

## Appendix

### ANALYTICAL METHODS

#### 1. Determination of reducing sugar content (Chaplin, 1994)

##### Chemicals

- Reagent A: Dissolve 15 g of sodium potassium tartrate and 30 g of anhydrous  $\text{Na}_2\text{CO}_3$  in about 300 ml water. Add 20 g  $\text{NaHCO}_3$ . Dissolve 180 g of anhydrous  $\text{Na}_2\text{SO}_4$  in 500 ml boiling water and cool. Mix the two solutions and make up to 1 litre with water.
- Reagent B: Dissolve 5 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 45 g anhydrous  $\text{Na}_2\text{SO}_4$  in water and make up to 250 ml.
- Reagent C: Mix reagent A (4 vol.) and B (1 vol.) just before use.
- Reagent D: Dissolve 25 g ammonium molybdate in 450 ml water. Carefully add 21 ml concentrated  $\text{H}_2\text{SO}_4$  with stirring. Dissolve 3 g  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  in 25 ml water and add to the molybdate solution. Incubate for 24-28 h at  $37^\circ\text{C}$  and store in a brown glass-stoppered bottle. Just before use, this reagent should be diluted with 2 vol. of 0.75 M  $\text{H}_2\text{SO}_4$  (4 ml concentrated  $\text{H}_2\text{SO}_4$  in 100 ml solution).

##### Method

MRP samples with an appropriate dilution (1.0 ml) was mixed with 1.0 ml of reagent C in small stoppered test-tubes. The mixture was heated at  $100^\circ\text{C}$  for 15 min, followed by cooling rapidly to room temperature. Then, reagent D (1.0 ml) was added and mixed well. Water (3.0 ml) was then added. The absorbance was measured at 520

nm. A standard curve was prepared with the reactant sugar at concentration range of 0-500  $\mu\text{mol}$ .

## **2. Determination of thiobarbituric acid reactive substance (TBARS) (Buege and Aust, 1978)**

### **Chemicals**

- TBA solution: 0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric acid were mixed thoroughly in 100 ml of distilled water.

### **Method**

Sample (1 g) was mixed with 10 ml of TBA solution. The mixture was heated for 10 min in a boiling water bath (95-100°C) to develop pink color, cooled with tap water, centrifuged at 5500 rpm for 25 min, and absorbance of the supernatant was measured at 532 nm. A standard curve was prepared with malonaldehyde bis (dimethyl acetal) (MDA) at concentration ranging from 0 to 3000  $\mu\text{M}$ . The quantity of TBARS in each sample was calculated as mg MDA/kg sample using standard curve.

## **3. Determination of peroxide value (IUPAC, 1979)**

### **Chemicals**

- Acetic acid: chloroform (3:2, v/v)
- Saturated potassium iodide solution
- 0.01N Sodium thiosulphate solution
- 1% Starch solution

### Method

Oil sample ( $\approx 1$  g) was mixed with a mixture of acetic acid and chloroform (25 ml), followed by addition of 1.0 ml of potassium iodide. The reaction mixture was left to stand for 5 min in the dark. Distilled water (75.0 ml) was added to the mixture. The mixture was titrated with sodium thiosulphate solution and shaken vigorously until color of mixture was light yellow, then 0.5 ml of starch solution was added. The mixture had blue color. Sodium thiosulphate was used to titrate until clear solution was obtained. Peroxide value was expressed as meq/kg oil using:

$$\text{Peroxide value (meq/kg oil)} = \frac{(a-b) \times N \times 100}{W}$$

Where

- a = volume (ml) of sodium thiosulphate of blank
- b = volume (ml) of sodium thiosulphate of sample
- N = concentration of sodium thiosulphate (Normal)
- W = weight of sample

#### 4. Measurement of conjugated diene (Frankel and Huang, 1996)

##### Chemicals

- Iso-octane

##### Method

Oil sample (0.1 g) was dissolved in 5.0 ml of iso-octane and the absorbance was measured at 234 nm. Conjugated diene was calculated as an increase in absorbance.

## **5. Determination of antioxidant activity in lecithin liposome system (Frankel *et al.*, 1997; Yi *et al.*, 1997)**

### **Chemicals**

- Soybean lecithin
- Cupric acetate (3.0  $\mu$ M)
- Butylated hydroxytoluene (BHT)
- TBA solution (15% TCA/ 0.375% TBA/ 0.025N HCl)
- Methanol

### **Method**

Lecithin (2.4 g) was suspended in deionized water at a concentration of 8 mg/ml by stirring with a glass rod and sonicating for 15 min. To test antioxidant activity, MRPs and decolorized MRPs were added to the lecithin liposome system to obtain final concentration of 100, 200 and 500 ppm. After addition of MRPs, the liposome suspension was sonicated again for 2 min. To initiate the assay, 20  $\mu$ l of cupric acetate (0.15M) was added. The mixtures were shaken (120 rpm) at 37°C in the dark. Liposome samples (1 ml) was added with 0.2% BHT (20  $\mu$ l) to stop the reaction. Liposome oxidation was monitored by determining thiobarbituric acid reactive substances (TBARS) and conjugated diene. For the control, the distilled water was added, instead of antioxidant in the assay system.

#### **1) Measurement of TBARS**

Liposome sample (1 ml) mixed with 20  $\mu$ l of butylated hydroxytoluene (0.2%) was added with 2 ml of TBA solution. The mixtures were then heated for 10 min in a boiling water bath (95-100°C) to develop pink color, cooled with tap water, and

centrifuged for 20 min at 5500xg. The supernatant containing the pink chromogen was quantified at 532 nm (Lee and Hendricks, 1997; Duh, 1998).

## **2) Measurement of conjugated diene**

Liposome samples (0.1 ml) were dissolved in methanol (5.0 ml) and conjugated dienes were measured at 234 nm (Frankel *et al.*, 1997).

## **6. Determination of antioxidant activity in $\beta$ -carotene-linoleic acid (Taga *et al.*, 1984)**

### **Chemicals**

- $\beta$ -carotene
- Chloroform
- Linoleic acid
- Tween 40

### **Method**

$\beta$ -carotene (1 mg) was dissolved in 10 ml of chloroform. A 3 ml aliquot of the solution was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed by purging with nitrogen. Oxygenated distilled water (50 ml) was added to the  $\beta$ -carotene emulsion and mixed well. 200 $\mu$ l of MRPs (100, 200 and 500 ppm) was mixed with oxygenated  $\beta$ -carotene emulsion (3 ml) and incubated at 50°C. Oxidation of the  $\beta$ -carotene emulsion was monitored spectrophotometrically at 470 nm after 0, 10, 20, 30 and 40 min incubation at 50°C. For the control, the distilled water was added, instead of antioxidant in the assay system.

## 7. Determination of hydrogen peroxide-scavenging activity (Ruch *et al.*, 1984)

### Chemicals

- 0.1M Phosphate buffer (pH 7.4)
- 43 mM Hydrogen peroxide

### Method

The MRPs (1.7 ml) was mixed with 1.7 ml of 0.1M phosphate buffer (pH 7.4) and mixed with 600 µl of 43 mM hydrogen peroxide. The absorbance of the reaction mixture was measured at 230 nm after 60 min. For each concentration of MRPs, a separate blank was prepared by leaving out Hydrogen peroxide from the assay system and used for background subtraction. For the control, the distilled water was added, instead of antioxidant in the assay system. Hydrogen peroxide scavenging activity (%) was calculated as follows:

$$\text{Hydrogen peroxide scavenging activity (\%)} = [1 - (A_{230 (60 \text{ min})} / A_{230 (0 \text{ min})})] \times 100$$

where  $A_{230 (60 \text{ min})}$  is the absorbance of sample after 60 min and  $A_{230 (0 \text{ min})}$  is the absorbance of the sample at time 0.

## 8. Determination of hydroxyl radical assay (Halliwell *et al.*, 1987)

### Chemicals

- Deoxyribose
- H<sub>2</sub>O<sub>2</sub>
- FeCl<sub>3</sub>
- EDTA
- Ascorbic acid

- Sodium hydrogen phosphate
- 1% w/v TBA
- 2% w/v TCA

### **Method**

The reaction mixture, containing 0.5 ml of MRPs or decolorized MRPs (100, 200 or 500 ppm), was incubated with deoxyribose (3.75 mM), H<sub>2</sub>O<sub>2</sub> (1 mM), FeCl<sub>3</sub> (100 μM), EDTA (100 μM) and ascorbic acid (100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37°C. The reaction was terminated by adding 1 ml of TBA (1% w/v) and 1 ml of TCA (2% w/v) and then heating the tubes in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture was measured at 532 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose. For each concentration of MRPs, a separate blank was prepared by leaving out deoxyribose from the assay system and used for background subtraction. For the control, the distilled water was added, instead of antioxidant in the assay system. Hydroxyl radical scavenging activity (%) was calculated as follows:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (A_{562} \text{ of sample} / A_{562} \text{ of control})] \times 100$$

## **9. Lipid extraction; Bligh and Dyer method (1959)**

### **Chemicals**

- Chloroform
- Methanol
- 43 mM Hydrogen peroxide
- Sodium sulfate anhydrous

## Method

Sample (50 g) was homogenized with 350 ml of chloroform: methanol: distilled water mixture (100: 200: 50) at the speed of 9,500 rpm for 2 min at 4°C using an IKA labortechnik homogenizer (Selangor, Malaysia). The homogenate was added with 100 ml of chloroform and homogenized at 9,500 rpm for 1 min. Then, 100 ml of distilled water was added and homogenized again for 30 sec. The homogenate was centrifuged at 2,000xg at 4°C for 20 min and transferred into a separating flask. The chloroform phase was drained off into the 125 ml Erlenmeyer flask containing about 2-5 g of sodium sulfate anhydrous, shaken very well, and decanted into a round-bottom flask through a Whatman<sup>®</sup> filter paper No.4. The solvent was evaporated at 25°C and the residual solvent was removed by flushing nitrogen.



## **Publications**

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Benjakul, S., Lertittikul, W. and Bauer, F. 2004. Antioxidant activity of Maillard reaction products from porcine plasma protein-sugar model system. Food Chem. In press.

Lertittikul, W., Benjakul, S. and Munehiko, T. 2004. Effect of pH on characteristics and antioxidative activity of Maillard reaction products from porcine plasma protein-glucose model system. Eur. Food Res. Technol. Submitted.

### **Conferences/ meeting**

Lertittikul, W., Benjakul, S. and Bauer, F. 2004. Maillard reaction products from porcine plasma protein-sugar model system: Characteristics and antioxidative activity. 30<sup>th</sup> Congress on Science and Technology of Thailand, Impact Exhibition and Convention Center, Muang Thong Thani, Bangkok. 19-20 October, 2004.