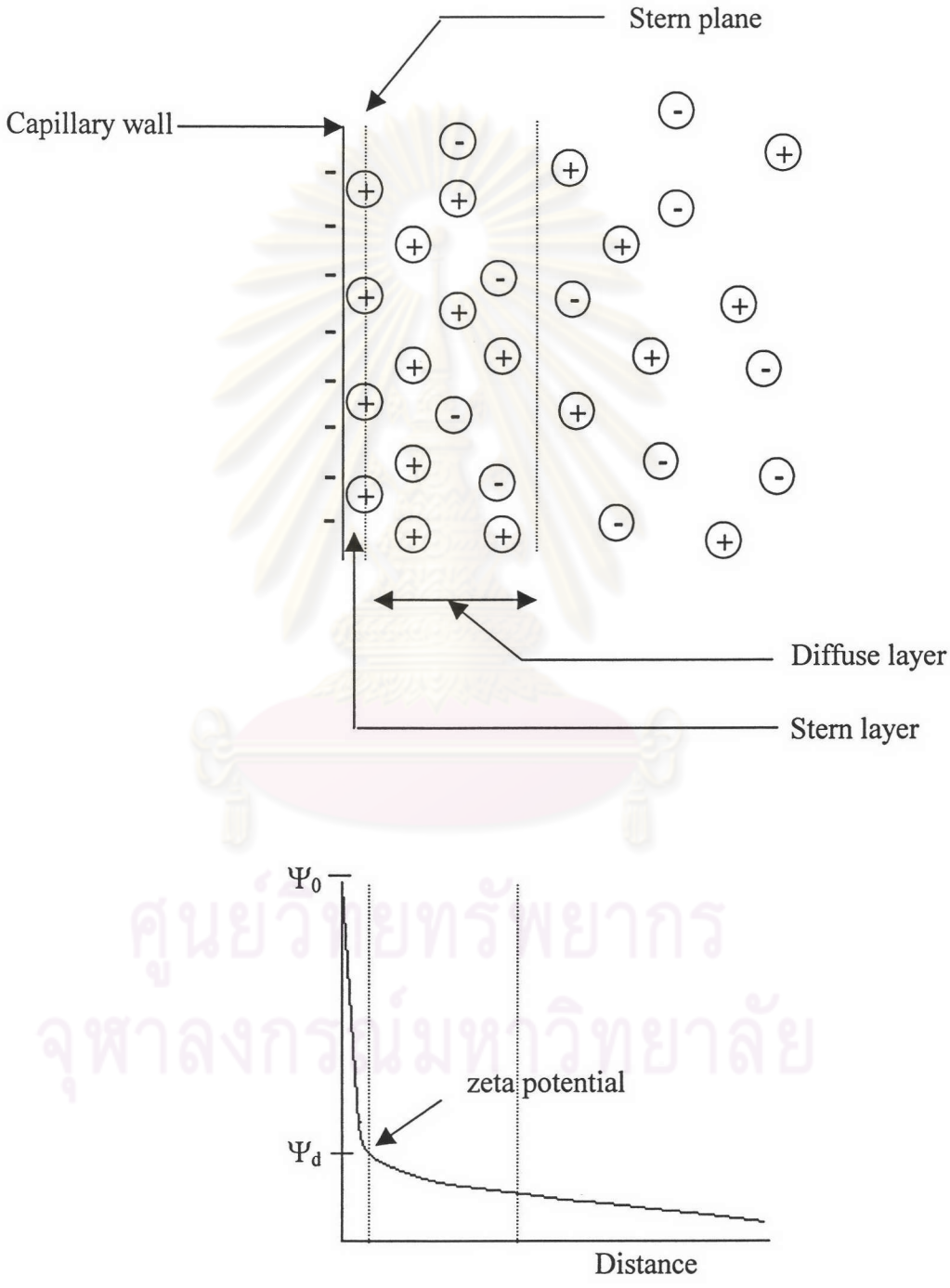


Figure 1.3 A model of a double electric layer and electrical potential. Adapted from Hiemenz *et al.* [1997].

From the internal capillary surface, the electroosmotic velocity increases with increasing distance, and is constant at the distance of approximately 15 nm from the wall [Grossman and Colburn 1992]. Typically, the capillary used in CE has 20 to 100 μm I.D. (20000 to 100000 nm). Thus, it can be said that the electroosmotic velocity is constant throughout the capillary radius. The electroosmotic velocity (ν_{ep}) is proportional to the zeta potential, as given by

$$\nu_{eo} = -\frac{\varepsilon\zeta}{4\pi\eta}E \tag{1.6}$$

where ε and η are the permittivity and the viscosity of the liquid in the double layer. These values may be different from those in the bulk solution [Kenndler 1998]. From Equation 1.6, dividing ν_{ep} by E gives **electroosmotic mobility** (μ_{eo} m^2 V^{-1} s^{-1}) as Equation 1.7.

$$\mu_{eo} = -\frac{\varepsilon\zeta}{4\pi\eta} \tag{1.7}$$

1.2.3 Flow profile and migration behaviour of analytes in CE

Since EOF is generated at the capillary wall, and the driving force of EOF is uniformly distributed along the capillary, a flow of bulk solution and analytes in CE has a flat profile which is contrast to the parabolic profile generated by laminar flow driven by a pressure gradient in HPLC, as shown Figure 1.4. Consequently, the peak width of the analyte in CE is typically narrower than that in HPLC, resulting the better peak efficiency in CE than that in HPLC.

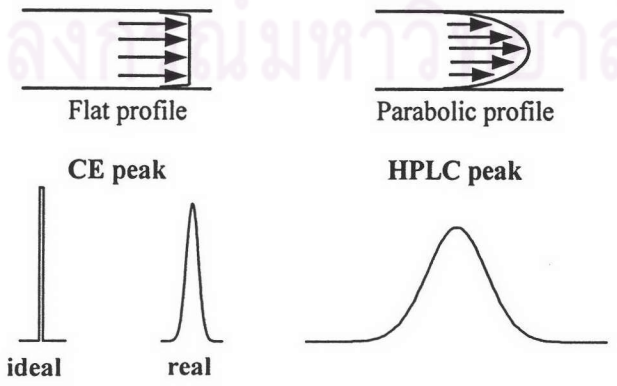


Figure 1.4 Flow profiles and peaks in CE and HPLC. Adapted from Chankvetadze [1997].

In the presence of the EOF, the net velocity, v_{net}, of the analyte is the sum of the electrophoretic velocity of the analyte and the electroosmotic velocity as Equation 1.8 and Figure 1.5:

$$v_{net} = v_{ep} + v_{eo} \tag{1.8}$$

In the presence of high EOF, both anions and cations migrate to the detection window. For cations, $v_{ep,+}$ and v_{eo} have the same direction to the cathode at the detection window. The higher the ion charges and the smaller the ion size, the faster the migration toward the cathode. For anions, $v_{ep,-}$ has the direction toward the anode. In the case where $v_{eo} > v_{ep,-}$, the anions can migrate to the cathode. The higher the ion charges and the smaller the ion size, the smaller the net velocity. Neutral molecules migrate toward the cathode only due to EOF, and cannot be separated.

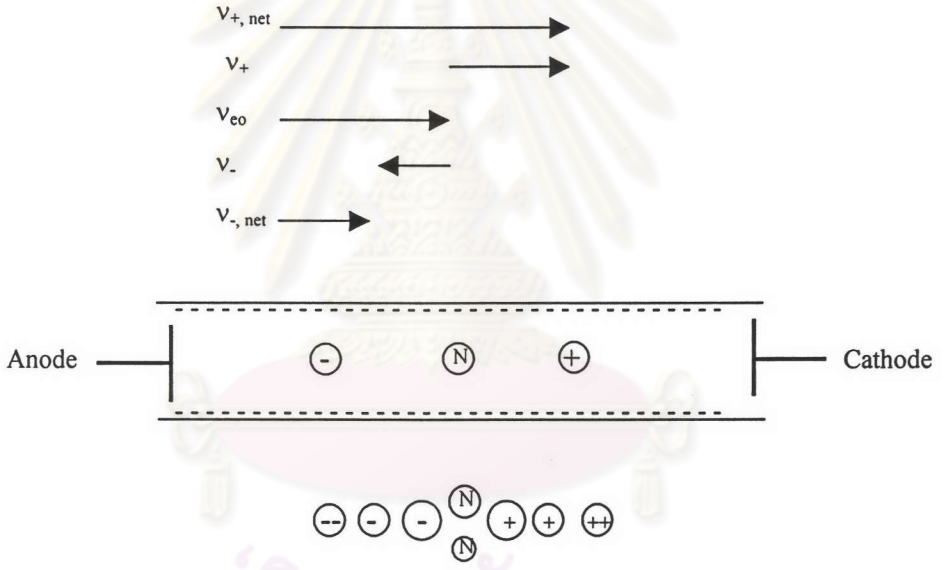


Figure 1.5 Migration behaviour of analytes. Adapted from Li [1992].

The net electrophoretic mobility ($\mu_{net} = \mu + \mu_{eo}$), μ_{net} and μ_{eo} can be calculated from an electropherogram using the following equations:

$$\mu_{net} = \frac{v_{net}}{E} = \frac{lL}{Vt_m} \tag{1.9}$$

$$\mu_{eo} = \frac{v_{eo}}{E} = \frac{lL}{Vt_{eo}} \tag{1.10}$$

$$\mu = \mu_{net} - \mu_{eo} = \frac{lL}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right) \tag{1.11}$$

where t_m and t_{eo} are the migration times of the analyte and the EOF marker, respectively, and l the length of the capillary to detector. Other parameters are previously mentioned.

1.2.4 Peak shape, efficiency and resolution in CE

Theoretically, when the solute migrates from the injection end to the detector both chromatographic and electrophoretic peaks are assumed to have a Gaussian shape with standard deviation, σ, in distance units or τ, in time unit as shown in Figure 1.6. The peak width at base, w_b, is equal to 4σ or 4τ. The peak width, w_h, at half height is given by [Dyson 1990]

$$w_h = 2.354\sigma \text{ or } w_h = 2.354\tau \tag{1.12}$$

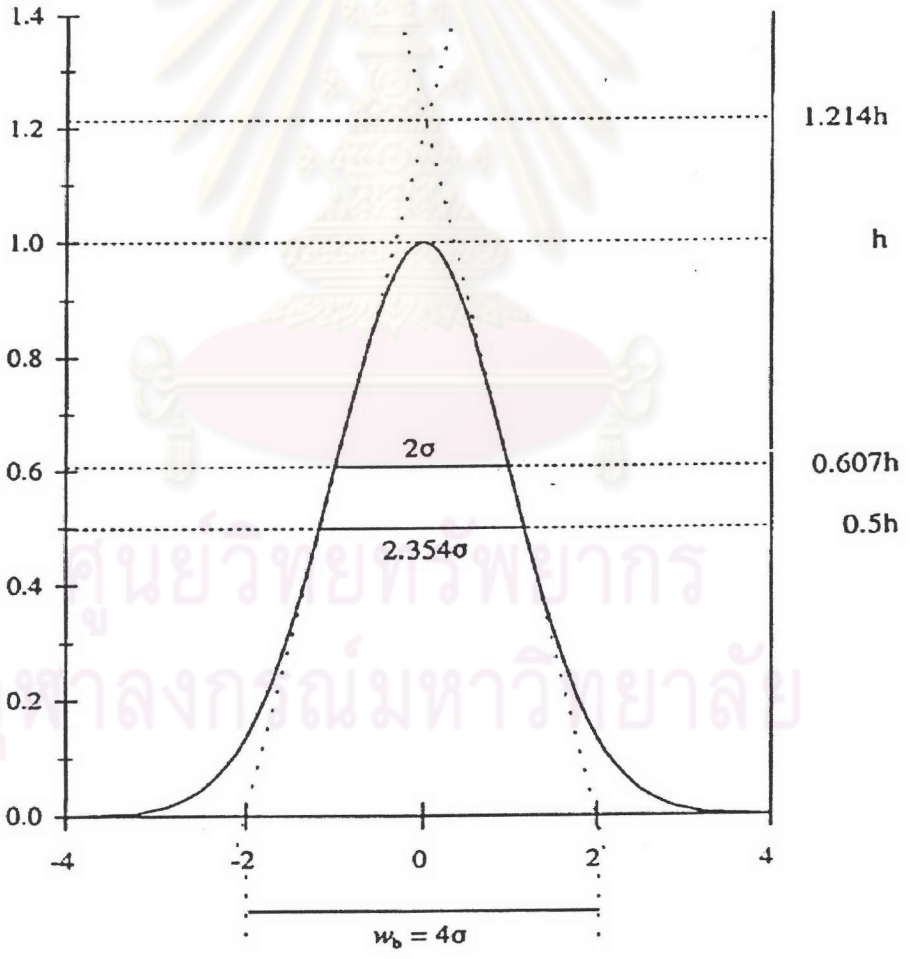


Figure 1.6 Gaussian peak. Adapted from Dyson [1990].

Peak efficiency in CE can be expressed similarly to that in HPLC in terms of the number of theoretical plates, N, which is equal to the square of the ratio of t_m or l to the standard deviation of peak

$$N = \left(\frac{t_m}{\tau}\right)^2 = \left(\frac{l}{\sigma}\right)^2 \tag{1.13}$$

N can be determined directly from the electropherogram using Equation 1.14 or 1.15

$$N = 5.54\left(\frac{t_m}{w_h}\right)^2 \tag{1.14}$$

$$N = 16\left(\frac{t_m}{w_b}\right)^2 \tag{1.15}$$

Resolution, R_s, is a measure of the degree of separation between two closed peaks. The resolution is defined as the ratio of the difference in their migration times to the average of their peak widths at base.

$$R_s = \frac{\Delta t_m}{0.5(w_1 + w_2)} \tag{1.16}$$

where w_1 and w_2 are peak widths at baseline for analytes 1 and 2, respectively. The resolution can be also calculated from the peak width at half height:

$$R_s = \frac{1.177\Delta t_m}{(w_{h,1} + w_{h,2})} \tag{1.17}$$

In CE, the resolution is related to the mobilities and the average number of theoretical plates $\overline{N}$ according to Equation 1.18

$$R_s = \frac{1}{4}\frac{\Delta\mu}{(\overline{\mu} + \mu_{eo})}\sqrt{\overline{N}} \tag{1.18}$$

where $\Delta\mu$ and $\overline{\mu}$ are the difference in the mobility and the average electrophoretic mobilities of the analytes, respectively.

1.2.5 Peak dispersion in CE [Grossman and Colburn 1992, Khaledi 1998]

The factors affecting the peak dispersion or band broadening include longitudinal diffusion, thermal dispersion, electromigration dispersion, injection length, detector width, wall adsorption and others. The total peak variance (σ^2) is given by the sum of the variances due to each contribution.

1.2.5.1 Longitudinal diffusion

When analyte migrates along the capillary, the diffusion of the analyte always occurs in the *x*-axial direction, causing concentration gradient $c(x,t)$, as shown in Equation 1.19

$$c(x,t) = \frac{Q_{inj}}{S\sqrt{4\pi Dt}} \exp\left(\frac{-(x-\bar{x})^2}{4Dt}\right) \tag{1.19}$$

where Q_{inj} is the amount of analyte injected, D the diffusion coefficient, and S the cross-sectional area of the capillary. This equation causes the Gaussian shaped peak having peak variance equal to

$$\sigma^2_{diff} = 2Dt \tag{1.20}$$

where t is the migration time t_m. Substituting t_m (Equation 1.9) in Equation 1.20 gives

$$\sigma^2_{diff} = \frac{2DlL}{(\mu + \mu_{eo})V} \tag{1.21}$$

with

$$D = \frac{\mu^\circ RT}{zF} = \frac{\mu^\circ kT}{ze} \tag{1.22}$$

where R is the gas constant, and k the boltzmann's constant.

1.2.5.2 Thermal dispersion

When the high voltage is applied across the capillary containing an electrolyte solution, heat is generated and called Joule heating. If heat transfer is less than its production, the temperature inside the capillary will increase. As a result, the temperature at the center of the capillary is higher than any other area due to the radial temperature gradient in the capillary. This problem influences a parabolic profile of sample zone causing band broadening. The peak variance from thermal dispersion is expressed by

$$\sigma_{th}^2 = 2D_{th}t_m \tag{1.23}$$

with

$$D_{th} = \frac{f_T^2 \kappa_c^2 E^6 r_c^6 \mu^2}{3072\lambda_s^2 D} \tag{1.24}$$

where D_{th} is the thermal dispersion coefficient, κ_c the electrical conductivity of the electrolyte solution, λ_s the thermal conductivity of the electrolyte solution, and f_T the temperature factor (0.02 to 0.03). From Equations 1.20 and 1.24, an increase of the electric field strength causes thermal dispersion, but reduces the longitudinal diffusion. In the absence of EOF, the optimum electric field strength giving the minimum sum of peak variance due to thermal dispersion and diffusion is given by using Equation 1.25 [Grossman and Colburn 1992]

$$E_{opt} = \frac{2.92}{r_c}\left(\frac{\lambda_s D}{\mu f_T \kappa_s}\right)^{\frac{1}{3}} \tag{1.25}$$

1.2.5.3 Electromigration dispersion

Electromigration dispersion (EMD) occurs due to lower conductivity of the BGE zone than that of the sample zone and mismatch of the electrophoretic mobilities between the analyte ion A and the BGE co-ion C. The BGE co-ion has the same charge as the analyte. For example, when the cationic analyte has higher μ than does the BGE co-ion, the cationic analyte in the leading edge of the sample zone will migrate faster into

the BGE zone which has lower conductivity or higher local electric field strength ($\nu_{ep} \propto E$ as shown in Equation 1.4), resulting the fronting triangular distribution in the distance domain, as shown in Figure 1.7a. When the cationic analyte has lower μ than does the BGE co-ion, the BGE co-ion will migrate faster into the tailing edge of the sample zone, resulting in the slower migration of the analyte in the tailing zone and the tailing triangular distribution of the analyte. In the presence of higher EOF and normal polarity, this situation is reversed when anions are analyzed instead of cations [Chankvetadze 1997, Nhujak 2001b]. Figure 1.8 shows the effect of electromigration dispersion on the analyte concentration distribution in the presence of diffusion. It is noted that the peak in the time domain is the mirror image of that in the distance domain.

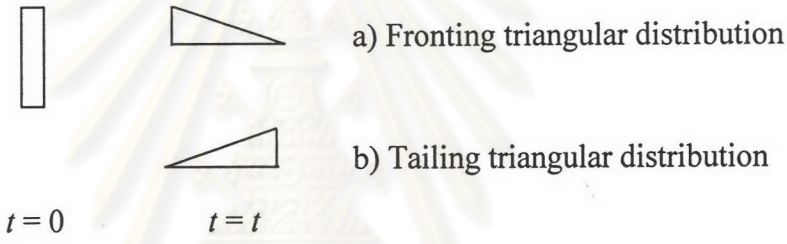


Figure 1.7 Concentration distribution of EMD in the distance domain. Adapted from Chankvetadze [1997].

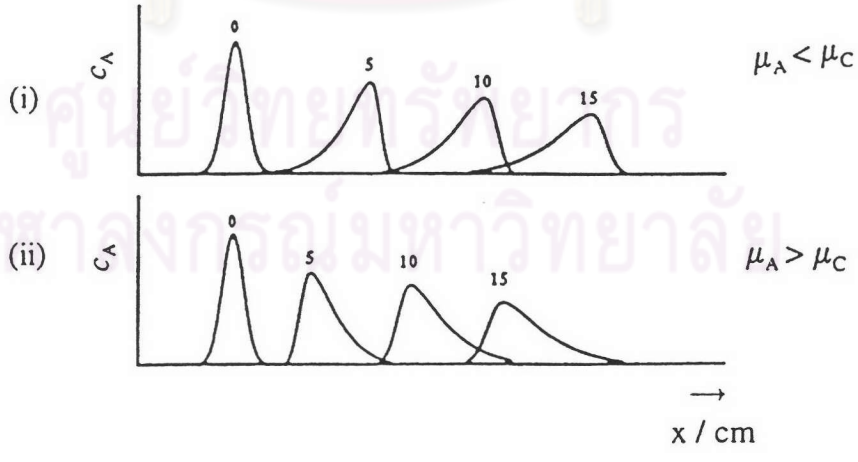


Figure 1.8 Schematic diagram illustrating concentration distribution in the presence of EMD. Evalution (i) tailing and (ii) fronting peaks in the distance domain as a function of time (min). Reproduced from Kuhn and Hoffstetter-Kuhn [1993].

The peak variance from EMD is given by [Erny *et al.* 2001]

$$\sigma^2_{EMD} = \left|\frac{2}{9} l l_{inj} c_A a_A \right| \left| \frac{\mu_A}{\mu_A + \mu_{eo}} \right| \tag{1.26}$$

with

$$a_A = \frac{\left(\frac{\mu_A}{\mu_C} - \frac{\mu_B}{\mu_C}\right)\left(1 - \frac{\mu_A}{\mu_C}\right)}{\frac{\mu_A}{\mu_C}\left(1 - \frac{\mu_B}{\mu_C}\right)c_C} \tag{1.27}$$

where subscripts A, B, and C are the analyte ion, the counter-ion BGE and the co-ion BGE, respectively, c_i the concentration of species i, and l_{inj} the injection length.

1.2.5.4 Detection width

For the use of UV-vis detector in CE, the shape of detection width is the rectangular with 200-800 nm width. The wider the detection width, the broader the peak shape. The wide detection width gives high sensitivity, but sometimes it affects the lose of resolution. The peak variance of the detection width can be expressed as the following:

$$\sigma^2_{det} = \frac{l^2_{det}}{12} \tag{1.28}$$

where l_{det} is the detection width.

1.2.5.5 Injection length

The peak variance from injecton length can be calculated from

$$\sigma^2_{inj} = \frac{l^2_{inj}}{12} \tag{1.29}$$

For the sample stacking as mentioned in Section 1.3.2.2, the injection length is decreased by a factor γ_{st}, thus the peak variance due to the injection length decreases as

$$\sigma_{inj}^2 = \frac{l_{inj}^2}{12\gamma_{st}^2} \tag{1.30}$$

1.2.5.6 Wall adsorption

The wall adsorption of analytes is caused by ionic interactions between analyte ions and the opposite charged capillary wall, leading to the tailing peak. To reduce solute-wall interactions, the capillary wall may be coated by dynamic or permanent coating. An increase of buffer concentration can reduce the wall adsorption, but may increase Joule heating.

1.3 CE Instrumentation

1.3.1 Basic apparatus of CE

The schematic of CE instrument is illustrated in Figure 1.9. A CE system consists of a high voltage power supply allowing voltages up to 30 kV, two electrodes (commonly platinum wire), a capillary column with 10-200 μm I.D. and 20-100 cm in length, a detector mostly used UV-vis detector, and a cooling system for controlling temperature of the capillary and reducing Joule heating.

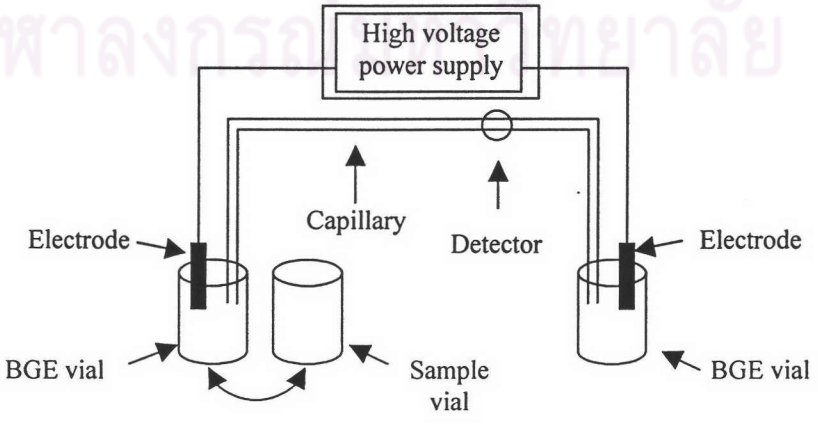


Figure 1.9 Basic schematic of CE instrument. Adapted from Weinberger [1993]

For the simple analysis, BGE is filled into the capillary. Then, the inlet BGE vial is replaced by the sample vial, and a sample solution is introduced into the capillary. After that, the high voltage is applied to the capillary. Analytes migrate and are separated because of the difference in their electrophoretic mobilities. Finally, the analytes will migrate to the detector.

1.3.2 Sample introduction

1.3.2.1 Sample injection methods [Khaledi 1998]

The two common methods of sample introduction into the capillary are electrokinetic and hydrodynamic injection. The electrokinetic injection is carried out by placing the inlet end of capillary in the sample vial and the outlet end in the buffer vial. A voltage is then applied for a short period of time, t_{inj}. Consequently, the analyte ions enter the capillary due to electrophoretic migration and electroosmotic flow of a solution in the capillary. The length of sample plug (l_{inj}) for electrokinetic injection is given by

$$l_{inj} = (\nu_{ep} + \nu_{eo})t_{inj} = (\mu + \mu_{eo})Et_{inj} \tag{1.31}$$

Hydrodynamic injection mostly used in CE injection can be classified into gravity (rising the sample vial the outlet vial for a fixed interval time), pressure (applying a pressure to the sample vial), or vacuum (applying a vacuum to the outlet vial). The most common approach is pressure injection. The length of sample plug injected into the capillary by pressure injection is given by

$$l_{inj} = \nu_{hf} t_{inj} = \frac{\Delta P r^2}{8\eta L} t_{inj} \tag{1.32}$$

where t_{inj} is the injection time. Thus, the volume (V_{inj}) and quantity (Q_{inj}) of the sample injected can be calculated from the following equations

$$V_{inj} = \frac{\Delta P \pi r^4}{8\eta L} t_{inj} \tag{1.33}$$

and $$Q_{inj} = \frac{\Delta P \pi r^4}{8\eta L} t_{inj} c \quad (1.34)$$

where c is the analyte concentration.

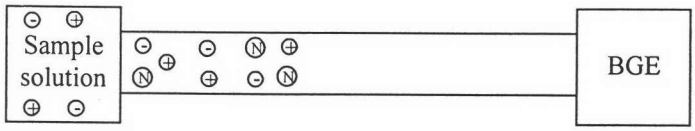
1.3.2.2 Sample stacking [Burgi and Chien 1991]

Since CE is a microanalytical separation technique, only small quantities of sample can be introduced into the capillary. Therefore, detection limit and sensitivity in CE are poorer than those in HPLC. A simple and cheap technique usually used to enhance CE sensitivity is on-column sample stacking. Typically, the sample stacking is carried out by dissolving the analyte with the diluted BGE. At the beginning of application voltage for separation, the electric field strength in the sample zone dramatically increases. This causes a large increase of electrophoretic velocity of the analytes ($v_{ep} \propto E$ as in Equation 1.4). For normal polarity of the applied voltage and high EOF, cationic analytes move rapidly and stack up at the front of the sample zone, whilst anionic analytes move and stack up to the inlet end of capillary, as shown in Figure 1.10. The effective sample zone length, l_{st}, after stacking is reduced by a factor γ_{st}, leading to an increase of the analyte concentration after stacking (c_{st}) by a factor γ_{st}.

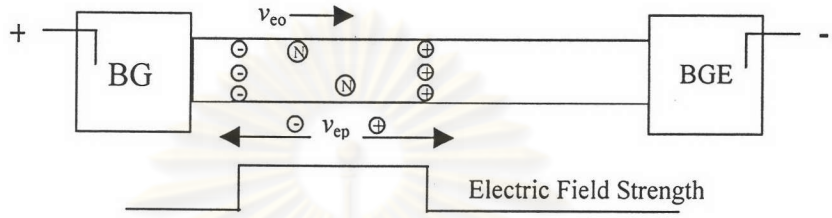
$$l_{st} = \frac{l_{inj}}{\gamma_{st}} \text{ and } c_{st} = \gamma_{st} c \quad (1.35)$$

For sample dissolved in diluted BGE, the γ_{st} can be estimated from the ratio of the conductivity between the BGE and the sample solution. However, diluted BGE of a sample solution causes mismatch between EOF in the sample plug and the BGE zone. This leads to laminar flow and a parabolic profile of the analyte zone, resulting in peak broadening. For the minimum peak variance due to the injection length and EOF mismatching, the analytes dissolved in ten times diluted BGE is recommended [Burgi and Chien 1991]

(a) Sample Loading



(b) Sample Stacking



(c) CZE separation

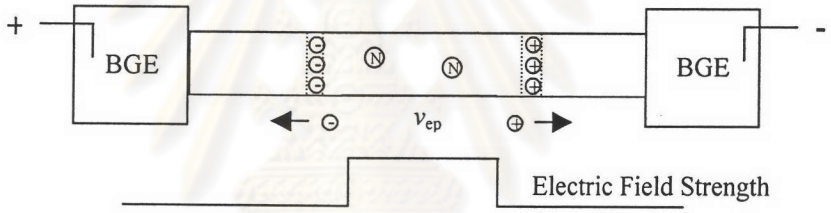


Figure 1.10 Sample stacking. Adapted from Khaledi [1998].

1.3.3 Detection [Khaledi 1998, Swinney and Bornhop 2000]

Detectors used in CE have been adapted from detection techniques used in HPLC, especially a UV-vis detector. For on-column UV-vis detection, a small section of polyimide coating near the outlet of the capillary is removed to form a cylindrical window serving as the detector cell. The window is positioned in the path of the light beam, and the analytes are detected when they flow through this window. Fluorescence detection is also used in CE due to its high sensitivity. Since not all compounds possess high fluorescence, the derivatization must be required for fluorescence detection. In addition, other types of detectors used in CE include electrochemical detectors, mass spectrometry (CE-MS), and nuclear magnetic resonance (CE-NMR). In this research, UV detection is used for analysis of gibberellic acid.

1.4 Modes of CE

The modes of CE are classified by the different separation mechanisms allowing CE techiques to be used for a wide variety of substances. These modes include

Capillary zone electrophoresis (CZE)

Micellar electrokinetic chromatography (MEKC)

Capillary electrochromatography (CEC)

Capillary gel electrophoresis (CGE)

Capillary isoelectric focusing (CIEF)

Capillary isotachophoresis (CITP)

The first two techniques, CZE and MEKC, used in this research are briefly discussed. Figure 1.11 shows separation mechanism in CZE and MEKC.

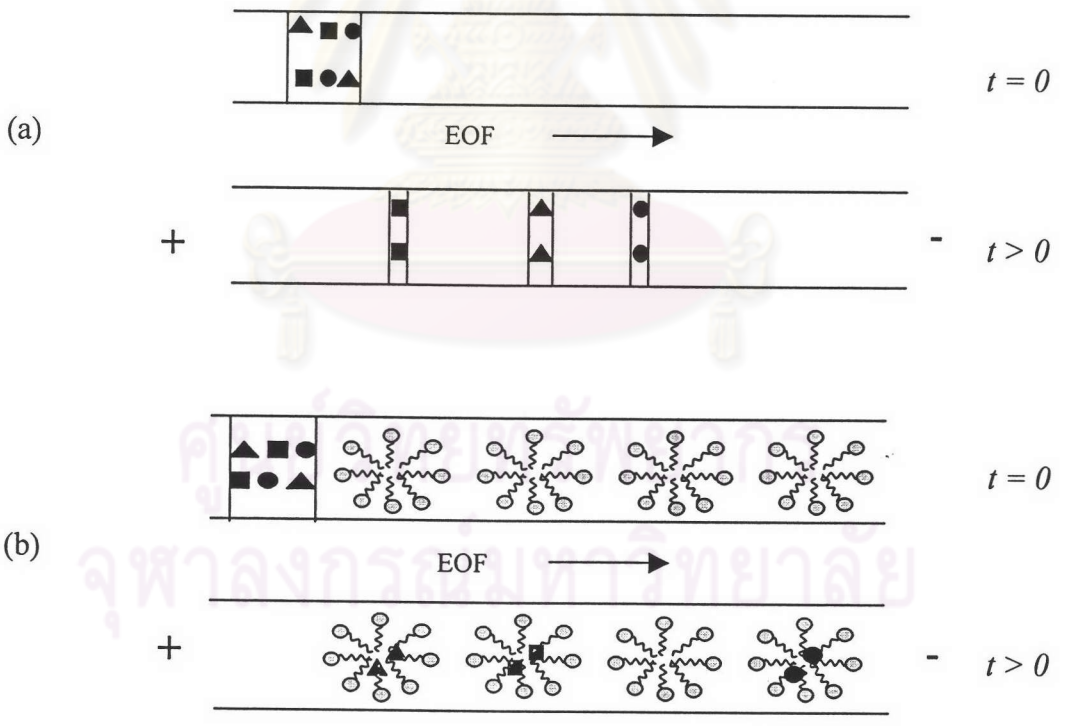


Figure 1.11 Separation mechanisms in (a) CZE and (b) MEKC. Adapted from Andrea and Brown [1997].

Capillary Zone Electrophoresis [Li 1992, Weinberger 1993] is the simplest and most widely used mode in CE. The BGE used in CE is common buffer such as phosphate and borate with or without organic solvent. The separation mechanism of CZE, shown in Figure 1.11a, is based on the difference in electrophoretic mobilities of the analytes due to the difference in the charge to size ratio of analyte (z/r as shown in Equation 1.5). When an electric field is applied, each species of analyte ions migrates in discrete zones toward the electrode of the opposite charge due to the electrophoretic mobility. In the presence of high EOF, both anions and cations migrate to the detection window. Neutral molecules migrate due to EOF, as previously mentioned in Section 1.2.3.

Micellar Electrokinetic Chromatography was introduced by Terabe *et al.* [1984]. MEKC is one of the modes of CE that is capable to separate uncharged compounds [Khaledi 1998, Patrick 1993]. In MEKC, sufactant, containing a long alkyl chain as the hydrophobic group and a polar or ionic head group as shown in Figure 1.12a, is added to the BGE. When concentration of the surfactant exceeds the critical micelle concentration (CMC), the surfactant monomers aggregate in an aqueous solution to form micelles, as shown in Figure 1.12b, that have spherical in shape which the hydrophobic tails of the surfactants are oriented toward the center and the polar head groups face outward into the electrolyte solution. The micelles serve as the micellar phase as pseudostationary phase. Thus, the separation mechanism of MEKC depends on the differential partitioning of the analytes between the micellar phase and the aqueous phase, as shown in Figure 1.11b, that is similar to reversed-phase HPLC.

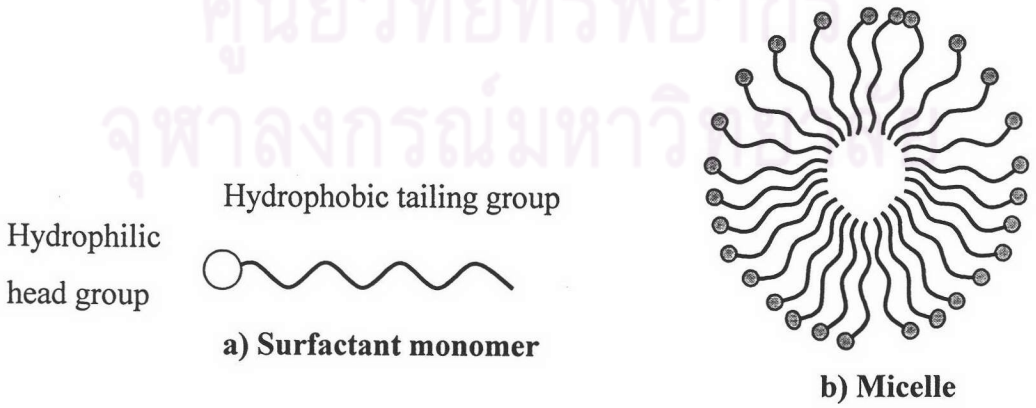


Figure 1.12 Schematic illustration of surfactant and micelle. Adapted from Khaledi [1998].

Sodium dodecyl sulfate (SDS) is the common surfactant widely used in MEKC due to no UV absorption, low CMC, available high purity, low cost and similar pseudostationary phase to C_8 and C_{18} stationary phase in HPLC. The SDS micelles have negative charge and migrate toward the positively charged electrode due to their electrophoretic mobility. Since the EOF is usually higher than the electrophoretic mobility of the micelles, eventually, the micelles are draged by the EOF toward the detector (at cathode electrode). When analytes are injected into the capillary, they partition between the micellar and aqueous phase. The analytes that spend in the micellar phase for long time will reach to detector late. Therefore, in MEKC using SDS as surfactant, analytes will migrate between the EOF marker and the micelle.

A typical elution order is shown in Figure 1.13, where t_m, t_{eo} and t_{mc} are the migation times of solute, EOF marker and the micelle, respectively. A peak of EOF represents a neutral molecule that has no interaction with the micelles, thus it will migrate at the velocity of the EOF. A micelle peak is a peak of the analyte mostly retained in micellar phase. It can be seen that the separation mechanism in MEKC is similar to that of the chromatographic technique. Therefore, the equations for the solute retention and resolution used in chromatography are also used in MEKC.

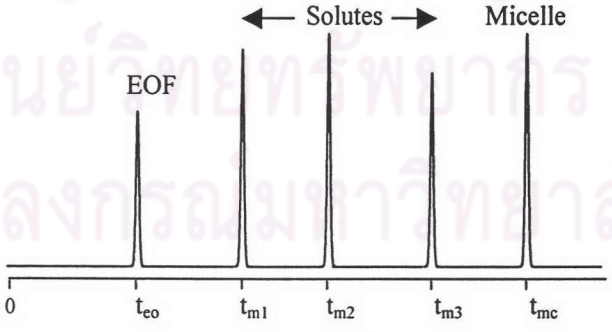


Figure 1.13 Typical elution order for analytes in MEKC. Adapted from Andrea and Brown [1997].

The retention factor, k', in MEKC is defined as the ratio of the amount of the analyte in the micellar phase (n_{mc}) to that in the aqueous phase (n_{aq}).

$$k' = \frac{n_{mc}}{n_{aq}} \tag{1.36}$$

k' may be calculated from an electropherogram using the equation

$$k' = \frac{t_R - t_0}{t_0\left(1 - \frac{t_R}{t_{mc}}\right)} \tag{1.37}$$

where t_R is the retention time of analyte and t_0 the migration time of the analyte due to electrophoretic mobility and EOF in BGE without micelle. The electrophoretic mobility of analyte:micelle complex is assumed to be equal to the electrophoretic mobility of the micelle (μ_{mc}). Therefore, the observed electrophoretic mobility of analyte ($\mu_{obs,A}$) is equal to

$$\mu_{obs,A} = x_{aq}\mu_A + x_{mc}\mu_{mc} \tag{1.38}$$

where x_{aq} and x_{mc} are mole fractions of analyte in aqueous and micellar phase, respectively. From Equations 1.36 and 1.38, $\mu_{obs,A}$ is given by the equation

$$\mu_{obs,A} = \frac{1}{1+k'}\mu_A + \frac{k'}{1+k'}\mu_{mc} \tag{1.39}$$

The resolution equation in MEKC for analytes migrating closely together is similar to that in conventional chromatography:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1+k'_{avg}}\right)\left(\frac{1 - \frac{t_0}{t_{mc}}}{1 + \left(\frac{t_0}{t_{mc}}\right)k'_{avg}}\right) \tag{1.40}$$

where α is the separation factor defined as the ratio k'_2 / k'_1. Although MEKC was originally developed for the separation of neutral compounds, it also enhances resolution for analysis of charged species.

1.5 Quantitative Analysis in CE

1.5.1 Peak area and corrected peak area [Mayer 2001]

The quantitative analysis in CE is obtained by using peak area which provides a greater linear response with respect to the amount of analyte. Sometimes the peak heigth can be used accurately for quantitative analysis when low concentration of sample is determined. This is because the higher the concentration of analyte, the higher the band broadening due to EMD causing a lower in peak height than it would be. Thus, peak height is not proportional to the amount of analyte at high concentration of the analyte.

In chromatography, the analytes eluting from the packed column move through the detector at the identical velocities driven by the constant flow rate of mobile phase. In contrast to chromatography, different analytes in CE do not pass the detection window with identical velocity because the analytes migrate with the different electrophoretic mobolities and identical EOF. The slowly migrating analyte remains longer in the detection window than does a rapidly migrating analyte, and gives detector response for long time, resulting in larger peak area. In multiple runs at a given CE conditions, a change in the migration time of the analyte may be due to alterations in the EOF, temperature and other parameters. Thus, in quantitative analysis in CE, corrected peak area, A_{corr}, defined as the peak area (A) divided by the migration time, is usually used.

$$A_{corr} = \frac{A}{t_m} \tag{1.41}$$

1.5.2 Internal standard [Grossman and Colburn 1990, Mayer 2001]

In HPLC and GC quantitative analysis, the internal standard is widely used to correct any error that may occur during the manual injection or sample preparation. For quantitative analysis in CE, the internal standard is mostly used to correct intrumental imprecision, primarily due to the injection process. In this work, sample introduction is carried out using pressure injection. It can be seen from Equation 1.33 that the

volume of the analyte injected by pressure is related to pressure difference, injection time and viscosity. It is noted that the viscosity parameter in Equation 1.13 is the average viscosity of a solution from the thermostatted section of the capillary and the unthermostatted sections in the vials [Nhujak 2001b]. Therefore, imprecision in the quantity of the analyte injected may be caused by the imprecision in the pressure difference and injection time and variation of the viscosity of a solution in unthermostatted sections of the capillary resulted from a change of ambient temperature. In addition, the variation of sample solution levels also results in the difference in the quantity of the analyte injected. Therefore, the internal standard should be used to compensate these errors. The fixed amount of internal standard is added into both sample and standard solutions. A calibration plot is established using the A_{corr} ratio of the standard and internal standard as a function of the standard concentration.

1.6 Quantitative Analysis of Gibberellic Acid

Gibberellic acid (GA_3) is a diterpenoid compound in a class of gibberellins, and its structure is shown in Figure 1.14. GA_3 is a plant hormone that promotes seed germination, stem elongation, premature flowering and cone production, and retards leaves and fruit senescence. GA_3 is also a metabolic product of the fungus *Gibberella fujikuroi*. Traditionally, GA_3 is produced by submerged fermentation by employing *Gibberella fujikuroi* [Perez *et al.* 1996, Kumar and Lonsane 1986 and Kumar and Lonsane 1986].

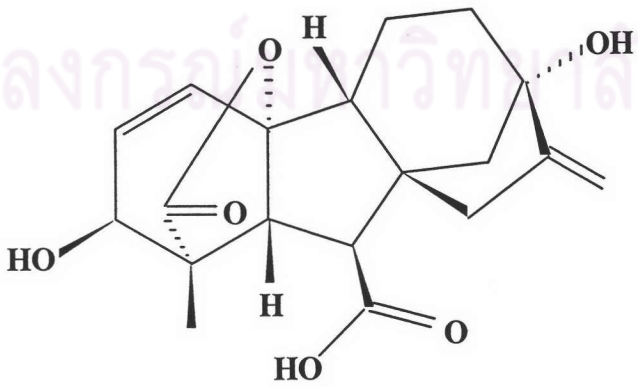


Figure 1.14 Structure of GA_3.

Conventional techniques used for determination of GA_3 and other plant hormones include spectrophotometry [Perez *et al.* 1996], fluorometry [Kavanagh and Kuzel 1958], high performance thin layer liquid chromatography (HPTLC) [Kumar and Lonsane 1986], gas chromatography (GC) [Du *et al.* 2000, Tuomi and Rosenqvisth 1995] and high performance liquid chromatography HPLC [Dupreez *et al.* 1993, Rotunno *et al.* 1999, Barendse *et al.* 1980]. Both spectrophotometry and fluorometry are non-separation techniques, and suffer interference of matrix for GA_3 analysis. In comparison with GC and HPLC, HPTLC is cheaper, but gives poorer resolution of GA_3 and other compounds, leading to inaccuracy of the amount of GA_3 determined. Since GA_3 is a non-volatile compound, it needs to be converted to be a volatile derivative for GC analysis. Therefore, GC is not a suitable choice for determination of the large amount of GA_3 in a sample. Among conventional techniques mentioned, HPLC is the most suitable technique for separation and analysis of GA_3. However, sample preparation, especially sample clean up, is required prior to injection into an HPLC column to avoid deposition of unwanted substances in the HPLC column.

Capillary electrophoresis is a powerful analytical tool and an alternative technique to HPLC. Advantages of CE over HPLC include high speed, high efficiency and resolution, low or no consumption of organic solvent, the small amount of sample requirement, advanced automation and no need of sample cleaning up. Two CE modes used for determination of GA_3 and other plant regulators have been reported to be CZE and MEKC. Yeo *et al.* [1992] were the first to develop CE for separation of GA_3 and other eight plant growth regulators, using a phosphate-borate buffer at pH 8.09 [Yeo *et al.* 1992]. However, baseline resolution of some plant hormones was not achieved. Improvement of resolution was carried out by using cyclodextrins and cholic acid as pseudo stationary phase. In addition, CZE was used for determination of GA_3 and other plant regulators in plant tissues such as *Chara vulgaris* thallus [Kazmierczak 1999a, 1999b, 2001] and banana leaves [Zheng *et al.* 1999]. The amount of GA_3 in *Chara vulgaris* determined by either CZE or bioassay was found to be in good agreement. MEKC using borate as a running buffer and sodium dodecylsulphate as micellar phase [Liu *et al.* 2002] was developed for determination of GA_3 and other plant hormones in tobacco flowers. Detection sensitivity was improved by using a bubble cell capillary to increase cell path length or large volume sample stacking as an on-line pre-concentration.

1.7 Aims and Scope

The institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University, has produced GA_3 by means of fermentation using the *Gibberella fujikuroi* fungus [Sukcharoen 1990 and Samappito 1994]. The commercial GA_3 product of IBGE is called CU Gibb as shown in Figure 1.15. During the fermentation process, the amount of GA_3 in the fermentation broth has to be determined for investigation of the process and efficiency of the fungus. A method currently used for quantitative determination of GA_3 in the fermentation broth is HPLC. Due to the fermentation broth containing substrates, products, metabolite by-products and cells of micro-organisms, liquid-liquid extraction with ethylacetate is used to remove unwanted substances that may be deposited in an HPLC column [Samappito 1994]. The disadvantages of the liquid-liquid extraction include consumption of organic solvent and time and GA_3 loss during extraction.

The aims of this work are to develop CE as a method for quantitative determination of GA_3 in fermentation broth and commercial products and to compare CE and HPLC for determination of GA_3. In previous work, CE was conducted for determination of GA_3 and other hormones in plant tissues [Yeo *et al.* 1992, Kazmierczak 1999a, 1999b, 2001, Zheng *et al.* 1999, Liu *et al.* 2002]. The CE method has not been previously reported for determination of GA_3 in fermentation broth and commercial products.

In this work no sample preparation, except for dilution and filtration, of GA_3 in fermentation broth is carried out for CE analysis. CZE and MEKC will be used in this work using a pH 9.2 $Na_2B_4O_7$ solution as BGE, an uncoated fused silica capillary and separation temperature 25 °C. For CZE analysis, opimisation of CZE conditions such as the BGE concentration and voltage for separation will be investigated. In MEKC, SDS is used as pseudostationary phase, and opimisation of MEKC conditions such as the SDS and BGE concentrations and voltage for separation will be studied. Validation of CE will be investigated on accuracy, precision, limit of detection and limit of quantitation. In addition, CE and HPLC will be compared for quantitative analysis of GA_3 in fermentation broth. Moreover, a developed CE method will be

used for determination of GA_3 in commercial GA_3 products. It is expected that CE could be an alternative technique to HPLC for determination of GA_3 in fermentation broth and commercial GA_3 products.

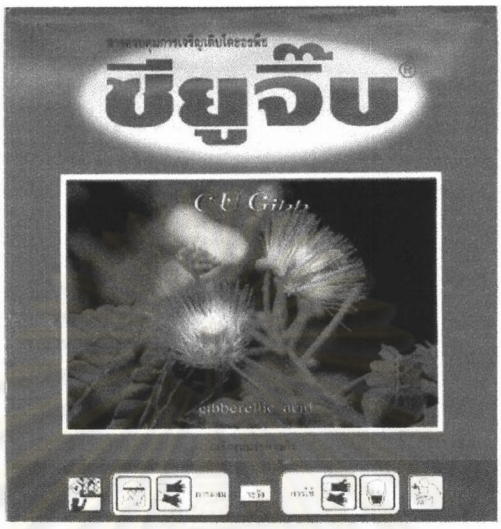


Figure 1.15 *CU Gibb.* (product of IBGE, Chula).