CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

Water spinach was purchased from four selected local hypermarkets in Negeri Sembilan. Organic water spinach (Figure 3.1 (a)) was presumed as pesticide-free and selected as a negative control for pesticide residue level determination. Non-organic samples were presumed to contain pesticide residue. The selection criterion for the non-organic samples were water spinach bundles displayed on rack without packaging prior to purchase as shown in Figure 3.1 (b)-(d). The plant materials were kept in an ice box and transported to the laboratory immediately within 2 hours.

(a) (b) (c) (d)

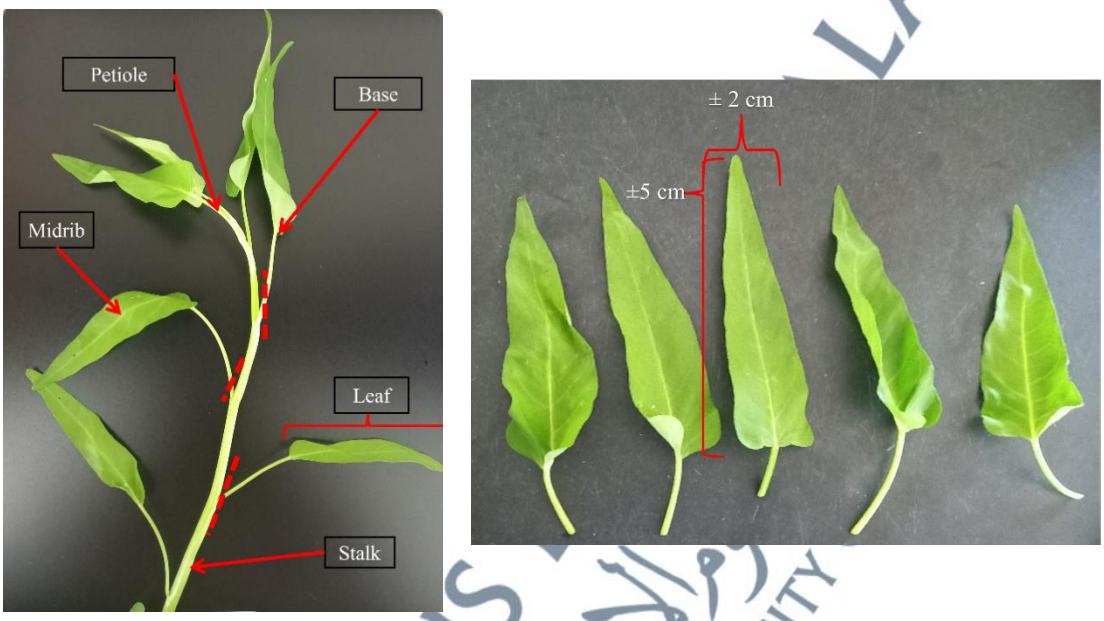
Figure 3.1: (a) Organic Sample and (b-d) Non-Organic Samples

3.2 Chemicals and Reagents

For the pesticide residue level determination, the pesticide standard diazinon, GC grade acetonitrile (C_2H_3N), sodium chloride (NaCl) and magnesium sulphate ($MgSO_4$) were purchased from Sigma Aldrich (St. Louis, USA). Primary secondary

amine (PSA) was obtained from Agilent Technologies (California, USA). Sterile peptone water (PW) used for dilution of the standard solution and plate count agar (PCA) from Oxoid (UK) were obtained for determination of microbial availability.

3.3 Sample Preparation



(a) (b)

Figure 3.2: (a) Section (As Marked) Where Water Spinach Leaves are Selected for Sampling and (b) Water Spinach Leaves Selected for Sampling

Fresh water spinach was cleaned and weighed. Leaves from the middle of water spinach, marked with dotted line as an example in Figure 3.2 (a) were cut with stainless steel scissors. Leaves and petiole with uniform colour and size (± 2.0 × 5.0 cm) were selected as shown Figure 3.2 (b).

3.4 Sample Treatment

Treatments were applied to water spinach leaves as follows; (F): fresh (untreated); (C1) : immersed in distilled water for 1 minute, (C7) : immersed in distilled water for 7 minutes, (C15):immersed in distilled water for 15 minutes, (F): fresh (non-treated); (U1) : ultrasonicated in distilled water for 1 minute, (U7) : ultrasonicated in distilled water for 7 minutes, (U15): ultrasonicated in distilled water for 15 minutes.

Table 3.2 summarises the duration and type of decontamination method applied on water spinach samples.

Table 3.1: Summary on Sample Treatment Methods

Treatment Duration (mins)	Method of Decontamination		
	F	C	U
0 **1** **7** **15**	No treatment (negative control)	Immersed in distilled water at 25°C (positive control)	In ultrasonic bath at 25°C

F: Fresh water spinach, C: water spinach immersed in distilled water, U: water spinach treated with ultrasonic waves

3.5 Ultrasonic Treatment

Treatment with ultrasonic wave was conducted at different duration using an ultrasonic bath (Power Sonic 405, Hwashin Technology, Korea). The tank was filled with approximately 3 L distilled water. A 600 mL glass beaker was filled with sterile saline water (SW). One sample leaf was immersed in the filled beaker and carefully placed in the centre of the ultrasonic bath tank before start of operation. Water temperature was set at 25°C throughout the treatment.

Samples were subjected to ultrasonic waves in water bath set at 40kHz frequency and 350W power output, for 1,7 and 15 minutes. After treatment, samples were stored in perforated polyethylene bags and stored at 4°C until analysis on the same day.

3.6 Pesticides Residues Determination

3.6.1 Stock Solution and Calibration Curve Preparation

For the calibration of GC-FID, stock solution of diazinon (1000 mg/L) was prepared. Pure standard was taken out of the refrigerator (2-8 °C) and the preparation was conducted in the dark room. Each 25 mg of standard was weighted into 25 mL volumetric flask and the volume was adjusted with acetonitrile. Labelled bottles were

stored at 4-6 °C. Standard stock solution (200 mg/L) was prepared by transferring 2 mL of stock solution using a 5 mL bulb pipette into 10 mL volumetric flask and adjusted with acetonitrile. Calibrations working standard concentrations of 200, 400, 600, 800 and 1000 ppm were prepared in 10 mL volumetric flask.

3.6.2 QuEChERS Extraction

50 g sample was weighed and homogenised with a blender (MX-337, Panasonic, Malaysia). 10 g homogenised samples were weighed in 50 mL centrifuge tubes and stored in freezer until further analysis. Samples were kept frozen in the next step to minimise possible loss of remaining pesticide residues due to its sensitivity to heat produced by the addition of salt during extraction process. 10 mL acetonitrile was added to the frozen sample followed by mixing in 8 g of $MgSO_4$ and 1 g of NaCl. The mixture was shaken vigorously using a vortex for 1 minute and centrifuged for 6 minutes at 4000 rpm.

3.6.3 Clean-Up Extraction

1.0 mL aliquot from the supernatant was transferred to 15 mL centrifuge tubes pre-filled with 150 mg of $MgSO_4$ and 50 mg of PSA. The tube was closed, shaken vigorously using a vortex for 30 seconds and then centrifuged for 4 minutes at 1000 rpm.

3.6.4 Chromatographic Analysis

To create the calibration curve and determine the level of pesticide residues, gas-chromatography coupled with flame ionisation detector (GC-FID) was used (Agilent 7890A GC System, USIM, Negeri Sembilan). GC-FID was injected with 1.5 mL of samples at temperature 300 ºC. The oven temperature program was programmed as

initial temperature 80 ℃ to 175 ℃ at 30 ℃/min to 225 ℃ (1 minute) at 15 ℃/min to 220 ℃ at 10 ℃/min to 240 ℃ (5 minutes) at 15 ℃/min and injector temperature was maintained at 230 ℃. The column used was HP-5 (30 m × 0.25 nm film thickness), carrier gas Helium purity (99.999% purity), and constant flow rate: 1 mL/min of the final extract were auto injected into the GC system.

3.7 Physical Quality Analysis

3.7.1 Physical Appearance Comparison

Physical appearance and colourimetric changes after each treatment of samples were compared and documented with photographs using a phone camera (Samsung Galaxy A12, Samsung, Korea).

3.7.2 Total Colour Changes (ΔE) Determination

A chromameter (Labscan XE, HunterLab, USA) was used for colour measurement determination. Zero calibration and white calibration was carried out beforehand. Colour parameters of samples (L*, a* and b*) was measured and the results were calculated as total colour change (ΔE) values measured in triplicate. Total colour changes (ΔE) were calculated as follows:

$$\Delta E = \sqrt{(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2} \quad (1.1)$$

where L_0^*, a_0^*, and b_0^* are colour values of water spinach prior to treatments.

3.7.3 Texture Analysis

Adapting methods from Oliveira et al.(2016), water spinach texture was determined by measuring the firmness. The instrument was set up by assembling a heavy-duty platform (HDP/90) with a 2 mm stainless steel cylindrical probe (P/2) and

using a 5 kg load cell attached to a texture analyser (TA.XTplus Texture Analyzer, Stable Micro Systems, Godalming, U.K.). Pre-test speed was set at 2 mms^{-1}, test speed was 1 mms^{-1}, and post speed was 10 mms^{-1}.

Figure 3.3: Set-Up for Leaf Firmness Measurement

Before starting the analysis, the petiole of one water spinach leaf (refer to Figure 3.2 (a)) was removed and the leaf placed on the platform as shown in Figure 3.3. The probe was positioned over the area to be compressed, avoiding the midrib region (refer to Figure 3.2 (b)). Analysis was then run on the sample and the maximum force required to penetrate the leaf was recorded using a software (Exponent Stable Microsystem Texture Expert, Stable Micro Systems Ltd. Software). Three replicates per treatment were carried out and the results were presented as averages of three values.

3.8 Microbiological Analysis

3.8.1 Total Plate Count

The plate count technique was done according to Bacteriological Analytical Manual (BAM) (FDA, 2001) with some modifications. About 25 g of samples was homogenized in 225 mL of 0.1% sterile saline water (SW) in stomacher bags for 2 minutes. Appropriate ten-fold dilutions were prepared in 0.1% SW and aliquots (0.1mL) plated on plate count agar (PCA) and were incubated at 35°C for 48 hours. The results were expressed as log colony-forming units per gram (log CFU/g).

3.9 Statistical Analysis

All procedures were run in triplicate. All values were reported as means ± SD (standard deviation) for each treatment of triplicates. Statistical analyses were conducted using IBM SPSS Statistics for Windows (SPSS 23, IBM Corp., USA). Analyses of variance was performed using ANOVA procedures and Tukey post-hoc test to assess the effects of treatment variables (duration of ultrasound exposure) on the responses (pesticide residue level, microbial availability, firmness and microbial availability). The results were considered statistically significant if the p-value obtained is less than 0.05.