CHAPTER 3 MATERIALS AND METHODS

3.1 Plant Materials

Fresh broccoli (*Brassica oleracea* L.) heads were purchased from The Royal Project Foundation, Chiang Mai province, Thailand. Broccoli heads were transported to the laboratory at King Mongkut's University of Technology Thonburi (KMUTT), Bangkhuntien, Bangkok by refrigerated truck within 1 night. Broccoli heads were sorted on the same day to eliminate those that were damaged or misshaped, and then cut into individual florets, using disinfected knifes. After that, broccoli florets were washed with tap water for 2 min followed by removing excess water with manual salad spinner.

3.2 Experimental Designs

3.2.1 Experiment I: Study on the Effects of Sanitary Treatments on Microbial Growth and Visual Quality in Fresh-Cut Broccoli Florets during Cold Storage

3.2.1.1 Effect of Hot Water (HW) Treatment on Microbial Growth and Visual Quality in Fresh-Cut Broccoli Florets

Fresh-cut broccoli florets prepared as described in 3.1 were directly treated with hot water at 50, 55 and 60 °C for 3 min. The treated florets were then cooled in tap water at 4 °C for 5 min and any excess water was removed by manual salad spinner. The untreated florets were rinsed with tap water for 2 min. Samples of approximately 120 g each were packed in clamshell box (polyethylene terephthalate; PET) and kept at 4 °C until the produce was not acceptable by the consumer. Treated broccoli samples were randomly taken for analyzing microbial populations, physical changes and visual quality every 3 days during storage as compared with the untreated florets.

3.2.1.2 Effect of Vapor Heat (VH) Treatment on Microbial Growth and Visual Quality in Fresh-Cut Broccoli Florets

Fresh broccoli florets prepared as described in 3.1 were treated with steaming treatment at 90 °C for 15, 30 and 45 sec. The untreated florets were rinsed with tap water for 2 min. The treated florets were then cooled in tap water at 4 °C and all treated samples were removed excess water by manual salad spinner. Samples of approximately 120 g each were packed in clamshell box (PET) and kept at 4 °C until the produce was not acceptable by the consumer. Treated broccoli samples were randomly taken for analyzing microbial populations, physical changes and visual quality every 3 days during storage as compared with the untreated florets.

3.2.1.3 Effect of Ozonated Water on Microbial Growth and Visual Quality in Fresh-Cut Broccoli Florets

Ozonated water was prepared by using an ozone generator (Ozonizer Model B6APT, Bangkok, Thailand) with a 2,500 mg/hr ozone production rate. When the ozone generator was turned on, the ozone gas was produced and flowed out via the outlet rubber tube that connected with four pieces of blue ball airstone to generate a small bubble, that facilitate dissolving of gaseous ozone into a glass jar containing 6 L of tap water at room temperature (25±2 °C). In this experiment, the ozone generator was operated at 25 °C for 5, 10, and 15 min. The concentrations of ozone dissolved in water were measured immediately by the indigo colorimetric method as described by Eaton et al. (2005) (3.3.20). The ozone concentrations in water were calculated, they were 0.56, 1.00, and 1.50 ppm, respectively.

Fresh broccoli florets prepared as described in 3.1 were immersed in ozonated water at different concentrations as described in above for 5, 10, and 15 min. The untreated florets were rinsed with tap water for 2 min. Thereafter, the excess of water in all treated fresh-cut broccoli florets was removed with a manual salad spinner. Samples of approximately 120 g each were packed in clamshell box (PET) and kept at 4 °C until the produce was not acceptable by the consumer. Treated broccoli samples were randomly taken for analyzing microbial populations, physical changes and visual quality every 2-3 days during storage as compared with the untreated florets.

3.2.1.4 Effect of Sodium Chlorite (SC) Solution on Microbial Growth and Visual Quality in Fresh-Cut Broccoli Florets

Fresh broccoli florets prepared as described in 3.1 were dipped into sodium chlorite (SC) solution at the concentrations of 500, 750 and 1,000 ppm for 1 min. The untreated florets were rinsed with tap water for 2 min. Excess water was removed from all samples using a manual salad spinner. Samples of approximately 120 g each were packed in clamshell box (PET) and kept at 4 °C until the produce was not acceptable by the consumer. Treated broccoli samples were randomly taken for analyzing microbial populations, physical changes and visual quality every 3 days during storage as compared with the untreated florets.

The parameters were examined in the experiment I shown as follows:

1. Microbial population
    1.1 Total bacteria counts
    1.2 Coliform counts
    1.3 *Salmonella* and *Shigella* spp. counts
    1.4 Yeast and mold counts
2. Physiological change
    2.1 The color of florets index: L*, a*, b* value, Hue angle
    2.2 Internal gas analysis in package: O_2, CO_2
    2.3 Respiration rate
    2.4 Weight loss
3. Sensory evaluation
    3.1 Overall visual quality
    3.2 Visual color
    3.3 Odor

3.2.2 Experiment II: Study on the Effects of Hot Water Combined with Sodium Chlorite on Microbial Growth and Visual Quality in Fresh-Cut Broccoli Florets during Cold Storage

The treatments of hot water (HW) and sodium chlorite (SC) solution from experiment I (3.2.1) shown the best effect to reduce microbial populations and improve the visual quality were selected to study in the experiment II. The prepared fresh-cut broccoli florets as described in 3.1 were dipped with HW at 45 °C for 1 min and then cooled in cold tap water at 4 °C for 3 min. After that, the excess solution was removed with a manual salad spinner. HW treated broccoli were then immersed in 100 and 300 ppm of SC solution for 1 min and the excess water was removed again by a manual salad spinner. Control florets were rinsed with tap water for 2 min and the excess water was removed by a manual salad spinner.

Samples of approximately 120 g each were packed in clamshell box (PET) and kept at 4 °C until the produce was not acceptable by the consumer. Treated broccoli samples were randomly taken for analyzing microbial population, physical changes and visual quality every 3 days as described in experiment I.

3.2.3 Experiment III: Study on the Effects of Hot Water Combined with Sodium Chlorite on the Changes of Bioactive Compounds in Fresh-Cut Broccoli Florets during Cold Storage

The combined treatments with HW at 45 °C for 1 min and 300 ppm of SC solution from experiment II (3.2.2) shown the best effect to reduce of microbial populations and improved the visual quality of fresh-cut broccoli were selected for studying in the experiment III. The prepared fresh-cut broccoli florets as described in 3.1 were dipped with HW at 45 °C for 1 min and then cooled in cold tap water at 4 °C for 3 min. After that, the excess solution was removed with a manual salad spinner. HW treated broccoli were then immersed in 300 ppm of SC solution for 1 min and the excess water was removed again by a manual salad spinner. Control florets were rinsed with tap water for 2 min and the excess water was removed by a manual salad spinner.

The effects of sanitary treatments on the changes of bioactive compounds in fresh-cut broccoli florets were investigated in compared with control. Samples of approximately 120 g each were packed in clamshell box and kept at 4 °C. Treated broccoli samples were randomly taken for analyzing bioactive compounds levels during storage as compared with the untreated as control. The data on bioactive compounds of fresh-cut broccoli florets were recorded every 2 days.

The parameters were examined in the experiment III shown as follows:

1. Ascorbic acid and dehydroascorbic acid content
2. Total chlorophyll content
3. Carotenoid content
4. Total phenolic content

5. Flavonoid content
6. Glucosinolate content
7. Antioxidant activity using DPPH$^{\bullet}$ assay

3.2.4 Experiment IV: Study on the Effects of Hot Water Combined with Sodium Chlorite on the Changes of Antioxidant Activities in Fresh-Cut Broccoli Florets during Cold Storage

The combined treatments with HW at 45 °C for 1 min and 300 ppm of SC solution from experiment II (3.2.2) shown the best effect to reduce of microbial populations and improved the visual quality of fresh-cut broccoli were selected for studying in the experiment IV. The prepared fresh-cut broccoli florets as described in 3.1 were dipped with HW at 45 °C for 1 min and then cooled in cold tap water at 4 °C for 3 min. After that, the excess solution was removed with a manual salad spinner. HW treated broccoli were then immersed in 300 ppm of SC solution for 1 min and the excess water was removed again by a manual salad spinner. Control florets were rinsed with tap water for 2 min and the excess water was removed by a manual salad spinner.

The effects of sanitary treatments on the changes of antioxidant activities in fresh-cut broccoli florets were investigated in compared with control. Samples of approximately 120 g each were packed in clamshell box and kept at 4 °C. Treated broccoli samples were randomly taken for analyzing antioxidant activities during storage as compared with the untreated as control. The data on antioxidant activities of fresh-cut broccoli florets were recorded every 2 days.

The parameters were examined in the experiment IV shown as follows:
1. Chlorophyllase (Chlase) activity
2. Catalase (CAT) activity
3. Peroxidase (POD) activity
4. Superoxide dismultase (SOD) activity
5. Ascorbate peroxidase (APX) activity
6. Hydrogen peroxide (H_2O_2) content
7. Glutathione reductase (GR) content

3.3 Analytical Parameters

3.3.1 Microbial Analysis

A 25 g sample of broccoli florets was homogenized in 225 mL of 1% sterile peptone water using a stomacher (IUL Instruments Masticator, Barcelona, Spain) for 2 min. Ten-fold dilution series were made in sterile peptone water as required for plating. The following culture media and conditions were used to enumerate the microbial growth: (1) Plate count agar (HiMedia, Mumbai, India) incubated at 37 °C for 24 h for total aerobic bacteria counts; (2) Eosin Methylene Blue agar, EMB (HiMedia, Mumbai, India) incubated at 37 °C for 24-36 h for coliform counts; (3) Xylose-Lysine Deoxycholate agar, XLD (HiMedia, Mumbai, India) at 37 °C for 24-48 h for *Salmonella-Shigella* spp. counts; and (4) Potato dextrose agar (HiMedia, Mumbai, India) incubated at 28 °C for 7 day for yeast and mold counts. Microbial counts were expressed as $\log_{10}$ CFU·g^{-1} (colony forming units per gram of sample).

3.3.2 The Florets Color

Color measurement of the florets was determined by measuring the Hue angle and L* value with a Minolta Chroma Meter (Model CR-100, Tokyo, Japan), which covered an area of 8 mm^2. Three positions on each of florets were measured for each treatment and storage time. Hue angle of 0, 90, 180 and 270 degree showed red-purple, yellow, bluish-green and blue color, respectively.

3.3.3 Internal Gas Analysis

The CO_2 and O_2 concentrations in clamshell box (PET) of broccoli florets were measured by a Gas Analyzer (OXYBABY® 6.0). A needle sensor was penetrated on the silicone rubber attached on the outer surface (on the top) of the lid for 10 sec. The CO_2 and O_2 concentrations were determined and expressed as percent.

3.3.4 Respiration Rate

Respiration rate was measured by Gas Chromatography (Model GC-8A, Shimadzu, Japan). Approximately 25 g sample was sealed in a 520 mL plastic polypropylene (PP) boxes (8.0 × 12.3 × 5.3 cm in dimension) with silicon sampling ports and tightly seal and kept at 4 °C for 2 h. A 1 mL of headspace gas samples were collected with a gastight plastic syringe and injected into Shimadzu-8A gas chromatograph equipped with the thermal conductivity detector was used for CO_2 analysis.

3.3.5 Sensory Evaluation (9-Point Hedonic Scale)

The sensory panel consisted of 8–10 untrained members. The sensory attributes were evaluated by visual characteristics such as overall visual quality, visual color, and odor. A rating score index of analyzed sample was given ranging from 9-1, overall visual quality score: 9 = fresh appearance, 1 = unusable. Color score: 9 = dark green, 1 = 100% yellow. Odor score: 9 = no off-odor, 1 = extreme off-odor. The sample was considered as unacceptable in terms of sensory characteristics when the rating score was lower than 5 (limit for customers).

3.3.6 Determination of Ascorbic Acid and Dehydroascorbic Acid Content

The determination of total ascorbic acid was carried out as described by Terada et al. (1978) cited by Fonseca et al. (2005) on the basis of coupling 2,4-dinitrophenylhydrazine (DNPH) with ketonic groups of dehydroascorbic acid through the oxidation of ascorbic acid by 2,6-dichloroindophenol (DCIP) sodium salt to form a yellow-orange color in acidic c onditions. Fresh-cut broccoli florets (0.5 g) were homogenized in 20 mL cold solution of 6% (w/v) meta-phosphoric acid. The homogenates were centrifuged at 9,000×g for 30 min at 4 °C. The supernatants were analyzed the ascorbic acid immediately. Briefly, 0.5 mL of the supernatant extract mixed with 0.2 mL of 0.02% (w/v) DCIP sodium salt in distilled water and then allow incubates at room temperature for 1 h. After that, adding 0.5 mL of 2% (w/v) thiourea in 5% meta-phosphoric acid and 0.25 mL of 2% (w/v) DNPH in 9 N sulfuric acid. The mixtures were kept in a water bath at 60 ºC for 3 h followed by an ice bath for 30 min. Then, the mixtures were stopped the reaction by slowly adding 1.25 mL of ice-cold 90% (v/v) sulfuric acid (H_2SO_4) and kept at room temperature for 30 min before reading the absorbance at 540 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). The 2,4-DNPH was added during the ice bath as a blank for a control. The amount of ascorbic acid concentrations were calculated using a standard curve generated using commercial L-(+)-ascorbic acid and then expressed as $mg \cdot 100g^{-1}$ FW.

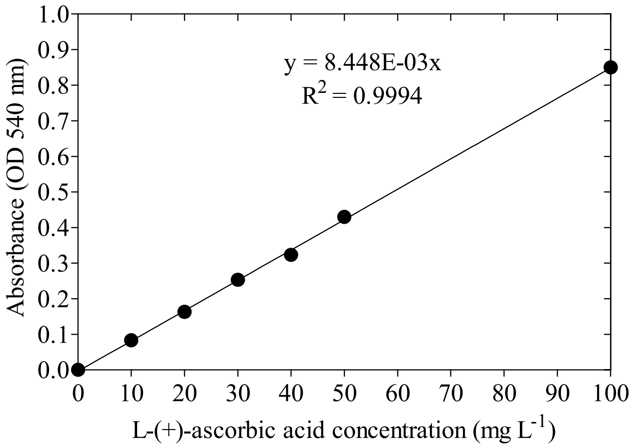

Figure 3.1 Calibration curve with L-(+)-ascorbic acid as standard

3.3.7 Determination of Chlorophyll and Carotenoid Content

Total chlorophyll (Chl) content was analyzed according to the method of Moran (1982). The florets of broccoli (0.5 g) were extracted in 20 mL of *N,N* dimethylformamide and kept overnight at 4 °C in the dark. An absorbance was read spectrophotometerically (UV-1800, Shimadzu, Japan) at 647 and 664 nm. Chl *a* and *b* were calculated with the following equations.

$$\text{Chl } a\ (\mu\text{g mL}^{-1}) = 12.64\ \text{OD}_{664} - 2.99\ \text{OD}_{647}$$

$$\text{Chl } b\ (\mu\text{g mL}^{-1}) = -5.6\ \text{OD}_{664} + 23.26\ \text{OD}_{647}$$

The unit of μg mL^{-1} is converted into mg 100g^{-1} FW of broccoli florets using the following equations:

$$\text{Chl } a\ (\text{mg } 100\text{g}^{-1}\ \text{FW}) = (\text{Chl } a\ (\mu\text{g mL}^{-1}) \times 20.5 \times (100/0.5) \times (1/1000)$$

$$\text{Chl } b\ (\text{mg } 100\text{g}^{-1}\ \text{FW}) = (\text{Chl } b\ (\mu\text{g mL}^{-1}) \times 20.5 \times (100/0.5) \times (1/1000)$$

3.3.8 Methanolic Extraction

The methanolic extraction was determined by the method of Volden et al. (2009), with some modifications. Fresh-cut broccoli florets (2.0 g) were extracted with 15 mL of methanolic extracts containing 10 mM HCl in methanol. The homogenized samples from the cold methanolic extracts were then centrifuged at 9,000×g for 20 min at 4 °C, for two times. The supernatants were transferred to vials, stored at –20 °C freezer and fully filled tightly capped storage tube prior used for total phenolic and flavonoid analysis. All samples were extracted in duplicate and analyzed in triplicated.

3.3.9 Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu assay by the method of Singleton and Rossi (1965) cited by Roy et al. (2009), with some modifications. A sample of the methanolic extract (125 µL) was added to 250 µL of distilled water and 125 µL of Folin-Ciocalteau reagent were put into a test tube. The mixture was vertexed and after 5 min at room temperature, 1.25 mL solution of 7% (w/v) sodium carbonate solution, 1 mL of distilled water was added. The reaction mixture was incubated for 90 min at room temperature. The absorbance was measured at 760 nm and total phenolic content was calculated by using gallic acid as a standard. Measurements were performed four replicated for each treatment at each storage times. The results were expressed as mg of gallic acid (GAE) $100g^{-1}$ FW.

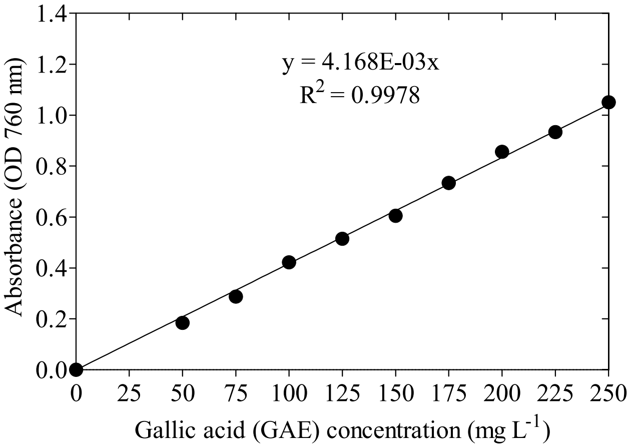

Figure 3.2 Calibration curve with gallic acid as standard

3.3.10 Determination of Total Flavonoid Content

Total flavonoid content was determined by modifying a colourimetric method of Dewanto et al. (2002) cited by Roy et al. (2009), with some modifications. Briefly, 0.2 mL of the methanolic extract, 0.8 mL of distilled water and 50 µL of a 5% $NaNO_2$ solution were mixed in a test tube. After 6 min, 100 µL of a 10% $AlCl_3{\cdot}6H_2O$ solution was added and allowed to stand for another 5 min before 0.5 mL of 1 M NaOH was added. Then, 850 µL of distilled water was added to bring the mixture to 2.5 mL and mixed well. The absorbance was measured immediately against the blank at 510 nm using a spectrophotometer (UV-1800, Shimadzu, Japan) in comparison with the standards prepared similarly with known quercetin concentrations (10–1,000 $\mu g{\cdot}mL^{-1}$).

Measurements were performed four replicated for each treatment at each storage times. The results were expressed as mg of quercetin equivalents (QE) $100g^{-1}$ FW.

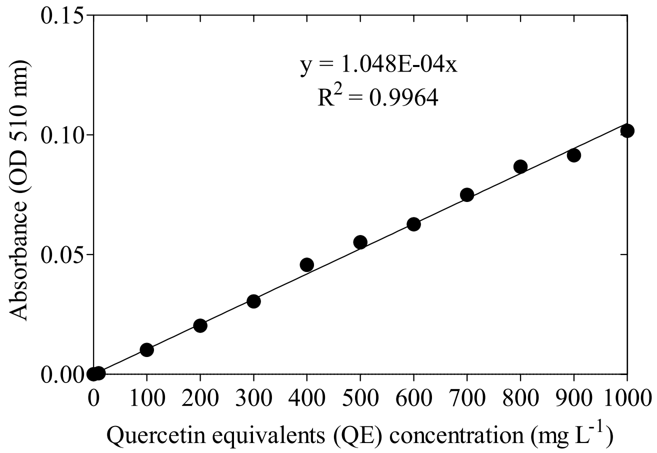

Figure 3.3 Calibration curve with quercetin as standard

3.3.11 Determination of Glucosinolate Content

Total glucosinolate content of the fresh-cut broccoli florets was determined by palladium test described by Barro et al. (2002) and Ippoushi et al. (2007) with slight modifications. About 500 mg of broccoli floret from each sample were overnight-dried at 60 °C (inactivate the enzyme myrosinase) and ground in a mortar. After this process, the powder was heated at 90 °C for 40 min and then 10 mL of 80% methanol (v/v) was added to the samples and incubated for 30 min at 90 °C. After centrifugation at 9,000×g for 15 min, the pellets were reextracted with 80% aqueous methanol (10 mL) for 20 min at 70 °C, and this procedure was repeated once more. The total extracts were combined and the methanol was removed by rotary evaporator. The residues of fresh-cut broccoli florets powders were dissolved and filled up to 20 mL with deionized water. Aliquots of 100 µL of the glucosinolate extracts were mixed with 2,000 µL of 2 mM sodium tetrachloropalladate (II) and incubated for 60 min at room temperature, the absorbance at 450 nm was measured on a spectrophotometer. The quantification of the total glucosinolates content was done by referring absorbance values to a calibration curve developed from solutions of sinigrin in water at different concentrations. Measurements were performed four replicated for each treatment at each storage times. The results were expressed as mg of $\text{sinigrin}\cdot g^{-1}$ FW.

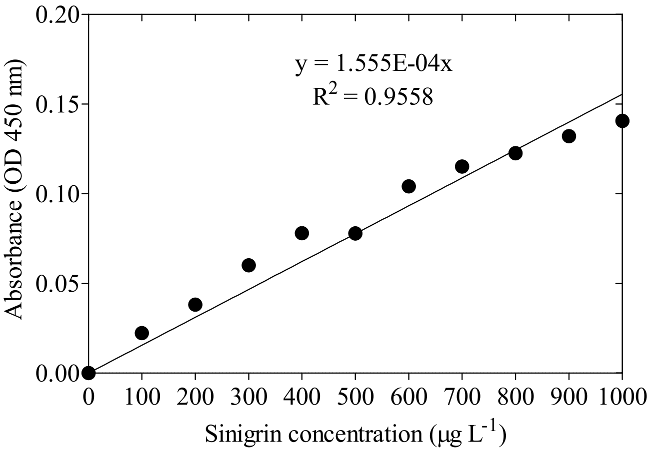

Figure 3.4 Calibration curve with sinigrin as standard

3.3.12 Antioxidant Capacity using DPPH• Assay

Fresh-cut broccoli florets (2.5 g) were extracted with 25 mL of methanol. The mixture was centrifuged at 9,000×g for 20 min at 4 °C for two times. The supernatants were transferred to vials, stored at –20 °C until used for antioxidant capacity analysis. The antioxidant capacity by DPPH• free radical scavenging assay of broccoli florets was according to the method described by Brand-Williams et al. (1995) and Thaipong et al. (2006) with some modifications. The stock solution was prepared by dissolving 24 mg 2,2 diphenyl-l-picrylhydrazyl (DPPH•) with 100 mL methanol and then stored at –20 °C until needed. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1±0.02 units at 515 nm using spectrophotometer. The supernatant extracts (50 µL) were added to test tubes containing 2,950 µL of the DPPH• solution for 30 min in the dark. The supernatant was measured at 515 nm. The DPPH• values were calculated using a standard curve generated using known concentration of Trolox. Additional dilution was needed if the DPPH• value measured was over the linear range of the standard curve. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity (Gülçin and Ak, 2008). Antioxidant activity was expressed as the percentage of DPPH• decrease using the equation (Robles-Sánchez et al., 2009):

$$\text{Radical-scavenging activity (\%)} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

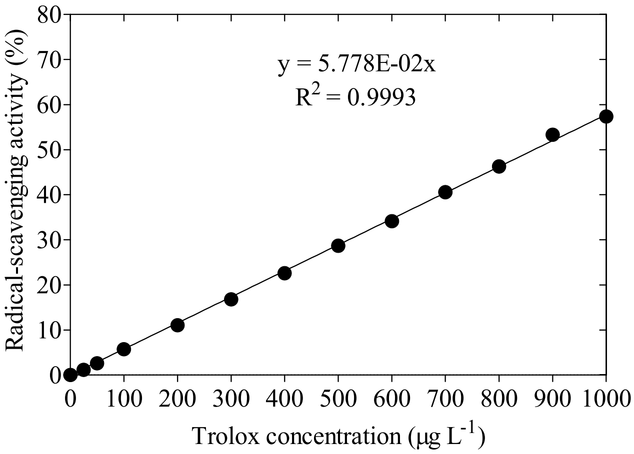

Figure 3.5 Calibration curve with trolox as standard

3.3.13 Chlorophyllase Activity

3.3.13.1 Preparation of Chlorophyll *a* as a Substrate of Chlorophyllase Assay

Chlorophyll *a* (Chl *a*) was extract from spinach (*Spinacia oleracea* L.) leaves according to the procedure of Yamauchi et al. (1997), with slight modifications. Fifteen grams of spinach were homogenized with 50 mL acetone for 3 min and filtered through two layers of cheesecloth. The homogenate was partially purified by adding 15 mL of distilled water and 6 mL of 1,4-dioxane to the acetone extract. The supernatant was incubating for 1 h at 4 °C. After 1 h, the homogenate was centrifuged at 10,000×g for 20 min. The clear supernatant was discarded immediately, and the pellet was dissolved with 7 mL of distilled water and 3 mL of 1,4-dioxane and then incubate for 1 h at 4 °C. After 1 h, the suspension was centrifuged again, and the pellet was dissolved in 5 mL of petroleum ether. The supernatant was used as the crude chlorophyll *a* extract. Individual pigments were purified from the petroleum ether extract for use as a standard.

Chl *a* was separated by sucrose column chromatography according to the method of Perkins and Roberts (1962). The powdered sugar which saturated with petroleum ether was poured into the chromatographic glass column and compressed by a plunger as to packed the tightly and uniformly sugar column. The total Chls were loaded on to the column by 10 mL per 500 g of powdered sugar and the developing solvent was changed to 0.5% (v/v) 2-isopropanol in petroleum ether after the non-adsorbed carotenes were gradually eluted. The Chl *a* zone (blue-green) was dug out of the sugar column and transferred to an evaporatory funnel with petroleum ether. The Chl *a* was evaporated as to removed petroleum ether under pressure and was dissolved in 5 mL of acetone solution according to Lichtenthaler (1987).

Chl *a* concentration was calculated with the following equations:

$$\text{Chl } a \text{ concentration} = 11.24A_{661.6} - 2.04A_{644.8}$$

3.3.13.2 Preparation Acetone Powder

Acetone powder was prepared by blending 10 grams of florets tissues were blended with 100 mL of 100% acetone. The homogenate was vacuum-filtered, and repeatedly washed the residue with chilled acetone until the color of residue was completely removed. After that, added 50 mL of 100% diethyl ether to pursue acetone and to obtain the acetone powder which were dried in desiccators of 3 h and ground to a powder. The acetone powder was stored at -20 ºC until used for analyzing.

3.3.13.3 Chlorophyllase Activity Assay

For enzyme assays, an acetone powder (3.3.13.2) (500 mg) was suspended in 15 mL of a 10 mM phosphate buffer (pH 7.0) containing with 50 mM potassium chloride (KCl) and 0.24% Triton X-100 for chlorophyllase (Chl) activities. A crude enzyme was stirred for 1 h at 0 ºC and then filtered through Whatman No. 2. Subsequently, the filtrate was centrifuged at 16,000×g for 15 min at 4 ºC. The supernatant was used as the crude enzyme extract (Funamoto et al., 2002).

Chlorophyllase activity was determined by modification of the method of Funamoto et al. (2002). The reaction mixture contained 0.5 mL crude enzyme solution, 0.1 mL 1.44% Triton X-100, 0.2 mL Chl *a* acetone solution (Chl *a*-100 ppm), and 0.5 mL of 100 mM phosphate buffer (pH 7.5). The mixture was incubated in water at 25 ºC for 40 min, and the enzyme reaction was stopped by the addition of 4 mL of acetone. The remaining (non-degraded) Chl *a* was extracted with 4 mL of hexane and assayed by reading the absorbance at 663 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). The activity was based on the decrease in absorbance by Chl *a* at 663 nm. Measurements were performed four replicated for each treatment at each storage times. The specific chlorophyllase activity was expressed as units·mg^{-1} protein.

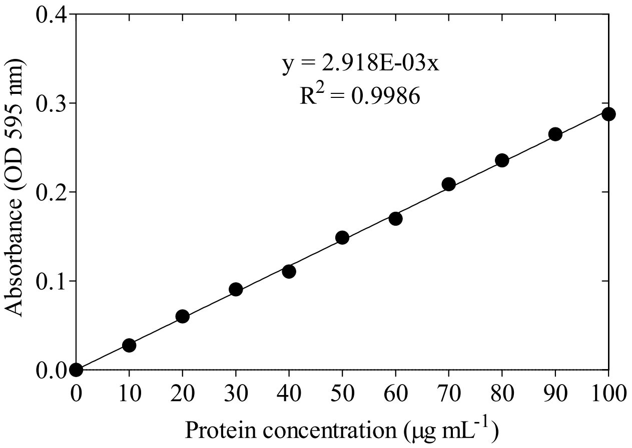

Figure 3.6 Calibration curve with Bovine Serum Albumin (BSA) as standard for Chlase

3.3.14 Determination of Catalase Activity (EC 1.11.1.6)

The catalase (CAT) activity was assayed according to Jiang et al. (2010). Frozen broccoli florets samples (1.0 g) were homogenized in 15 mL of 100 mM sodium phosphate buffer (pH 7.0) contained 0.1 mM EDTA, 0.1% Trition X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) polyvinylpolypyrrolidone (PVPP) at 4 °C. The homogenates were centrifuged at 9,000×g for 30 min at 4 °C and the supernatants were used for assays of enzyme activity and protein content. The reaction mixture (3.0 mL) contained 0.15 mL of enzyme extract and 2.85 mL of substrate mixture contained 85 μL of 30% H_2O_2 in 20 mL of 50 mM sodium phosphate buffer (pH 7.0) (freshly prepared). CAT activity was assayed by the disappearance of H_2O_2 in the enzyme reaction mixture by estimating the residual H_2O_2 using titanium reagent. Record the decrease in H_2O_2 concentration by reading the absorbance at 240 nm for 3 min. Measurements were performed four replicated for each treatment at each storage times. One unit of CAT enzymatic activity was calculated using the extinction coefficient of H_2O_2 (ε) (39.4 $mM^{-1}\cdot cm^{-1}$ at 240 nm) and then expressed as units·mg^{-1} protein.

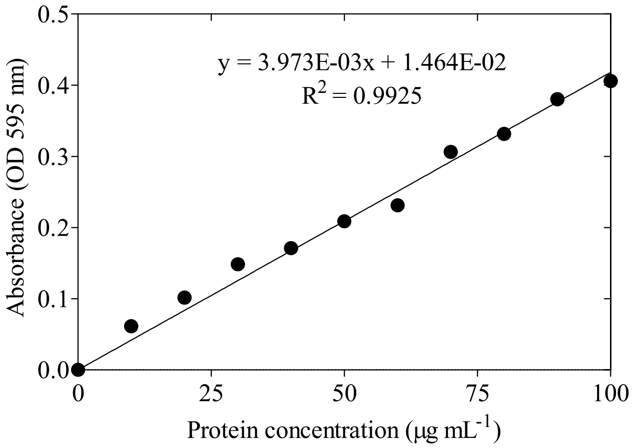

Figure 3.7 Calibration curve with Bovine Serum Albumin (BSA) as standard for CAT

3.3.15 Determination of Peroxidase Activity (EC 1.11.1.7)

Peroxidase (POD) activity using guaiacol as a substrate. POD was assayed according to Jiang et al. (2010). Frozen broccoli florets samples (1.0 g) were homogenized in 15 mL of 100 mM sodium phosphate buffer (pH 7.0) contained 0.1 mM EDTA, 0.1% Trition X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) polyvinylpoly-pyrrolidone (PVPP) at 4 °C. The homogenates were centrifuged at 9,000×g for 30 min at 4 °C and the supernatants were used for assays of enzyme activity and protein content. The reaction mixture (3.0 mL) contained 0.1 mL of enzyme extract and 2.9 mL of substrate mixture contained 100 mL of 50 mM sodium phosphate buffer (pH 7.0), 10 mL of 1% guaiacol and 10 mL of 0.3% H_2O_2 (from 30% H_2O_2). The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 2 min. The reaction was initiated by adding enzyme extract. One unit of POD enzymatic activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per minute. The specific POD activity was expressed as units·mg^{-1} protein.

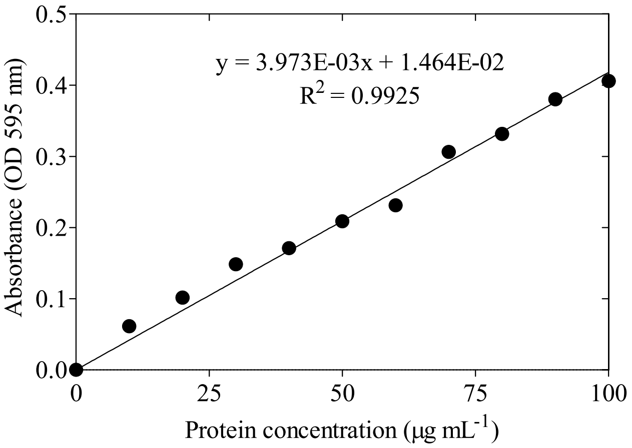

Figure 3.8 Calibration curve with Bovine Serum Albumin (BSA) as standard for POD

3.3.16 Determination of Superoxide Dismutase Activity (EC 1.15.1.1)

The superoxide dismutase (SOD) activity was determined on the basis of the photochemical reduction of nitroblue tetrazolium (NBT) by superoxide radicals ($O_2^{-\bullet}$) at 560 nm (Beauchamp and Fridovich, 1971). Frozen broccoli florets samples (1.0 g) were homogenized in 15 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA-Na_2, 2% (w/v) polyvinylpolypyrrolidone (PVPP) at 4 °C. The homogenates were centrifuged at 9,000×g for 30 min at 4 °C and the supernatants were used for assays of enzyme activity and protein content. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 0.66 mM EDTA, 10 mM L-methionine, 33 μM nitro blue tetrazolium (NBT) in a total volume of 2.75 mL and 0.2 mL of enzyme extract. The reaction was started by adding 0.05 mL of 0.2 mM riboflavin and then keeping the mixture under a light source of 36 W fluorescent lamps at room temperature for 15 min. Then, the absorbance of the assay mixture was measured at 560 nm. Non-irradiated assay mixtures did not developed color and used as the control. The reaction mixture without the enzyme extract developed maximum color, which taken as 100%. One unit of SOD activity was defined per gram of fresh weight causing 50% inhibition of the reduction of NBT photoreduction at 560 nm. Measurements were performed four replicated for each treatment at each storage times. The specific SOD activity was expressed as units·mg^{-1} protein. Measurements were performed four replicated for each treatment at each storage times.

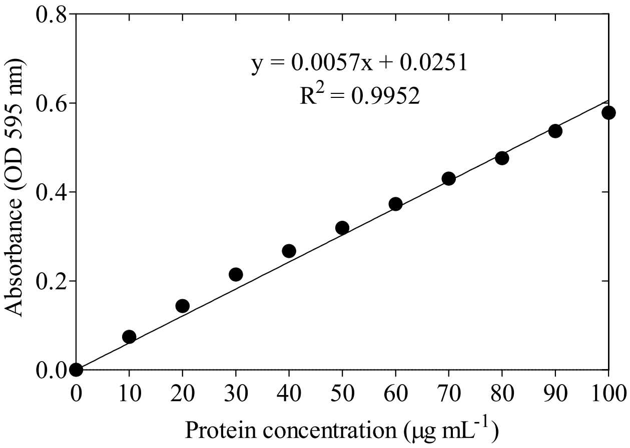

Figure 3.9 Calibration curve with Bovine Serum Albumin (BSA) as standard for SOD

3.3.17 Determination of Ascorbate Peroxidase Activity (EC 1.11.1.11)

The ascorbate peroxidase (APX) activity measurement was assayed according to Vicente et al. (2006). Approximately 1 g of frozen broccoli florets were homogenized with 15 m of 50 mM sodium phosphate (pH 7.0) containing 0.1 mM EDTA, 2 mM ascorbate, 2% (w/v) polyvinylpolypyrrolidone (PVPP) at 4 °C. The homogenates were centrifuged at 9,000×g for 30 min at 4 °C and the supernatants were used for assays of enzyme activity and protein content. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 5 mM ascorbate, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.3 mL of 3 mM H_2O_2 and 0.3 mL of enzymatic extract in a final volume of 3.0 mL. The reduction of decrease in absorbance at 290 nm recorded for 3 min. The APX enzyme activity was calculated using the extinction coefficient of NADPH (ε) (2.8 $mM^{-1}\cdot cm^{-1}$ at 290 nm) and then expressed as units·mg^{-1} protein. Measurements were performed four replicated for each treatment at each storage times.

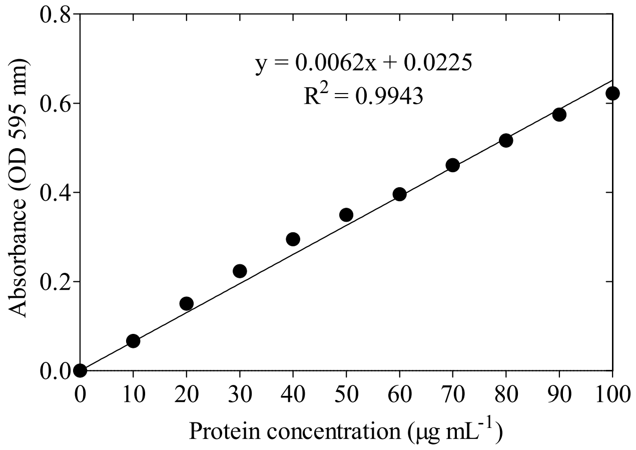

Figure 3.10 Calibration curve with Bovine Serum Albumin (BSA) as standard for APX

3.3.18 Determination of Hydrogen Peroxide Content

The assay of hydrogen peroxide (H_2O_2) content was determined as described by Patterson et al. (1984), Fresh-cut broccoli florets (4.0 g) were homogenized with 10 mL of acetone on ice. The homogenate was centrifuged at 9,000×g for 15 min at 4 °C. The supernatant (1 mL) extracted was mixed with 2 mL of 50 mM sodium phosphate buffer (pH 6.8), 0.5 mL of 0.1% titanium sulphate in 20% (v/v) H_2SO_4 and 0.5 mL ammonia (NH_4OH) solution. The intensity of the yellow color of the supernatant was measured at 410 nm. Measurements were performed four replicates for each treatment at each storage times. The H_2O_2 level was calculated using the extinction coefficient (ε) (0.28 μM^{-1} cm^{-1}) and then expressed as mg $H_2O_2 \cdot 100g^{-1}$ FW.

3.3.19 Determination of Glutathione Reductase Activity (EC 1.6.4.2)

The glutathione reductase (GR) activity was assayed according to Jiang et al. (2010). Frozen broccoli florets samples (1.0 g) were homogenized in 15 mL of 100 mM sodium phosphate buffer (pH 7.0) contained 0.1 mM EDTA, 0.1% Trition X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) polyvinylpolypyrrolidone (PVPP) at 4 °C. The homogenates were centrifuged at 9,000×g for 30 min at 4 °C and the supernatants were used for assays of enzyme activity and protein content. The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.8), 5 mM GSSG, 1.2 mM $NADPH \cdot Na_4$ in a total volume of 2.70 mL and 0.3 mL of enzyme extract. Record oxidation of NADPH by reading a decrease in absorbance at 340 nm was recorded for 3 min. One unit of GR enzymatic activity was calculated using the extinction coefficient of NADPH (ε) (6.22 $mM^{-1}\cdot cm^{-1}$ at 340 nm) and then expressed as units·mg^{-1} protein. Measurements were performed four replicated for each treatment at each storage times.

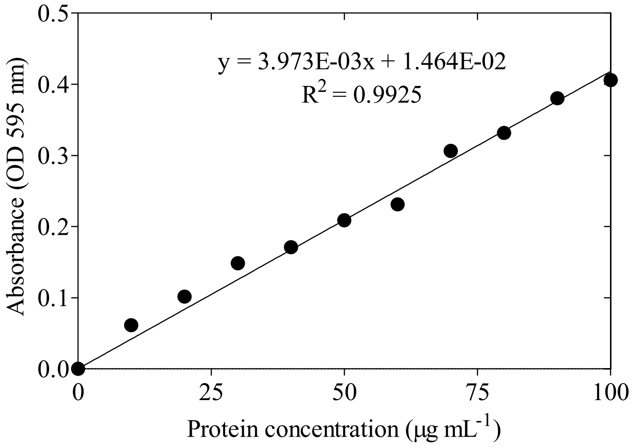

Figure 3.11 Calibration curve with Bovine Serum Albumin (BSA) as standard for GR

3.3.20 Protein Concentration

Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

3.3.21 Statistical Analyses

Experiments were performed according to a completely randomized design (CRD). The data obtained from three replicates were analyzed using analysis of variance (ANOVA), and mean differences were determined using Duncan's multiple range tests. The least significant difference (LSD) test was used to analyze significant differences ($P \leq 0.05$) between the means of different treatments using the SPSS 12.0 (SPSS, SPSS Inc., Chicago. IL, USA). All data are presented as means and standard errors.

3.3.22 Determination of O_3 Concentration in Water

Ozone (O_3) residual in water is determined by the indigo method. Residual ozone decays rapidly that depending on water quality, ozone residual half-life may be several seconds to a few minutes (Eaton et al., 2005). The concentrations of O_3 dissolved in water were measured immediately using the indigo colorimetric method according to Eaton et al. (2005).

3.3.22.1 General

The indigo colorimetric method is quantitative, selective and simple; it replaces methods based on the measurement of total oxidant. The method is applicable to lake water, river infiltrated, manganese-containing ground waters, extremely hard ground waters, and even biologically treated domestic wastewater.

a. *Principle*: In acidic solution, ozone rapidly decolorized indigo. The decrease in absorbance is linear with increasing concentration. The proportionality constant at 600 nm is 0.42 ± 0.01 $cm^{-1}\cdot mg^{-1}\cdot L^{-1}$ ($\Delta\varepsilon = 2950/M\cdot$cm at 258 nm).

b. *Interferences*: Hydrogen peroxide (H_2O_2) and organic peroxides decolorize the indigo reagent very slowly. H_2O_2 does not interfere if ozone is measured in less than 6 h after adding reagents. Organic peroxides may react more rapidly. Fe(III) does not interfere. Mn(II) does not interfere but it is oxidized by ozone to forms that decolorize the reagent. Correct for this decolorization by making the measurement relative to a blank in which the ozone has been destroyed selectively. Without the corrective procedure, 0.1 mg L^{-1} ozonated manganese gives a response of about 0.08 mg L^{-1} apparent ozone. Chlorine also interferes. Low concentrations of chlorine (<0.1 mg L^{-1}) can be masked by malonic acid. Bromine, which can be formed by oxidation of Br^{-}, interferes (1 M HOBr corresponds to 0.4 M ozone). In the presence of HOBr or chlorine in excess of 0.1 mg L^{-1}, an accurate measurement of ozone cannot be made with this method.

c. *Minimum detectable concentration*: For the spectrophotometric procedure using thermostated cells and a high-quality photometer, the low-range procedure will measure down to 2 μg O_3 L^{-1}. The practical lower limit of residual measurement is 10 to 20 μg L^{-1}.

d. *Sampling*: React sample with indigo as quickly as possible, because the residual may decay rapidly. Avoid loss of ozone residual due to off-gassing during sample collection. Do not run sample down side of flask. Add sample so that completely decolorized zones are eliminated quickly by swirling or stirring.

3.3.22.2 Apparatus

Photometer: Spectrophotometer on filter colorimeter for use at 600 ± 10 nm.

3.3.22.3 Reagents

a. *Indigo stock solution*: Add about 500 mL distilled water and 1 mL conc. phosphoric acid to a 1 L volumetric flask. With stirring, add 770 mg potassium indigo trisulfonate, $C_{16}H_7N_2O_{11}S_3K_3$ (use only high-grade reagent, commercially available about 80 to 85% purity). Fill to mark with distilled water. A 1:100 dilution exhibits an absorbance of 0.20±0.010 cm at 600 nm. The stock solution is stable for about 4 mouths when stored in the dark. Discard when absorbance of a 1:100 dilution falls below 0.16 cm^{-1}. Do not change concentration of dye for higher ranges of ozone residual. Volume of dye used may be adjusted.

b. *Indigo reagent I*: To a 1 L volumetric flask add 20 mL indigo stock solution, 10 g sodium dihydrogen phosphate (NaH_2PO_4), and 7 mL conc. phosphoric acid. Dilute to mark. Prepare solution fresh when its absorbance decreased to less than 80% of its initial value, typically within a week.

c. *Indigo reagent II*: Proceed as with indigo reagent I, but add 100 mL indigo stock solution instead of 20 mL.

d. *Malonic acid reagent*: Dissolve 5 g malonic acid water and dilute to 100 mL.

e. *Glycine reagent*: Dissolve 7 g glycine in water and dilute to 100 mL.

3.3.22.4 Procedure

a. *Spectrophotometric, volumetric procedure:*

1) Concentration range 0.01 to 0.1 mg O_3 L^{-1}: Add 10.0 mL indigo reagent I to each of two 100 mL volumetric flakes. Fill one flask (blank) to mark with distilled water. Fill other flask to mark with sample. Measure absorbance of both solutions at 600 nm as soon as possible but at least within 4 h. Preferably use 10 cm cells. Calculate the ozone concentration from the difference between the absorbance found in sample and blank (3.3.20.5a below).

Note: A maximum delay of 4 h before spectrophotometric reading can be tolerated only for drinking water samples. For other sample types that cannot be read immediately, determined the relationship between time and absorbance.

2) Range 0.05 to 0.5 mg O_3 L^{-1}: Proceed as above using 10 mL indigo reagent II instead of reagent I. Preferably measure absorbance in 4 or 5 cm cells.

3) Concentrations greater than 0.3 mg O_3 L^{-1}: Proceed using indigo reagent II, but for these higher ozone concentrations use a correspondingly smaller sample volume. Dilute resulting mixture to 100 mL with distilled water.

4) Control of interferences: In presence of low chlorine concentration (<0.1 mg L^{-1}), place 1 mL malonic acid reagent in both flasks before adding sample and/or filling to mark. Measure absorbance as soon as possible, within 60 min (Br^{-}, Br_2, and HOBr and only partially masked by malonic acid).

   In presence of manganese prepare a blank solution using sample, in which ozone is selectively destroyed by addition of glycine. Place 0.1 mL glycine reagent in 100 mL reagent volumetric flask (blank) and 10 mL indigo reagent II in second flask (sample). Pipette exactly the same volume of sample into each flask. Adjust dose so that decolorization in second flask is easily visible but complete bleaching does not result (maximum 80 mL).

   Insure that pH of glycine/sample mixture in blank flask (before adding indigo) is not below 6 because reaction between ozone and glycine becomes very slow at low pH. Stopper flasks and mix by carefully inverting. Add 10 mL indigo reagent II to blank flask only 30 to 60 s after sample addition. Fill both flasks to the mark with ozone-free water and mix thoroughly. Measure absorbance of both solutions at comparable contact times of approximately 30 to 60 min (after this time, residual manganese oxides further discolor indigo only slowly and the drift of absorbance in blank and sample become comparable). Reduced absorbance in blank flask results from manganese oxides while that in sample flask is due to ozone plus manganese oxide.

5) Calibration: Because ozone is unstable, base measurements on known and constant loss of absorbance of the indigo reagent ($f = 0.42 + 0.01$ cm^{-1} mg^{-1} O_3 L^{-1}). For maximum accuracy analyze the lot of potassium indigo trisulfonate (no commercial lot has been found to deviate from $f = 0.42$) using the iodometric procedure.

   When using a filter photometer, readjust the conversion factor, f, by comparing photometer sensitivity with absorbance at 600 nm by an accurate spectrophotometer.

b. *Spectrophotometric , gravimetric procedure:*

1) Add 10 mL indigo reagent II to 100 mL volumetric flask and fill flask (blank) to mark with distilled water. Obtain tare weight of a second flask (volumetric or Erlenmeyer). Add 10 mL indigo reagent II to second flask. Fill directly with

sample (do not run water down side), and swirl second flask until blue solution has turned to a light blue color. Weight flask containing indigo and sample.

2) Preferably using 10 cm cells, measure absorbance of both solutions at 600 nm as soon as possible, but at least within 4 h. Note: A maximum delay of 4 h before spectrophotometric reading is suitable only for drinking water samples. For other sample types, test the time drift.

3.3.22.5 Calculations

a. *Spectrophotometric, volumetric method:*

$$\text{mg } O_3 \text{ L}^{-1} = \frac{100 \times \Delta A}{f \times b \times V}$$

Where:

ΔA = difference in absorbance between sample and blank,

b = path length cell, cm,

V = volume of sample, mL (normally 90 mL), and

f = 0.42

The factor f is based on a sensitivity factor of 20,000 cm^{-1} for the change of absorbance (600 nm) per M of added ozone per liter. It was calibrated by iodometric titration. The UV absorbance of ozone in pure water may serve as a secondary standard: the factor $f = 0.42$ corresponds to an absorption coefficient for aqueous ozone, $\varepsilon =$ 2950/M•cm at 258 nm.

b. *Spectrophotometric, gravimetric method:*

$$\text{mg } O_3 \text{ L}^{-1} = \frac{(Ab \times 100) - (As \times Vt)}{f \times Vs \times b}$$

Where:

Ab, As = absorbance of blank and sample, respectively,

Vs = volume of sample, mL = [(final weight – tare weight) g × 1.0 mL/g] −10 mL,

Vt = total volume of sample plus indigo, mL = (final weight – tare weight) g × 1.0 mL/g,

b = path length of cell, cm, and

f = 0.42