CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and instruments as shown in appendix A and B

2.2 Preparation of *Centella asiatica* extract

Fresh whole plants of *Centella asiatica* were purchased from local markets in Amphur Muang, Chiang Mai, Thailand. After washing with tap water, they were cut up into small pieces, and minced with distilled water at the ratio of 1:2 (weight:volume ; g:ml) by a blender. The mixture was soaked in an ice bath for 4 hours and then centrifuged at 4,000 g at 0°C for 15 min. The supernatant was filtered through filter paper (Whatman no.1), the filtrate was freeze dried using a lyophilizer, and stored at -20°C until used. The scheme of the extraction is shown in Figure 5.

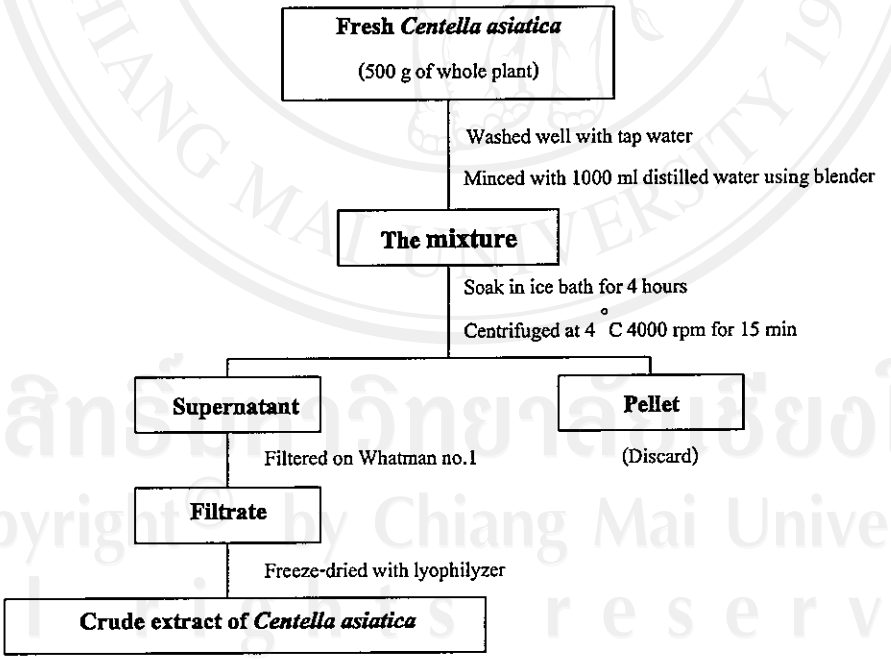

Figure 5 Preparation of *Centella asiatica* extract

2.3 Effect of *Centella asiatica* extract on aflatoxin B_1-albumin adduct formation after a single dose of aflatoxin B_1 exposure.

2.3.1 Animals

Male Wistar rats, weighing 70-90 g, were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The rats were allocated into five groups of 39 animals each, and were housed three per cage. The temperature ($26\pm1^{\circ}C$), humidity ($50\pm10\%$), and lighting (12 hr. day/night cycle) were constantly controlled. The rats were acclimated to the diet and environment for a period of one week after their arrival before beginning the experiment.

Table 1 Protocol for *Centella asiatica* extract treatment and AFB_1 administration

Group	Treatment		
	Day 1-5	Day 6	Day 7-11
1	Distilled water, daily	40 μg AFB_1/kg body weight Distilled water	Distilled water
2	10 mg CA extract/kg bodyweight (low dose), daily	10 mg CA extract/kg bodyweight 40 μg AFB_1/kg body weight	10 mg CA extract/kg bodyweight (low dose), daily
3	100 mg CA extract/kg bodyweight (high dose), daily	100 mg CA extract/kg bodyweight 40 μg AFB_1/kg body weight	100 mg CA extract/kg bodyweight (high dose), daily
4	Distilled water, daily	40 μg AFB_1/kg body weight 10 mg CA extract/kg bodyweight	10 mg CA extract/kg bodyweight (low dose), daily
5	Distilled water, daily	40 μg AFB_1/kg body weight 100 mg CA extract/kg bodyweight	100 mg CA extract/kg bodyweight (high dose), daily

C. asiatica extract was fed by gavage 8.00 a.m. after 9.00 a.m., the rats were fed by gavage with AFB_1. Water was fed *ad libitum*.

2.3.2 The study of effects of *Centella asiatica* (CA) extract on aflatoxin B_1-albumin adduct formation in rats.

The rats were randomly divided into five groups of 36 rats. The first group was fed with distilled water only. The second and third group received 10 mg/kg body weight (low dose) and 100 mg/kg body weight (high dose) of CA extract, respectively, before and after aflatoxin B_1 (40

μg/kg body weight) treatment. The last two groups received low dose and high dose CA extract, respectively, after aflatoxin B_1 treatment. As shown in Table 1, three rats from each group were sacrificed by cervical dislocation 0, 2, 4, 6, 8, 12, 18, 24, 36, 48, 72, 96 and 120 hours after aflatoxin B_1 treatment. Blood samples were collected and serum obtained was quantified for the aflatoxin B_1-albumin adducts by using competitive ELISA.

2.3.2 Aflatoxin-albumin adduct analysis

The level of aflatoxin-albumin adducts was determined by the ELISA method developed by Wild *et al.* (1990). Albumin was isolated from 0.5 ml of serum by precipitation and hydrolyzed with proteinase K. The hydrolysate was then passed through a Sep-Pak C18 cartridge and the bound AFB_1-albumin adduct was purified and analyzed. The analysis is shown schematically in Figure 6.

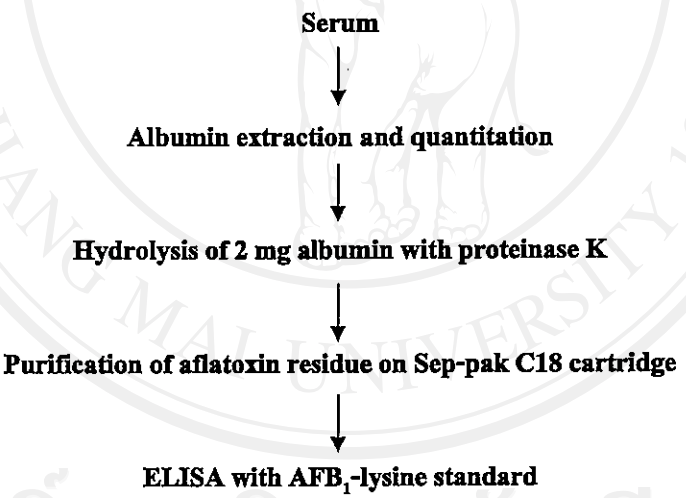

Figure 6 Diagram of AFB_1-albumin adduct analysis by ELISA

2.3.3.1 Albumin extraction

Five hundred microlitres of cold sera were slowly combined with 1.5 volume of cold (4°C) saturated ammonium sulfate. The mixture was vortexed and centrifuged at 9,000 g, 0°C for 15 min to remove precipitated immunoglobulins. The supernatant containing the albumin was transferred to the other tube and 100 μl of 1 M acetic acid was added to adjust the solution to pH 5 and precipitate the albumin. The precipitate was collected by centrifugation at 9,000 g, 0°C for

15 min. The albumin was redissolved in 500 ml of PBS pH 7.4. The extraction protocol is shown in Figure 7.

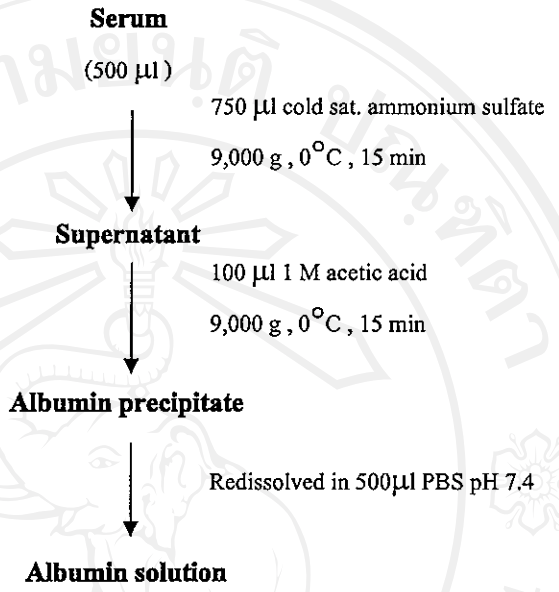

Figure 7 Scheme of albumin extraction

2.3.3.2 Protein quantification

Albumin was quantified by a standard protein Coomassie dye binding assay using bovine serum albumin standards. Albumin solution was generally diluted 1:50 in distilled water for protein analysis and assayed as follows: 20 µl of albumin solution was diluted to 1.0 ml with distilled water and 100 µl of diluted sample was placed into a test tube. A standard calibration curve was prepared (Table 2).

Table 2 Preparation of standard calibration curve

Standard albumin, (µl) (500 µg/ml)	0	20	40	60	80	100
Distilled water, (µl)	100	80	60	40	20	0
Corresponding to the following in mg albumin/ml serum	0	5	10	15	20	25

Coomassie Blue reagent was added into the tube. The mixture was vortexed gently. After 15 min, the optical density was measured at 595 nm using a spectrophotometer. The albumin content of the sample was calculated from the standard calibration curve.

2.3.3.3 Albumin hydrolysis prior to ELISA

Two milligrams of albumin from the prepared albumin solution were digested with 0.67 mg of proteinase K in a total volume of 0.8 ml PBS pH 7.4. The mixture was incubated in a water bath at 37°C for 15 hours. Ten milligrams of bovine serum albumin (100 mg/ml stock solution in PBS) were added to improve precipitation of proteinase K and any remaining incompletely hydrolyzed albumin. Proteins were then precipitated by the addition of two volumes of cold ethanol and samples were placed at -20°C for 2 hours. Precipitated proteins were removed by centrifugation at 1000 g, 0°C for 15 min. The supernatant was removed and diluted to a final volume of 30 ml with PBS pH 7.4. This was essential to reduce the final ethanol concentration to 5% in order that the AF residues could bind to the Sep-pak C18 cartridge. The albumin hydrolyzation protocol is shown in Figure 8.

2.3.3.4 Sep-Pak C18 purification

At first the cartridge was activated with 5 ml of 80% methanol and washed with 10 ml double-distilled water. Then 30 ml of the hydrolysate from 2.3.3.3 was loaded onto the cartridge. The cartridge was washed with 5 ml of double-distilled water and 5 ml of 5% methanol. Then the AFB_1-residue was eluted by 5 ml of 80% methanol and dried using a Speed Vac concentrator. The AFB_1-lysine was reconstituted in 0.5 ml PBS pH 7.4 with 1% fetal calf serum and quantified by ELISA.

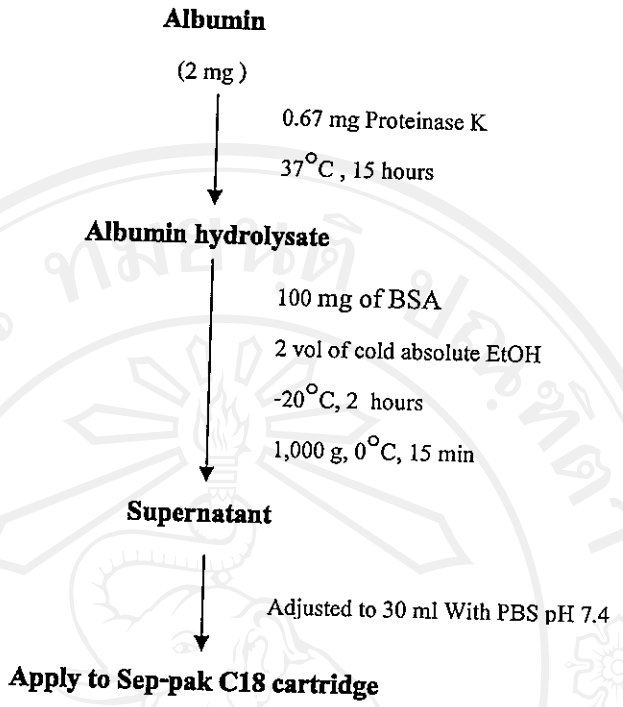

Figure 8 Scheme for albumin hydrolysis

2.3.3.5 Quantitation of AFB_1-lysine adduct by competitive ELISA

The immunoassay used is a competitive ELISA using AFB_1-ovalbumin as the coating antigen and AFB_1-lysine as the inhibitor for generation of standard curves.

2.3.3.5.1 Plate coating

Maxisorp plates (Nunc, immunoplate) were coated with 2.5 ng of AFB_1-ovalbumin conjugate in PBS pH 7.4.

2.3.3.5.2 ELISA for AFB_1-lysine adduct

The coated plates were washed 6 times with PBS-Tween. One hundred microlitres of 5% skim milk powder were added to each well and the plates were incubated at room temperature for 60 min in the dark. Then the plates were washed 6 times. After that 25 µl of test samples were added to each well and quantified relative to a standard AFB_1-lysine inhibitor control (0% inhibition and blank). The standard AFB_1-lysine inhibitors, (0.5-6 fmole/25 µl of sample) were analyzed in each plate for generation of standard curve in each assay. Then 25 µl of optimum

dilution of antiserum were added to each well. The plates were incubated at room temperature in the dark for 90 min on an ELISA plate shaker. The plates were washed 6 times with PBS-Tween. Then fifty microlitres of goat anti-rabbit IgG enzyme conjugate was added into each well. Plates were incubated at room temperature in the dark for 90 min, washed 6 times with PBS-Tween followed by one wash in double-distilled water and dried. Then fifty microlitres of TMB substrate solution were added into each well. The reaction was terminated by addition of HCl and the absorbance was read at 450 nm. The percentage of inhibition for the samples were calculated as described in 2.3.3.5.3

2.3.3.5.3 Calculation

The percent inhibition of antibody binding to immobilized antigen was calculated using the following equation:

$$\%Inhibition = \left[1 - \left[\frac{ODs - ODb}{ODc - ODb}\right]\right] \times 100$$

whereas :

OD_s = The mean absorbance value for sample

OD_c = The mean absorbance value control

OD_b = The mean absorbance value blank

The values for the sample were derived from the standard inhibition curve (obtained from the values of standard AFB_1-lysine inhibitors). A percent inhibition value of 20% was considered as the limit for detection of a positive sample, and equivalent to 1.00 fmole/0.1 mg albumin or 5 pg/mg albumin. Each sample was assayed in triplicate.

2.3.3.6 Statistics

The significance of differences in AFB_1-lysine adduct level in each group were analyzed by a Mann-Whitney U test.

2.4 Effect of *Centella asiatica* extract on aflatoxin B_1-metabolism after multiple dose of aflatoxin B_1 exposure.

2.4.1 Animals

Male Wistar rats, weighing 70-90 g, were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The rats were allocated into five groups of 30 animals, and were housed five per cage. The temperature (26±1°C), humidity (50+10%), and lighting (12 hour day/night cycle) were constantly controlled. The rats were acclimated to the diet and environment for a period of one week after their arrival before beginning the experiment.

2.4.2 The study of effects of *Centella asiatica* extract on aflatoxin B_1-albumin adduct formation and liver function in rats after multiple dose AFB_1 exposure.

The experiment was designed as shown in Figure 9, and lasted for 28 weeks. In the control group (Group I) and the group given *Centella asiatica* (CA) extract only (Group II), rats were gavaged with distilled water or high dose of the extract (100 mg/kg bodyweight /day, daily), respectively, throughout the experiment. In the group given AFB_1 alone (Group V) or together with the high dose of the extract (Group III) or together with low dose (100 mg/kg bodyweight /day, daily) of the extract (Group IV), rats were first given distilled water or the extracts, respectively, via gavage for 4 weeks followed by administration of AFB_1 (400 mg/kg bw, once a week by gavage) or the extract plus AFB1 for another 24 weeks. Five rats from each group were scarified within 24 hours after 4, 8, 12, 16, 20 and 24 doses of AFB_1. Blood samples were collected from the portal vein for isolation of serum then stored at -20°C until analysis. Serum AFB_1-albumin adducts were determined as in 2.3.3.5. Livers were removed, washed with ice cold 0.9% NaCl and weighed. Liver tissue was homogenized in buffer A without Triton X-100, then stored at -80°C until analysis for 8-OHdG.

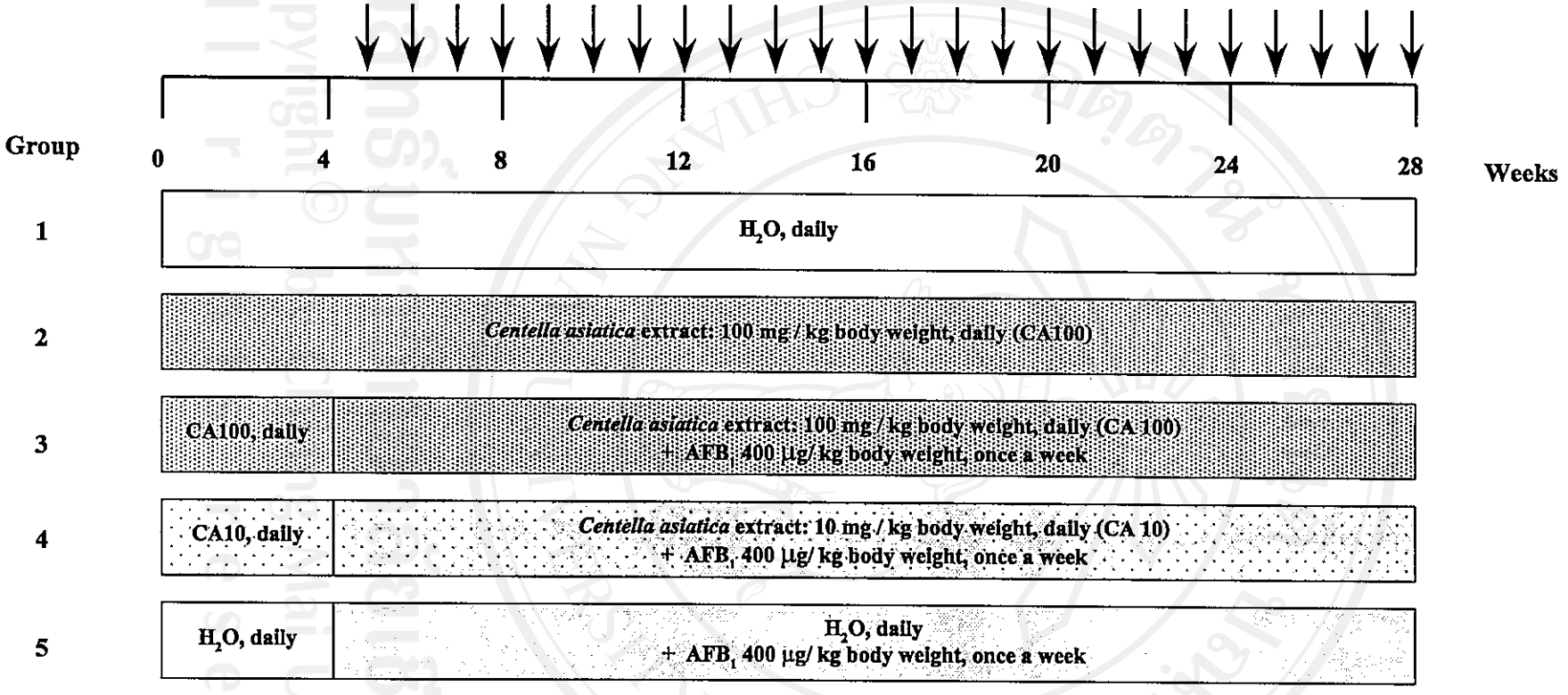

Figure 9 Schematically treatment protocol for studying effect of *C. asiatica* on AFB_1-metabolism in multiple dose of AFB_1-treated rat.

↓ AFB_1 400 µg/kg body weight, by gavage

2.4.3 Determination of serum γ-glutamyl transpeptidase (GGT) by kinetic photometric method

γ-Glutamyl transpeptidase (GGT) is one of a large group of enzymes known as peptidases. A peptidase is an enzyme that catalyzes the hydrolytic cleavage of peptides to form amino acids or smaller peptides. GGT catalyzes the transfer of γ-glutamyl groups from peptidases or peptide like compounds to an acceptor peptide molecule. Although renal tissue has the highest level of GGT, the major source of the enzyme present in serum is of hepatic origin. Elevated levels of GGT are found in association with hepatobiliary and pancreatic disorders; in alcoholics and heavy drinkers, in patients with myocardial disorders, and in diabetics (Tietz, 1976).

Methods for determining GGT are based on the use of glutamyl derivatives of aromatic amines as the substrate (Tietz, 1986). Orlowski and Meiser introduced γ-glutamyl-*p*-nitroanilide as a substrate in 1963 (Orlowski *et.al.*, 1963). To increase the speed of reaction, glycylglycine was added (Kulhanek *et.al.*, 1966).

2.4.3.1 Principle of the method

The γ-glutamyl group is transferred from *L*-γ-glutamyl-*p*-nitroanilide to glycylglycine in the presence of GGT in the serum. The change in the absorptivity of *p*-nitroaniline at 405 nm under the specified conditions is proportional to the GGT activity in the sample.

2.4.3.2 Procedure

One millilitre of GGT reagent was pre-warmed for 5 min at 37°C. The instrument was adjusted to zero at 405 nm with distilled water. Serum was added to the reagent. The mixture was incubated at 37°C for 30 seconds. After 30 seconds preincubation, Absorbance (A_1) was recorded. The tube was returned to the 37°C incubator. After exactly one minute, absorbance (A_2) was again recorded.

2.4.3.3 Definition of enzyme unit

One international unit (IU) of GGT activity is the amount of enzyme that transfers 1 μmole of glutamate per minute per liter of sample with the concurrent release of 1 μmole of *p*-nitroaniline during the specific interval under the specific conditions of the procedure.

2.4.3.4 Calculation

The activity of GGT calculated by the following equation

$$\text{GGT activey} = \frac{\Delta A/\text{min} \times 10^3 \times TV \times 1000}{\varepsilon \times SV \times LP}$$

$\Delta A/\text{min}$	=	Change in absorbance per minute
10^3	=	Conversion of millimoles to micromoles
TV	=	Total reagent volume (1.05 ml)
ε	=	Molar absorptivity of p-nitroaniline at 405 nm (9.9×10^3 liters/mole$\times$ cm)
SV	=	Sample volume (0.05 ml)
LP	=	Light path (1 cm)

Thus

$$\text{GGT activey} = \frac{\Delta A/\text{min} \times 10^3 \times 1.05 \times 1000}{9.9\times 10^3 \times 0.05 \times 1}$$

2.4.4 Measurement of liver DNA damage

8-Hydroxy-2 -deoxyguanosine (8-OHdG) is one of the major oxidative damage products and the most commonly measured marker of oxidative DNA damage. Capillary electrophoresis offers an alternative to liquid chromatography for measuring 8-OHdG concentration in biological samples.

2.4.4.1 Capillary electrophoresis

Capillary electrophoresis (CE) is a high-resolution analytical technique that has been applied to a wide variety of different types of molecules. The potential value of this technique for analysis of biopolymers, such as proteins, peptides, and nucleic acids, is widely recognized (Khur, 1990; Novotny, 1990). There has been an increasing number of reports of CE being used to analyze attomole or zeptomole quantities of analyte (Linhardt, 1994; Novotny and Sudor, 1993; Oefner and Chiesa, 1994; Oefner *et al.*,1994). In addition to its high sensitivity, CE has several advantages over a variety of other analytical methods, including an extremely high separation efficiency; online detection; simple operation; short analysis time; automated and reproducible analysis; and very low consumption of sample and buffers.

2.4.4.2 Principle

The unique characteristics of CE are that the separation is obtained by differential migration of solutes in an electric field and electrophoresis is performed in narrow-bore capillaries filled with electrolyte (Oda *et al.*, 1997). As shown in Figure 10, a principle of capillary electrophoresis was described. Migration of samples in CE is driven by two forces including the electrophoretic migration and the electroosmotic flow (EOF). The EOF or 'bulk flow' results from the charge on the inner walls of the capillaries during application of an electric field. The fused silica capillaries contain silanol groups (pI=1.5), which are easily ionized under most conditions, resulting in the negative charge of the inner wall (Camilleri, 1998). Positively charged ions from the electrolyte are attracted to the negatively charged wall and an electrically charged double layer is formed. Upon applying a voltage, cations carrying water migrate toward the cathode. Therefore, an inner flow of the electrolyte toward the cathode is obtained. In a typical CE instrument, samples are introduced at the anode and are detected at the cathode; cations migrate first with the highest velocities toward the cathode by the combined effects of electrophoretic flow and EOF. Anions migrate last by the force difference of the EOF toward the cathode and the electrophoretic flow from the anode in the opposite direction. Neutral compounds migrate out from the capillary by the effect of EOF and are not well separated (Weston and Brown, 1997). The EOF flow causes the flat profile during a separation, thus the driving force of flow is uniformly distributed, without the pressure drop from the pump.

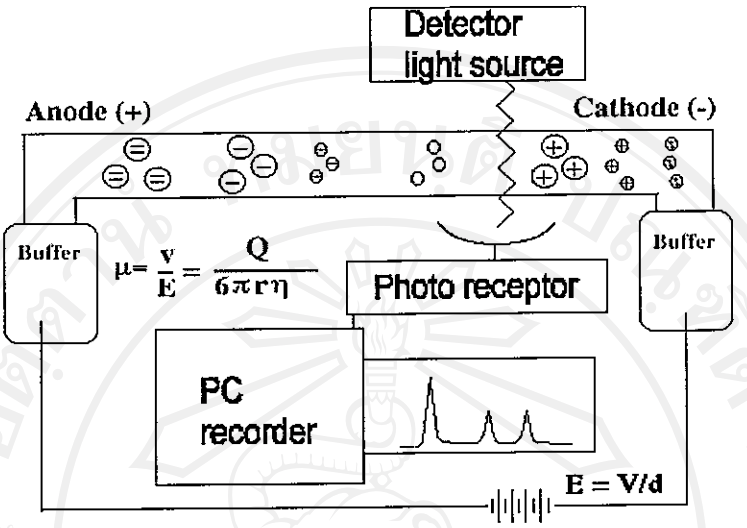

Figure 10 Principle of capillary electrophoresis

(Derived from http://chemi.muni.cz/~analytika/ce/cze.html)

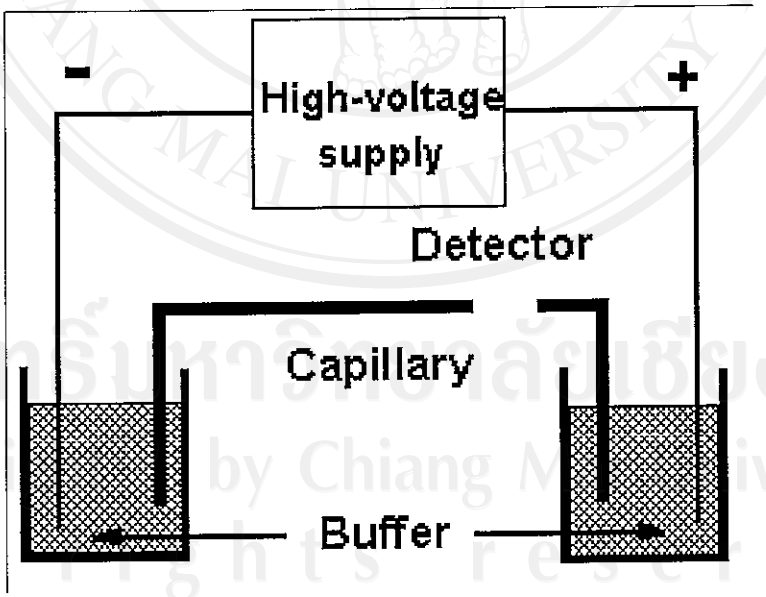

Figure 11 Schematic of capillary electrophoresis

(Derived from http://www.chemistry.adelaide.edu.au/external/soc-rel/content/cap-el.htm)

2.4.4.3 Instrument

The instrumentation of CE is shown in Figure 11, and consists of a capillary tube, electrolyte reservoirs, electrodes, detector and voltage power supply. Most capillary tubes are made of silica since it is inert and inexpensive. A typical tube is 25 75 cm long with an outer diameter of 300 400 m and inner diameter of 25 75 m. Sample injection in CE can be performed by hydrodynamic, electrokinetic injection and on-capillary sample concentration. Hydrodynamic injection is based on differences in pressure at the inlet and the outlet, which can be achieved by applying pressure at the inlet, applying vacuum at the outlet or by elevating the inlet (siphon effect). In electrokinetic injection, a low voltage of 5 10 kV is applied during injection. The injection voltage is typically 3 5 times lower than the separating voltage (Weston and Brown, 1997).

2.4.4.4 Mode of CE

Several modes of CE are available for separation of various types of samples: (1) capillary zone electrophoresis (CZE), (2) micellar electrokinetic chromatography (MEKC), (3) capillary gel electrophoresis (CGE), (4) capillary isoelectric focusing (CIEF), (5) capillary isotachophoresis (CITP), (6) capillary electrochromatography CEC) and (7) non-aqueous CE (Camilleri, 1998). CZE is the simplest and most versatile CE mode, in which the separation is based on differences in the charge-to-mass ratio and analytes migrate into discrete zones at different velocities (Weston and Brown, 1997). Anions and cations are separated by CZE due to electrophoretic migration and the EOF, while neutral species co-elute with the EOF. MEKC, a hybrid technique between electrophoresis and chromatography, is a CE mode in which surfactants above the critical micelle concentration (CMC) are added into the electrolyte to form micelles. Surfactants are molecules that contain both polar head groups (e.g. cationic, anion, neutral or zwitter ionic) and non-polar hydrocarbon tails. The hydrophilic polar head groups point outward, whereas the hydrophobic non-polar tails point toward the center of the micelles. During MEKC separation, non-polar portions of neutral solutes are incorporated into the micelles and migrate at the same velocity of the micelles, while the polar portions are free and migrate at the EOF velocity. The distribution coefficient between the micellar and non-micellar phase greatly influences the migration velocity of the analytes (Monning and Kenedy, 1994).

2.4.4.5 CE and Oxidative DNA damage

CE offers an alternative to liquid chromatography (LC) as separation is based on the charge-to-size ratio of the analyte rather than partitioning and it typically provides higher separation efficiency. Capillary electrophoresis with electrochemical detection (CEEC) allows high efficiency separation needed for complex biological samples as well as excellent selectivity and limit of detection afforded with electrochemical detection. CE also has been studied as a separation tool for analysis of DNA adducts (Geldart and Brown, 1998). CE is the most suitable method for separation of charged analytes, so adducts can be detected as nucleotides. Lin *et al.* employed CEEC for analysis of purine bases, ribonucleotides and ribonucleosides in plasma (Lin, 1997). Hadwiger *et al.* demonstrated that while quantification of isopropanol was not possible with CE-UV because of insufficient detection limits, a detection limit of 13 nM could be achieved using CEEC (Hadwiger, 1996). In addition, detection limit for catechol standards has been reported to be in the range of 2-5 nM (Park *et al.*, 1995; Park and Lunte, 1995). Nowadays, several modes of CE have been developed for 8-OHdG detection, including capillary electrophoresis with electrochemical detection with 50 nM as limit of detection (Weiss *et al.*, 2000), CE with end-column amperometric detection with 20 nM as the limit of detection (Mei *et al.*, 2003).

2.4.4.6 DNA isolation

Liver (~ 0.5 g) was homogenized in 3 volumes of buffer A. The homogenate was centrifuged at 1,500 g, 4°C for 10 min. The pellet containing nuclei was dispersed in buffer A (containing 1% Triton X-100) and centrifuged at 1,500 g, 4°C for 10 min. Afterward the nuclear pellet was dispersed in 600 µl of buffer B, and 35 µl of 10% SDS were added. Then RNA residues were digested by incubation (50°C, 15 min) with 3 µl of 100 mg/ml RNase A, after cooling to room temperature, 30 µl of protease (20 mg/ml) was added to the mixture. The mixture was gently vortexed for 10 sec, incubated at 37°C for 1 hour, then the mixture was transferred to a fifteen milliliters centrifuge tube, 1.2 ml of NaI solution were added, and the tube was vortexed vigorously for 30 second. Then 2 volumes of 100% isopropanol were added and gently inverted several times for mixing. This was followed by centrifugation at 5,000 g in 20°C for 5 min. The supernatant was discarded and the DNA pellet was washed with 40% isopropanol. Centrifugation

was performed at 5,000 g in 20°C for 5 min. The pellet was rinsed with 70% ethanol (at -20°C). Ethanol was removed as completely as possible and the pellet was partially dried in the tube for 5 min. The pellet was dissolved in 0.1 mM desferoxamine mesylate in an amount appropriate for hydrolysis. DNA solution was qualified and quantified by spectrophotometer. The solution was immediately stored at -80°C. The DNA extraction protocol is shown in Figure 12.

2.4.4.7 DNA hydrolyzation prior to capillary electrophoresis

About 100 μg of DNA was resuspended in 100 μl 20 mM sodium acetate, pH 4.8, and digested to nucleotides with 4 U of nuclease P_1 at 65°C for 12 min. The mixture was incubated at 37°C for 60 minutes. The hydrolyzed DNA solutions were centrifuged at 10,000 g for 10 min. The supernatant was removed and used for 8-OHdG determination by CE. The DNA hydrolyzation protocol is shown in Figure 13.

2.4.5.8 Quantitation of 8-OHdG by capillary electrophoresis

One hundred microlitres of DNA hydrolysate was separated on a P/ACE MDQ capillary electrophoresis system equipped with a photodiode array (PDA) detector (Beckman Instruments Inc., Fullerton, CA). A fused-silica capillary of 75 μm i.d. and 40 cm length to the detector (50.2 cm total length) was used. New capillaries were first rinsed with 1.0 M NaOH (5 min, 20 psi), followed by rinsing with the separation electrolyte buffer (10 min, 20 psi). The capillary was then left to equilibrate overnight in the separation electrolyte buffer prior to use. Each separation was preceded by a 2-min rinse with 0.1 M NaOH, followed by a 4-min rinse with the separation BGE. The sample was introduced using a low-pressure (0.5 psi) injection for up to 5 sec. All separations were carried out at 25°C, and absorbance detection was made with a Beckman P/ACE UV detector at 254 nm. Data were collected using the System Gold software (Beckman). The concentration ratio, 8-OHdG to dG, in each DNA sample was determined based on the peak area, in comparison with dose of known amounts of authentic 8-OHdG and dG. The result was expressed as 8-OHdG/10^3 dG.

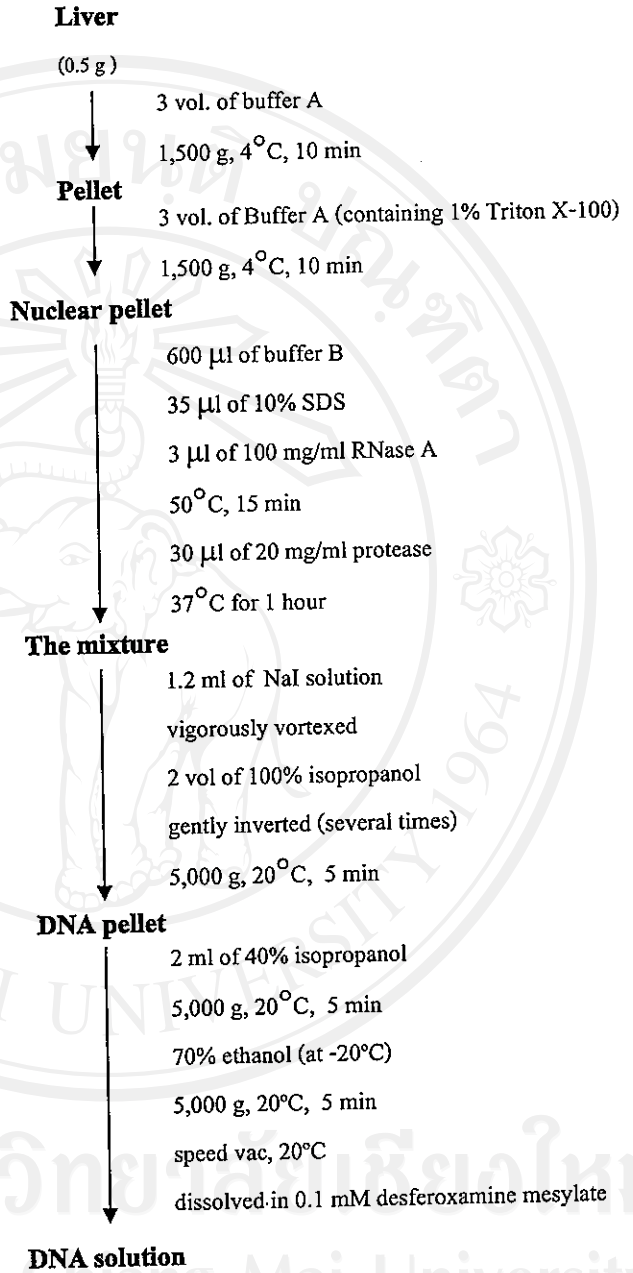

Figure 12 Scheme of DNA extraction

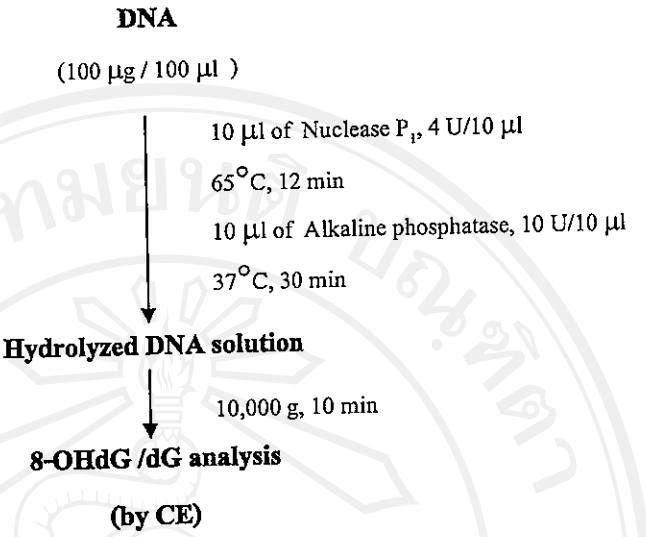

Figure 13 Scheme of DNA hydrolyzation