CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Pesticide Residue Determination

4.1.1 Pesticide Standard

This research focused on the presence of diazinon on water spinach samples from Negeri Sembilan. The chromatogram for 1000 ppm diazinon pesticides standard was shown in Figure 4.1. Diazinon peaked at 13.794 minutes, with a sharp peak, no fronting and tailing.

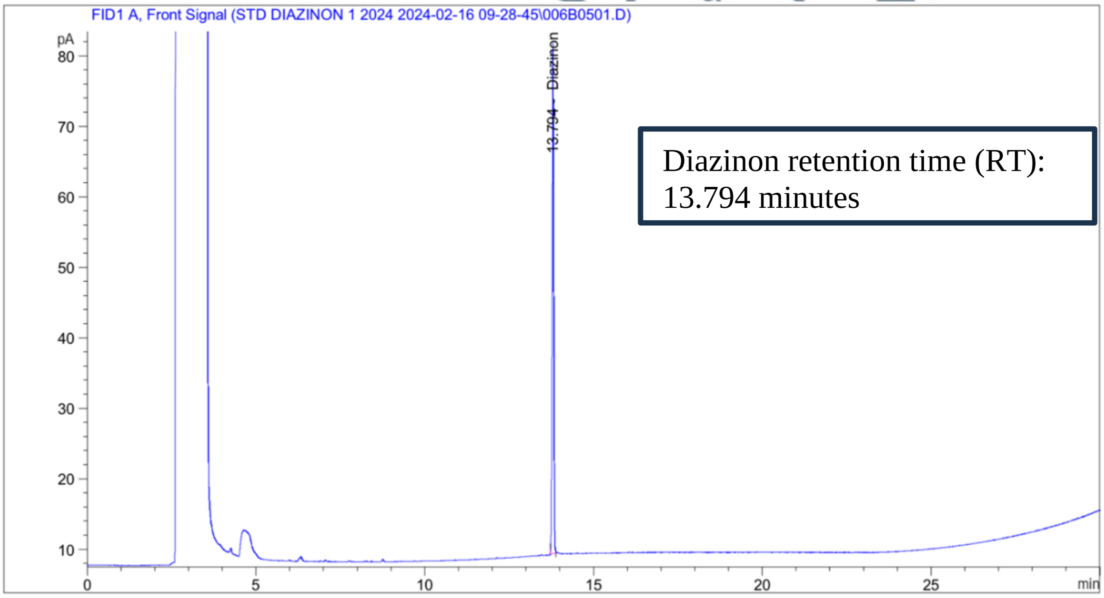


Figure 4.1: Chromatogram of Standard Diazinon

4.1.2 Method Validation

Method validation is performed to obtain results that are reliable, accurate and consistent within a sample analysis' statistical limits (FAO/WHO, 2017). Calibration curve reflected the relationship between the analyte concentration in the sample and the analytical signal as accurate as possible. Standard concentrations 200, 400, 600, 800 and 1000 ppm were used to construct the calibration curve (Figure 4.2). The linearity was measured, and the correlation coefficient (R2) obtained was higher than 0.99 (Table 4.1). However, the use of this value as a quality of fit can be misleading because the significance is greater if high concentrations of standards are chosen to construct the curve (FAO/WHO, 2017).

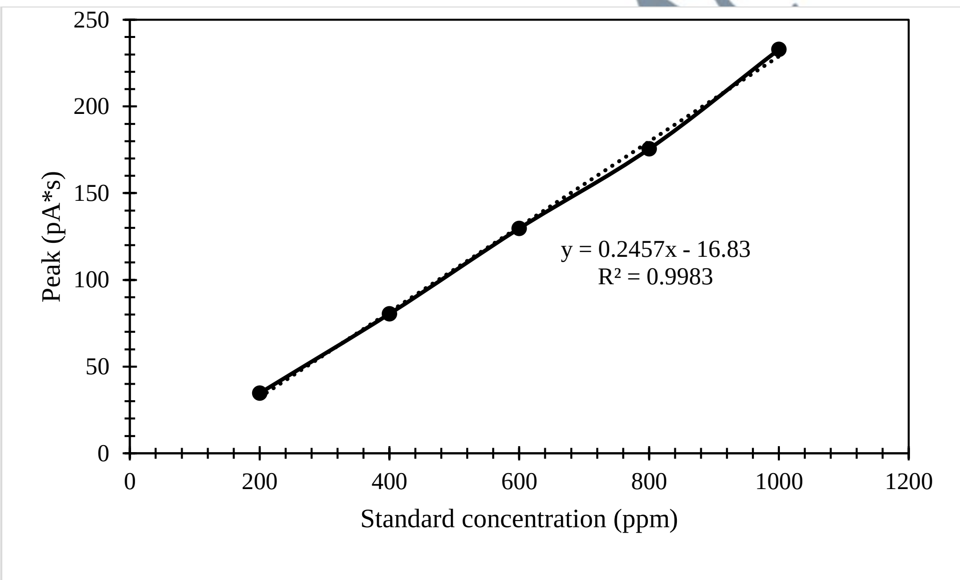


Figure 4.2: Calibration Curve for Diazinon Standard

The range of standards (200-1000mg/kg) used to construct the calibration curve for this study was high. For GC-FID, range as low as 0.1-1.5µg/kg has been used to detect diazinon in apple samples (Kilicel et al., 2018). Based on a previous study that successfully detected diazinon residue in water spinach, the range for the calibration curve was between 2 to 10 mg/kg (Md Sa'at, 2020). The Guidelines on Performance Criteria for Methods of Analysis for The Determination of Pesticide Residues in Food and Feed recommended the use of weighted-linear regression in determining low pesticide concentration down to part per billion (μg/kg) and reducing errors in residue level calculation by obtaining value of the intercept close to zero.

Table 4.1: Validation Data for Pesticides Residue Detection Using GC-FID

Organophosphorus pesticide	Diazinon
Retention Time (RT) (min)	13.794
Equation	y = 0.2457x - 16.83
Coefficient of Detection (R^2)	0.998
Limit of detection (LOD) (ppm)	52.3
Limit of quantification (LOQ) (ppm)	158
Relative Standard Deviation (RSD) (n=5) %	0.246

According to Sanagi et al. (2009), limit of detection (LOD) is generally defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test whereas limit of quantification (LOQ) is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test. LOD and LOQ are the minimum amount of analyte that can be detected and quantified using an analytical method or instrument without being confused with the analytical noise (Armbruster & Pry, 2008).

As recovery spike was not performed, reagent blank (acetonitrile) was measured directly by the instrument. The extraction method was validated by calculating the LOD and LOQ based on the standard deviation of the response of the standard blank and the slope of calibration curve at signal-to-noise ratio of 3 and 10, respectively (Appendix 1). The LOQ value should be at or below the maximum residue level (MRL) to draw significant conclusions about pesticide residues in a sample (Shrestha et al., 2024). As summarised in Table 4.1, LOD for diazinon was 52.3 ppm whereas LOQ value was 158 ppm. Therefore, any conclusion of detection of pesticide residue in this study may be not significant as the LOQ value was above the MRL value for diazinon.

It is difficult to ascertain the amount of diazinon present in the water spinach sample, therefore method validation is required to determine the efficacy of QuEChERS extraction performed in this study. Spiking is a method to determine if the extraction method is efficient by spiking a sample with an analyte of a known concentration, performing the extraction procedure and measuring the spiked analyte concentration. (Magnusson & Örnemark, 2014). The amount successfully retrieved is measured as a percentage of the amount of original spiking analyte added to the sample with contains either no detectable level of the analyte or a known detectable level. This method is known as recovery experiments, which measures the accuracy, precision and trueness of the method (FAO/WHO, 2017). Therefore, in the case of this study, pesticide-free water spinach samples can be spiked with various concentration of diazinon, then extraction is performed and the recovery percentage of spiked diazinon is determined.

The numerical values of RSD in Table 4.1 did not exceed 10% independently of the composition of the pesticides in analysed products which indicates good reproducibility in the subsequent repetitive measurement.

4.1.3 Detection and Quantification of Diazinon Residue

Diazinon was not detected in the organic water spinach samples tested as negative control (Table 4.2). Organic water spinach is supposed to be pesticide-free and the organic samples' results confirmed the authenticity of the pesticide-free claim. However, organic vegetables are susceptible to be tainted by pesticide residue from soil shared with nearby non-organic farms that liberally used pesticides (Farina et al., 2018).

The average concentration of diazinon for each sample was tabulated in Table 4.2. One sample (U1) was found to have traces of diazinon at 3.07 mg/kg (Appendix 2), which exceeded the permitted maximum residual limit (MRL) of 0.2 mg/kg set in the

Sixteenth Schedule [Regulation 41] Pesticide Residue of Food Regulations 1985. The U1 sample was treated by ultrasonic treatment for 1 minute and there was no pre-treatment value (F) to compare with this sample to evaluate the efficacy of ultrasonic treatment in removing diazinon residues on water spinach. The value was also lower than LOD calculated in section 4.1.2.

Table 4.2: Average Concentration of Pesticide Residue Found in Water Spinach Samples (n = 4)

Treatment	Average Concentration (mg/kg)	
	Organic	Non-organic
F	ND	ND
C1	ND	ND
C7	ND	ND
C15	ND	ND
U1	ND	3.07
U7	ND	ND
U15	ND	ND

(F): fresh (non-treated); (C1): immersed in distilled water for 1 minute, (C7): immersed in distilled water for 7 minutes, (C15): immersed in distilled water for 15 minutes; (U1): ultrasonicated in distilled water for 1 minute, (U7): ultrasonicated in distilled water for 7 minutes, (U15): ultrasonicated in distilled water for 15 minutes; ND: not detected.

Due to issues with method validation discussed in section 4.1.2, there are possibilities that the detection and quantification of diazinon in water spinach samples were not as accurate and precise as expected. However, it is also possible that there may not be any presence of diazinon in the samples acquired. All samples were sourced from hypermarkets, where the distribution chain is complex because between the farm and the centralised distribution centre, there are multiple parties involved such as collectors or transporters, wholesalers and middleman (Man et al., 2009). Due to this circumstance, it was almost impossible to trace the samples to the farms and found out the pesticides used on the water spinach prior to harvesting.

The absence of diazinon in water spinach samples in this study does not mean that they are pesticide-free. Presence of other pesticides that possess health risk cannot be ruled out, as mentioned by (Ling & Mahat, 2014). The findings in this study also corroborated the disadvantages of single-residue method as compared to multiresidue method in pesticide analysis in ensuring the safety of leafy vegetables (Villaverde et al., 2014).

4.2 Effects of Ultrasonic Treatment on Physical Qualities

4.2.1 Effects of Ultrasonic Treatment on Physical Appearance

The physical appearance of water spinach leaves after various treatments for organic and non-organic samples were shown in Figures 4.3 and 4.4. All samples treated as control or ultrasonicated appeared firmer compared to untreated samples.

Figure 4.3: Difference Between Ultrasonic-Treated Leaf After (a) 7 Minutes and (b) 15 Minutes Treatment Duration

Prolonged ultrasonic treatment after 7 minutes caused physical damage to the leaves treated with ultrasonic waves for 15 minutes as presented in Figure 4.3. The ultrasonic-treated leaf (Figure 4.3(b)) exhibited damage along the edges on the left side of the leaf while no damage at the edges was observed with the leaf treated with ultrasonic waves for 7 minutes (Figure 4.3(a)).

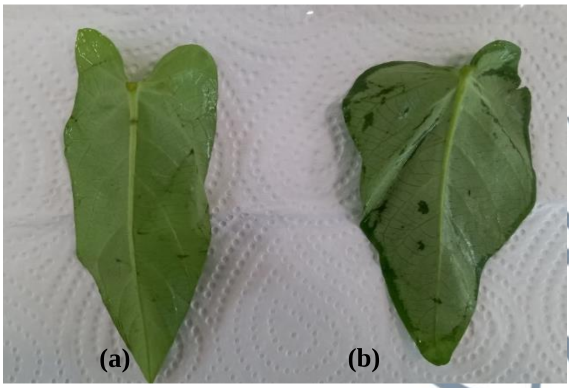
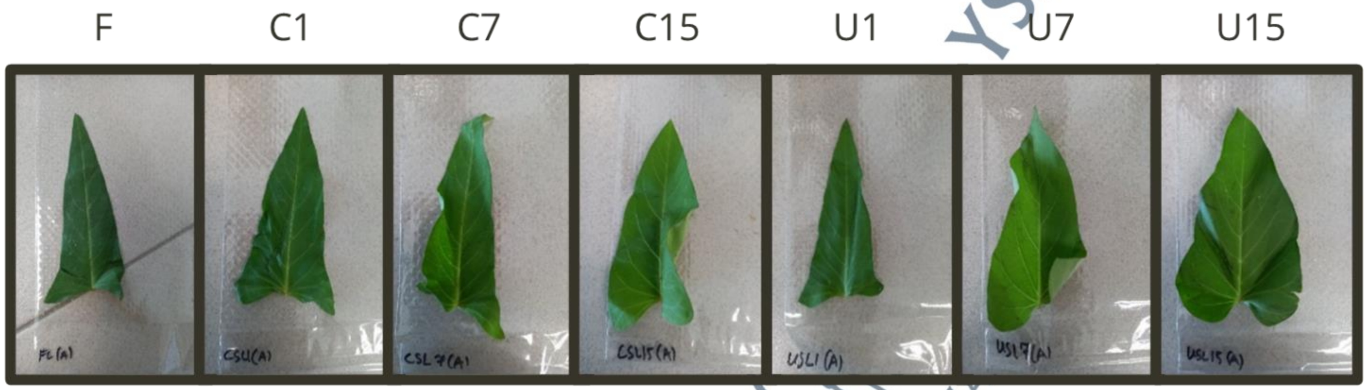


Figure 4.4: Difference Between (a) Control Leaf and (b) Ultrasonic-Treated Leaf After 15 Minutes Treatment Duration

Comparison of physical damages between control samples and ultrasonicated samples after 15 minutes' treatment was shown in Figure 4.4. The ultrasonicated leaf exhibited physical damage on the left side of the leaf while no clear physical damage was observed on the control sample.

The physical damage to ultrasonicated leaves can be attributed to several mechanisms identified in ultrasonic-assisted extraction (UAE). One mechanism is facilitated mass transfer caused by fragmentation during ultrasonic treatment, where implosion of acoustic bubbles onto the surface of plant matrix causes erosion and leads the solvent into the water spinach plant cells (Shen et al., 2023). Other possible mechanisms include sono-capillarity and sonoporation, process, which enhance liquid penetration via the channels produced by the bubble implosion and the alteration of the permeability of the cell membranes, respectively (Medina-Torres et al., 2017).

Physical damage not only led to reduction in consumer appeal towards treated water spinach, but also potentially shorten the shelf-life and affects nutritional contents (Zhu et al., 2018).



(F): fresh (non-treated); (C1): immersed in distilled water for 1 minute, (C7): immersed in distilled water for 7 minutes, (C15): immersed in distilled water for 15 minutes; (U1): ultrasonicated in distilled water for 1 minute, (U7): ultrasonicated in distilled water for 7 minutes, (U15): ultrasonicated in distilled water for 15 minutes.

Figure 4.5: Visual Comparison of Organic Samples

(F): fresh (non-treated); (C1): immersed in distilled water for 1 minute, (C7): immersed in distilled water for 7 minutes, (C15): immersed in distilled water for 15 minutes; (U1): ultrasonicated in distilled water for 1 minute, (U7): ultrasonicated in distilled water for 7 minutes, (U15): ultrasonicated in distilled water for 15 minutes.

Figure 4.6: Visual Comparison of Non-Organic Samples

The comparison between organic and non-organic samples across all treatments are recorded by digital imaging in Figures 4.5 and 4.6. This comparison is not statistically tested to prove any statistical differences between the treatments. Therefore, qualitative analysis involving imaging-based technique can be applied to be able to perform statistical tests. One of the techniques involving feature extraction from segmented grey image of sample followed by feature analysis of the coefficients of wavelet transform (Dutta et al., 2016). Another type of analysis is by machine learning and deep learning approaches (Meenu et al., 2021).

4.2.2 Effects of Ultrasonic Treatment on Water Spinach Colour

Colour influences consumers' choice of vegetables as it indicates freshness and flavour.(Barrett et al., 2010). Table 4.3 summarised the changes of brightness or lightness (L*), the green colour represented by negative values of a* scale and influence of blue (positive b* scale) of water spinach across all treatments (Kutlu et al., 2022).

For organic samples, only three treatments (C1, U7 and C15) produced higher L* value than the pre-treatment value. All treatment on non-organic samples recorded higher L* values between 37.29 and 39.31 compared to the pre-treatment value of 36.97. The lightness of water spinach leaves was not significantly different between pre-treatment (F) and post-treatment samples, indicating that both the control and ultrasonic treatment between 0 to 15 minutes did not significantly alter the lightness of water spinach.

Table 4.3: Changes in Colour Parameters

Treatment	L*		a*		b*	
	Organic	Non-organic	Organic	Non-organic	Organic	Non-organic
F	37.05 ±0.69[ab]	36.97 ±2.25[ab]	-7.28 ±0.02[a]	-9.10 ±1.12[ab]	14.68 ±0.19[b]	20.82 ±3.86[ab]
C1	37.68 ±0.56[ab]	38.35 ±1.75[ab]	-9.65 ±0.52[ab]	-8.90 ±1.92[ab]	20.09 ±0.77[ab]	21.21 ±3.65[ab]
C7	35.83 ±1.11[ab]	38.67 ±2.73[ab]	-10.23 ±0.21[ab]	-9.73 ±0.88[ab]	21.89 ±0.72[a]	22.97 ±1.82[a]
C15	40.19 ±0.29[a]	38.92 ±1.31[ab]	-10.28 ±0.29[ab]	-9.25 ±2.02[ab]	22.50 ±0.48[a]	23.21 ±3.40[a]
U1	34.38 ±1.33[b]	37.46 ±2.18[ab]	-9.30 ±0.85[ab]	-9.29 ±0.68[ab]	19.83 ±1.10[ab]	21.42 ±2.56[a]
U7	38.46 ±1.53[ab]	39.31 ±1.39[a]	-10.75 ±1.39[b]	-9.70 ±1.57[ab]	23.63 ±2.20[a]	22.57 ±3.05[a]
U15	36.85 ±0.62[ab]	37.29 ±1.84[ab]	-8.14 ±0.48[ab]	-9.12 ±0.10[ab]	18.64 ±0.93[ab]	21.54 ±1.32[a]

(F): fresh (non-treated); (C1): immersed in distilled water for 1 minute, (C7): immersed in distilled water for 7 minutes, (C15): immersed in distilled water for 15 minutes; (U1): ultrasonicated in distilled water for 1 minute, (U7): ultrasonicated in distilled water for 7 minutes, (U15): ultrasonicated in distilled water for 15 minutes; ND: not detected.

The a* value was indicative in the change of the green colour of water spinach as chlorophyll is present in all parts of water spinach excluding the root part. The green colour appearance gives the positive indication of freshness of the vegetable (Saba et al., 2018). All post-treatment organic samples recorded higher negative a* values (between -8.14 and -10.75) than the pre-treatment value of -7.28. There was no significant difference between treatment type and treatment duration. Non-organic samples exhibit similar pattern, except for C1 treatment that recorded lower negative a* value.

The b* scale hovers between the intensity of yellow (negative) and blue (positive). Yellowing of leafy vegetable is undesirable as it indicated quality deterioration (Yuan et al., 2021). For all treatments, the post-treatment values were higher than the pre-treatment value. There was a significant increase in b* value between pre-treatment value of organic sample and U7 treatment. All treatment did not cause yellowing of water spinach as the increase in positive values moves away from the increase in yellow appearance.

Total colour change (ΔE) is a quantitative indicator on how post-harvest treatment alters the colour of water spinach pre- and post-treatment. Hartyáni et al. (2011) constructed the range as follows: values between 0 to 0.5 indicates that the colour change is not noticeable, values between 0.5 and 1.5 denotes slightly noticeable colour change, noticeable (1.5–3.0), well visible (3.0–6.0) and great difference (6.0–12.0). The results were presented in Table 4.4.

There was no significant difference in treatment duration for all control samples in this study and non-organic ultrasonicated samples. Therefore, ultrasonic treatment of water spinach did not alter the colour significantly compared with untreated or soaked water spinach. Unlike disinfectants which has strong oxidising effects, ultrasonication has no strong oxidizing properties, therefore the colour of the fruits and vegetables was not degraded by oxidation effects (Lee & Feng, 2010).

Table 4.4: Effects of Ultrasonic Treatment on Total Colour Change (ΔE)

Treatment duration (mins)	**ΔE**			
	Organic		Non-Organic	
	Control	Ultrasonicated	Control	Ultrasonicated
1	3.40 $\pm0.28^{ab}$	3.76 $\pm1.07^{ab}$	2.04 $\pm0.76^{cd}$	2.46 $\pm1.12^{bcd}$
7	4.46 $\pm0.16^{a}$	2.15 $\pm0.23^{cd}$	1.22 $\pm0.49^{d}$	1.12 $\pm0.65^{d}$
15	5.22 $\pm0.30^{a}$	2.03 $\pm0.24^{cd}$	2.01 $\pm0.85^{cd}$	1.95 $\pm1.11^{cd}$

According to this the following ranges were applied on which base the colour parameters could be distinguished as follows: not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great difference (6.0–12.0) (Hartyáni *et al.,* 2011). Values are means of three replicates ± SD. For each sample, mean values with different superscript letters in each column indicate significant differences ($p<0.05$, ANOVA).

Non-organic water spinach treated with ultrasonic waves for 7 minutes showed lowest total colour change values of 1.12 under the range of slightly noticeable (0.5-1.5). The mechanical action of ultrasonic waves may have contributed to the inhibition of chlorophyllases, an enzyme that degrades chlorophyll and able to maintain the colour of fresh greens such as water spinach. This finding further supports the role of ultrasonic treatment in preserving the green colour in vegetables as previously discovered by Xu et al. (2020) and C. Zhang et al. (2023).

Organic samples showed well visible differences in colour (between 3.40 to 5.22) according to the scale, especially the control treatment samples. There is a possibility of senescence process taking place between the pre-treatment analysis and post-treatment analysis, which causes chlorophyll degradation. It has been reported that senescence can occur in a few hours for water spinach due to its sensitivity to the environment (Hu et al., 2015).This senescence process may be accelerated by the exposure to several warm conditions during the experiment, as previously discovered by (Wijerathne et al., 2018). Firstly, the water used for soaking the control samples are set at 25 °C, which is warmer than the average temperature of tap water in Malaysia between 16-19°C (Ahmad et al., 2013). Another condition is the air-drying after the soaking treatment in ambient temperature up to 30 °C.

4.2.3 Effects of Ultrasonic Treatment on Leaf Firmness

Ultrasonic treatment produces physical actions by cavitation effects to remove contaminants from the surface of water spinach leaves (W. Zhou et al., 2022). The action may cause structure alteration or property changes of enzymes and proteins, leading to textural changes such as loss of firmness, which is an indicator of low-quality fresh produce (São José et al., 2014).

Firmness of water spinach leaves was determined as the resistance to penetration with a 5mm-probe. The results were presented in Figure 4.7. All organic samples showed increased firmness from the initial pre-treatment value of 5.02N with increased treatment duration (both control and ultrasonic) up to 7 minutes. For organic samples treated by both control and ultrasonic treatment, treatment for 7 minutes gave the highest increase in firmness for water spinach leaves, with an increase by 2.06 N and 0.59N respectively.

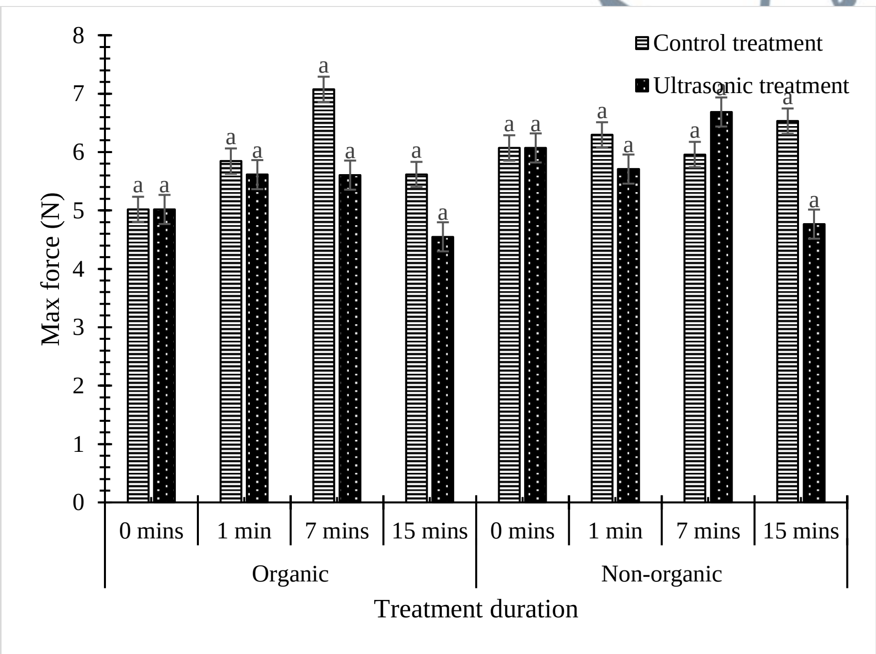


Figure 4.7: Maximum Force Required to Penetrate Water Spinach Leaves

Non-organic samples showed fluctuations in the maximum force value with increasing treatment time for both control and ultrasonicated samples. This was due to the destructive nature of the compression test where it is not possible to use the same leaf to measure the firmness pre-and post-treatment.

However, a modification to the methodology may give a better representation of the effects of both control and ultrasonic treatment on the leaf firmness. By having a batch of leaves stacked on top of each other up to 1 cm thickness and setting the compression test to penetrate approximately 15% of the height, the damage on the leaf will be minimised in the pre-treatment measurement and there will be undamaged leaf in the same batch to be used for post-treatment measurement. This modification will be able to allow the use of the same (batch) of leaves for pre-and post-treatment measurement.

All ultrasonicated water spinach treated for 15 minutes showed reduction in firmness from the pre-treatment values. For the organic sample, the firmness post-treatment was 4.65 N, showing a reduction of 0.47 N. The non-organic sample post-treatment showed reduction by 1.30 N, from pre-treatment value of 6.07 N to 4.47 N. It has been hypothesised that prolonged ultrasonication imposes destructive effects on plant cell wall stability, causing cell injury and loss of water leading to loss in firmness (Muzaffar et al., 2016). Moisture loss by evaporation from surface layer of ultrasonic-treated Barhi dates has also been reported to contribute to loss of firmness with increasing treatment duration due to increase in operating temperature during treatment (Abdelkarim et al., 2022).

Based on Figure 4.7, differences in leaf firmness are not statistically significant between organic and non-organic samples as well as samples treated at different durations for both control and ultrasonicated samples. There were also no statistical differences between pre-treated (0-minute treatment) and post-treated water spinach samples, similar to findings on fresh-cut cucumbers by C. Zhang et al. (2023).

4.3 Effects of Ultrasonic Treatment on Microbial Availability

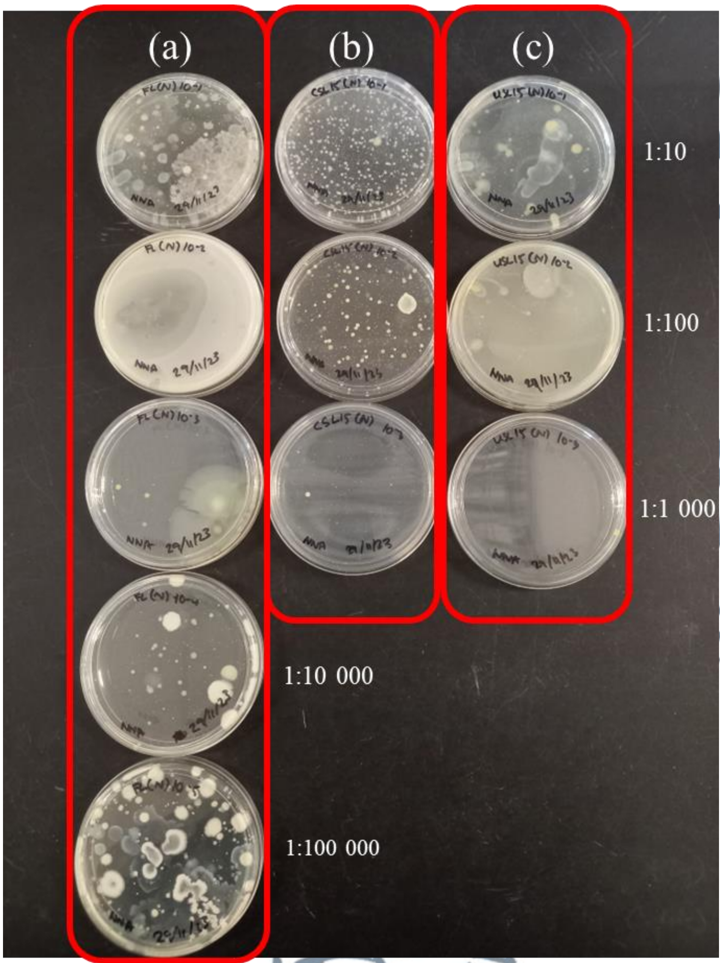


Figure 4.8: Representative Visual Comparison of Microbial Availability Between (a) Pre-Treatment Samples, (b) Control Samples and (c) Ultrasonicated Samples

Reduction in microbial availability ensures prolonged shelf-life and makes raw or minimally processed vegetables safer for human consumption. Figure 4.8 visualised comparison between pre-treatment and post-treatment samples. The pre-treatment samples shown in Figure 4.8 (a) has high microbial availability up to 1 $\times 10^5$ cfu/g compared to post-treatment samples, where the microbial availability has been successfully reduced by more than 2 log reduction as shown in Figure 4.8 (b) and (c). The increase in colony forming units in 10^4 and 10^5 dilutions in Figure 4.8 (a) may be caused by cell aggregation due to absence of pre-treatment to separate bacterial cell flocs (Di Caprio, 2020) or due to clumping of colonies caused by inadequate mixing of the sample with the melted agar during pour plate preparation (Sanders, 2012).

There was currently no internationally acknowledged microbiological standards for raw vegetables nor there is a Malaysian standard, however there are recommendations of a safe level for consumption (Badosa et al., 2008). For example, in Ireland, the acceptable level of microorganism in vegetables which is less likely to cause foodborne illness is below 10^5 cfu/g (Institute of Medicine (US) and National Research Council (US) Committee on the Review of the Use of Scientific Criteria and Performance Standards for Safe Food, 2003). Based on the pre-treatment findings in this study, all water spinach samples have acceptable levels of microorganisms.

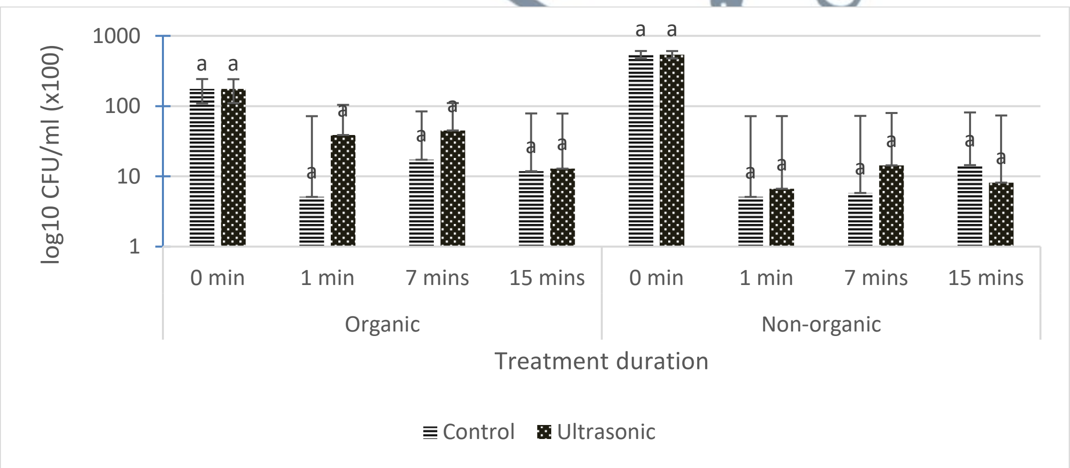


Figure 4.9: Total Plate Count Representing Microbial Availability in Water Spinach Leaves

Figure 4.9 presented the comparison between pre-treated and post-treated water spinach leaves, which presented reduction of total microbial availability after both immersion (control) and ultrasonic treatment. There is no significant difference in the reduction of microbial availability between control and ultrasonicated samples. This finding supported the outcome of Microbiological Risk Assessment Series No. 44

conducted by the Food and Agriculture Organization of the United Nations and World Health Organization that currently no postharvest treatment for leafy vegetables produces significant microorganisms' reduction (FAO & WHO, 2023).

Table 4.5: Comparison Between Control and Ultrasonic Treatment in Reducing Microbial Availability

Treatment time (mins)	Reduction (log)			
	Organic		Non-organic	
	Control	US	Control	US
1	1.54	0.66	2.03	1.91
7	1.01	0.59	1.97	1.58
15	1.17	1.14	1.57	1.82

The comparison of log reduction between the control treatment and ultrasonic treatment was presented in Table 4.5. The reduction of microorganisms was much higher in all control samples for all treatment duration except for the non-organic samples treated for 15 minutes. The highest log reduction for both control and ultrasonicated treatment were 2.03 and 1.91 respectively at 1 minute treatment. The discrepancies in the total plate count data collection may be due to varying degrees of intermediate precision and reproducibility and counts were done manually, causing large total error (Fusco et al., 2021).

The 15-minutes treatment of non-organic water spinach samples by ultrasonic treatment showed a higher log reduction (1.82) compared to control treatment (1.57). Although the data in this study was not statistically significant, there are findings that longer ultrasonic treatment duration resulted in significant decrease of microbial availability on cherries (Muzaffar et al., 2016; Pinheiro et al., 2015).The lower values of log reduction for almost all ultrasonic-treated organic and non-organic samples in this study may be attributed to difficulty in removing viable microorganisms due to high affinity of adhesion to vegetable surface resulting from long periods of time between harvesting and post-harvest cleaning (Lúcia et al., 2018).

Washing and soaking can remove microorganisms from raw water spinach physically, however there is possibility for bacteria to thrive via mechanical damage on leaves that allows easier penetration into nutrient rich cell cytoplasm. With ultrasonic waves applied during washing or soaking, it provides additional action to ensure bacterial damage that affects the viability by action on cell membrane and inactivation of intracellular esterase (Traore et al., 2019).