CHAPTER 3

MATERIALS AND METHODS

3.1 Extraction And Determination of Pestcide's Residues in Date Fruits Using QuEChERS Extraction Method and HPLC-DAD Analysis

3.1.1 Chemicals and Reagents

HPLC grade acetonitrile, and methanol were obtained from Merck (Germany), pesticides standards were of high purity from Sigma-Aldrich, Pestanal™. Dimethoate (98.0 %), carbendazim (98.9 %), chlorpyrifos, (98 %). Anhydrous sodium acetate, magnesium sulfate was from MERCK, KGaA, EMSURE® and glacial acetic acid were purchased from Fisher Scientific.

3.1.2 Instrumentation

Agilent 1200 HPLC system with diode array detector DAD, Agilent Zorbax XDB C18 5 mm 4.6 x 250 mm 5 m column, 95 % MeOH + 5 mM Ammonium formate, and 5 % H_2O + 5 mM Ammonium formate mobile phase, 20 µl injection volume, 0.2 ml/min flow rate.

3.1.3 Preparation of Pesticides' Standard Solutions for Chromatographic Analysis

Individual pesticide standards at various concentrations were made as stock solutions in methanol and stored at 5°C (Dimethoate 1mg /mL, chlorpyrifos 1mg /mL and carbendazim 0.25 mg /ml). Dilution of the stock solutions yielded mixed standard

solutions containing the specified pesticides at various concentration levels, which were utilized for spiking samples. All solutions were kept at a temperature of 5°C.

3.1.4 Selection and Preparation of Date Fruits Samples

Date fruits samples were collected from stores in Selangor, Malaysia, classified and labeled according to the species name, the exporting country, manufacturing, and expiration date. Each sample was divided into two portions. First portion was used as blank or control sample for equality control and determination the pesticide residues and heavy metals contents. The second portion was used for spiking with pesticide standards to calculate the recovery percentage for each pesticide. The list of samples used in this study are shown in Table 3.1.

Table 3.1: Characterization of Date Fruits Samples

Sample no.	Sample variety	Sample case	Country of issue	Expiration date
1	Ajwa	Loose	KSA	23/12/2018
2	Deglet Nour	Packed	TUNISIA	12/2018
3	Lulu	Packed	UAE	13/11/2019
4	Mariami	Loose	UAE	22/1/2019
5	Zahdi -1	Packed	UAE	11/5/2019
6	Medjool	Packed	UAE	2/12/2018
7	Zahdi. -2	Packed	UAE	19/1/2019
8	Mabroom	Packed	UAE	26/7/2018
9	Khudri	Packed	KSA	20/2/2018
10	Lamry	Packed	-	3/2/2019
11	Palm dates	Packed	UAE	28/12/2018
12	Nomades (Promel)	Packed	TUNISIA	-
13	Safawi	Packed	UEA	6/2/2019
14	Khasouei	Packed	KSA	29/1/2019
15	Khalas-Ihsa	Packed (paste)	KSA	2/2018
16	Frad	Packed	UAE	-
17	Roshdi	Packed	KSA	-
18	Mashrook	Packed	KSA	2017
19	Rabi	Packed	IRAN	19/9/2018
20	Safawi	Packed	KSA	23/3/2020

3.2 Optimization of Acetate Buffered Method (AOAC)

QuEChERS methodology was built on separating compounds in two immiscible liquids depending on their different solubilities using a single step with solvent extraction and salting-out of the water using dehydrating salts, followed by a depressive solid-phase extraction (d-SPE) for cleanup. The method is employed for pesticide extraction from food stuff with water content >80 %. As the date fruits have a low water content of <20% therefore, there was need for modification of the QuEChERS methodology regarding to the factors involved in the extraction and clean-up conditions (acetic acid %, Acetonitrile (CH_3-CN), water (H_2O), magnesium sulfate ($MgSO_4$), sodium acetate ($NaCOCH_3$) primary secondary amine (PSA), and C18, in conventional analytical methods, the significance of interactions between factors is often not considered. Hence, conventional optimization approaches for analytical methods often not succeed to achieve exact designs. However, in this study, the chemometric method namely, Minitab17.0 software, Plackett-Borman design (P–B design) statistical technique was used in screening the above-mentioned factors for modifying QuEChERS method to achieve the highest recoveries of dimethoate carbendazim, and chlorpyrifos in date fruits using high performance liquid chromatography and DAD detector.

3.2.1 Screening Design of The Extraction Variables

In this study, the chemometric method namely, Plackett-Burman design (P–B design) statistical technique (Manav et al., 2019) was used in screening the above-mentioned factors for modifying QuEChERS method to achieve the highest recoveries of dimethoate, carbendazim, and chlorpyrifos in date fruits using high performance liquid chromatography and DAD detector. Seven factors of the extraction and clean up

solution composition were chosen i.e. (Acetic acid, 0.75-1.25% v/v), Acetonitrile (10 - 15 mL), Water (5-10 mL), Anhydrous magnesium sulfate (4000 - 6000 mg), Anhydrous sodium acetate (1000-3000 mg), Primary secondary amine, (20-50 mg), C18 (20 - 50mg) (Table 3.2). The tested weighs and volumes ranges were chosen to include the minimum and the maximum quantities reported in literature for extraction and cleanup of fruits and vegetables. The main effect of each factor was investigated in 13 runs (12+ 1 center point), and analysis of variance (ANOVA) and test at a 95% confidence level were employed. The Plackett-Burman design (P–B design) experiments were run in a random manner to decrease the influence of uncontrolled variables. Moreover, the effectiveness and applicability of the developed method were evaluated in real samples. Figure 3.1 showed the flowchart of Sample Preparation of Modified Buffered and unbuffered QuEChERS.

Table 3.2: Plackett-Burman Design (P-B Design)

Std. Order	Run order	Pt. Type	Blocks	Acetic acid	MeCN	H_2O	$MgSO_4$	NaOAc	PSA	C18
11	1	1	1	0.75	15	5	4000	1000	50	50
2	2	1	1	1.25	15	5	6000	1000	20	20
6	3	1	1	1.25	15	10	4000	3000	50	20
13	4	0	1	1	12.5	7.5	5000	2000	35	35
5	5	1	1	1.25	15	5	6000	3000	20	50
7	6	1	1	0.75	15	10	6000	1000	50	50
4	7	1	1	1.25	10	10	6000	1000	50	20
12	8	1	1	0.75	10	5	4000	1000	20	20
8	9	1	1	0.75	10	10	6000	3000	20	50
9	10	1	1	0.75	10	5	6000	3000	50	20
3	11	1	1	0.75	15	10	4000	3000	20	20
10	12	1	1	1.25	10	5	4000	3000	50	50
1	13	1	1	1.25	10	10	4000	1000	20	50

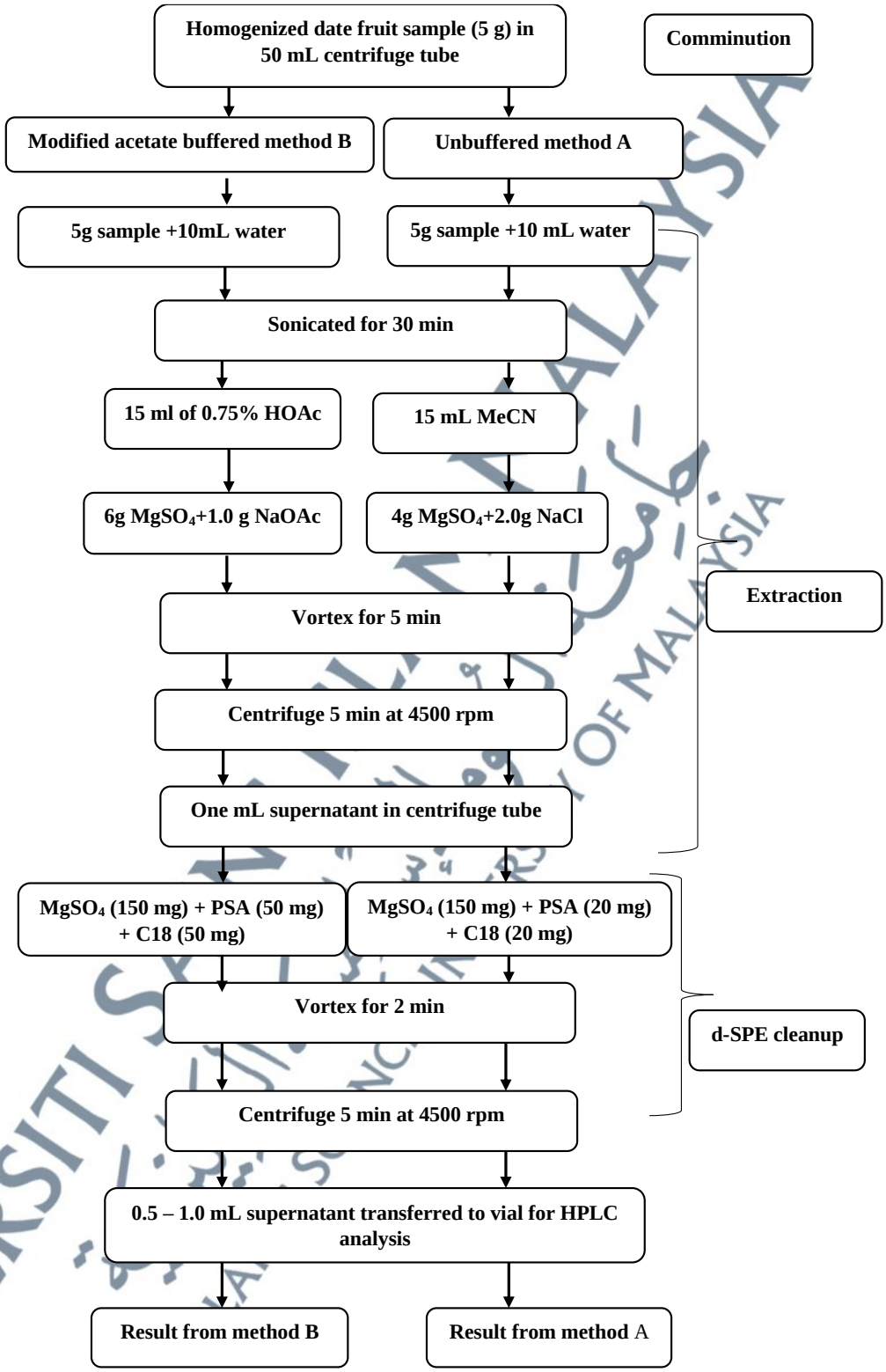

Figure 3.1: Flowchart of Sample Preparation of Modified Buffered and unbuffered QuEChERS

3.3 Chromatographic Analysis

For separation and simultaneous determination of the targeted pesticides in date fruits, high performance liquid chromatography equipped with diode array detector (HPLC-DAD) was used. HPLC is always preferred for such function because of its high resolution, short analysis time and reproducible retention time (Wang et al., 2003) in addition to low operation temperature to avoid degradation of any of pesticide residue.

3.3.1 HPLC-DAD Method Development for Separation of the Target Pesticides

The objective of developing an analysis method is to give substantial advantages in precision and to reduce bias errors, method creation and validation can offer significant improvements in precision and bias error reduction in addition to other benefits such as low cost and short analysis time. In this study three major effective factors were optimized for selection the best operation conditions namely, wavelength, mobile phase composition and flow rate.

3.3.1.1 Optimization Detection Wavelength in HPLC- DAD

In terms of robustness and sensitivity, determining the optimal wavelength at which to detect the response of the analyte is critical. Achieving this is normally performed by the examination of the UV spectra for reference standards or by analyzing the individual UV spectra for each component inside the chromatogram generated by diode-array detector. Molecular absorption bands can be affected by changes in the sample solvent's composition, its pH, and its temperature. The optimal wavelengths for determination of the three pesticides, dimethoate, carbendazim and chlorpyrifos were determined using the standard solution. All other chromatographic conditions (mobile

phase composition and flow rate were kept constant while the wavelength was examined at 249, 254 and 270 nm. The wavelength with less interference and strongest absorption for each of the tested pesticides was chosen as working wavelength.

3.3.1.2 Optimization of Mobile Phase Composition

In order to improve the separation, mobile phases with varied ratios of acetonitrile, methanol, and water with and without ammonium format were tested. Acetonitrile water systems were found not suitable mobile phase because of poor separation for the target compounds. Isocratic elution of methanol water 95:5 with 5 mM ammonium formate was found suitable for separation of dimethoate, carbendazim and chlorpyrifos in standard and sample extract (Tsochatzis, et al., 2012; Abdallah, et al., 2018). However, the isocratic elution was chosen for the following advantages: it is a cost-effective and simple method of separation as compared to gradient elution, no specific HPLC pump is necessary to flow the mobile phase, it is simple to transfer the method and between consecutive injections, there is no need to re-equilibrate with the initial mobile phase composition (Emara et al., 2015).

3.3.1.3 Optimization of The Mobile Phase Flow Rate

Solvent flow rate is contributing to column efficiency. If the solvent flow rate is excessively fast, the compounds will not have enough time to reach equilibrium and will be driven down the column, leaving a long tail. When the solvent flow rate in column chromatography is exceedingly slow, diffusion mechanisms cause band broadening. The ideal rate for smaller diameter columns is lower than for bigger diameter columns. As a result, larger columns can be run at a higher flow rate than smaller columns (https://chrominfo.blogspot.com/2019/09/effect-of-flow-rate-on-

column.html). For flow rate optimization in this work, the flow rate was varied at the following rates: 1, 0.8, 0.5 and 0.2 mL/min. While fixing the other factors such as mobile phase composition and detection wavelength. The flow rate that gave the best resolution for the three pesticides was chosen as optimal flow rate.

3.3.2 Validation of HPLC Developed Method

Method validation is an important part of creating reference procedures and assessing a laboratory's capacity to produce trustworthy analytical records. Analytical method validation, which considers the most important processes for verifying the best parameters of analytical methods and employs a plethora of relevant overall performance indicators such as linearity, range, limit of detection (LOD), limit of quantification (LOQ) (sensitivity), accuracy, precision, and matrix effect, was performed according to method recommended by (SANCO/12571/2013). Each one of these factors are discussed in detail below.

3.3.2.1 Linearity of Calibration Curve

Three sets of mixed standards of dimethoate, carbendazim and chlorpyrifos were prepared. The first set consists of the following concentrations: 2, 4, 6, 8 and 10 µg/mL. The second set was built up from the following medium concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 µg/mL. And the third set consist of the following: 0.02, 0.04, 0.06, 0.08 and 0.10 µg/mL. Each set of standards was injected in triplicated into the HPLC -DAD systems. The calibration equation and correlation coefficient (R^2) for each pesticide were calculated using linear regression analysis of peak area and concentration.

3.3.2.2 Limit of Quantification and Limit of Detection

The sensitivity of HPLC- DAD method was expressed as limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ of the three pesticides mixtures were calculated from the calibration curves as following: LOD = 3.3*σ/S and LOQ = 10*σ/S. Where, σ is standard deviation of regression line and S is the slope the calibration curve (Szepesi, 1990; Zalat et al., 2014).

3.3.2.3 Accuracy

The accuracy of an analytical method is identified as the similarity of the calculated value to the true value obtained. The accuracy and the precision are important quantitative parameters to assess the errors associated with the analysis process (Manoli and Samara, 1999). Therefore, identifying the sources of errors influencing the accuracy is necessary to obtain a smart technique to minimize the effect of the errors. The accuracy of the developed method was evaluated through recovery studies by spiking known amounts of standard solutions in the date samples. Unspiked samples (controls) from all samples was prepared by the same way and analyzed. Recovery experiments were performed at three standard concentrations 0.05, 0.1, 0.15 mg/kg. It was done in accordance with the European Commission Guidance Documents for Monitoring of Pesticide Residue Analysis (SANCO, 2013), which defined mean recoveries for initial validation in the range of 70-120 % and RSD of < 20 %.

3.3.2.4 Precision

Precision is the degree of agreement between individual tests performed on different batches of a standardized sample when the procedure is used. As a rule, it's reported as the standard deviation (SD) or relative standard deviation (RSD %) based on more than five standard replication measurements at different concentrations - low,

medium, and high concentration (Green, 1996; Miller and Miller, 2005). The system suitability of the method was evaluated by intra-day and inter-day Precision. Concentration of (2 µg/mL) from each pesticide were spiked and analyzed five times on the same day and on three consecutive days (Ganzera et al., 2002). The relative standard deviation was calculated as following; RSD = SD/X, where SD is the standard deviation and X is the mean of obtained concentrations.

3.3.2.5 Matrix Effect

The effect of the date fruit matrix (ME), suppression or enhancement, on the pesticides studied was determined by comparing the slopes of the solvent calibration curve (SCC) and the slope of the matrix matched calibration curve (MMCC), both of which were created using five concentration levels (0.5, 2, 4, 6, 8 µg/mL) and the formula described by Ferrer et al., (2011) and Abdallah et al., (2018).

ME (%) = (slope of MMCC – slope of SCC)/ (slope of SCC) × 100

(3.1)

Based on the calculated ratio from the above equation, the matrix effect was classified as soft, medium, or strong, i.e. 0–20, 20–50 and >50%, respectively (Kmellar et al., 2008; Abdallah et al., 2018).

3.3.3 Comparison of the Efficiency of the Modified Acetate Buffered QuEChERS Method and the Original Unbuffered QuEChERS

The modified acetate buffered QuEChERS method (AOAC) was compared with the original unbuffered QuEChERS method regarding to the obtained recoveries of the examined pesticides at spiked concentration of 0.05, 0.5 and 0.15 mg/kg. Table 3.3 shows the quantities of components of the modified AOAC and the original QuEChERS

Table 3.3: Comparison of the Modified Acetete Buffered QuEChERS (AOAC) Method and the Original unbuffered QuEChERS

EXP.	Acetic acid %	CH_3CN	Water	$MgSO_4$	NaOAc	PSA	C18	NaCl
Original	0	15 mL	10 mL	4 g	0.0 g	20 mg	20 mg	2.0 g
Modified Buffered QuEChERS	0.75 %	15 mL	10 mL	6 g	1.0 g	50 mg	50 mg	3.4 g

3.4 Analysis of Real Samples

In this study, according to the screening result of (P-B Design), the modified QuEChERS acetate method was applied according to the optimization result. The components shown in Table 3.3 which gave the highest recovery and lower RSD were used for extraction and clean-up of the real samples as following. 5 g homogenized date fruit samples were weighed and put into 50 mL centrifuge tubes and then 10 mL of deionized water were added to each tube then sonicated for 30 min. 15 mL of acidified acetonitrile (0.75 % glacial acetic acid), vortexed for 2 min. Then, a mixture of $MgSO_4$ 6g and CH_3COONa 1.0 g was added to the tube, vortexed again for 2 min and centrifuged at 4500 rpm for 5 min. One mL of the supernatant was transferred to 2 mL Eppendorf tube containing 50 mg PSA, 50 mg C18 and 150 mg of anhydrous $MgSO_4$. The mixture was immediately shaken and vortexed for 2 min and then centrifuged for 5 min at 4500 rpm. The supernatant was filtered through Agilent 0.20 µm Nylon filter into 1.5 mL HPLC vials for chromatographic analysis.

3.5 Health Risk Assessment for Examined Pesticides

Health risk assessment for examined pesticides was calculated using the method reported by Kumari & John (2019). For each pesticide, the estimated daily intake (EDI, mg/kg/day) was defined using the following formula recommended by FAO/WHO (1997):

$$EDI = C * FER/BW \quad (3.2)$$

where EDI is the pesticide's estimated daily intake, C is the pesticide's mean residual concentration, FER is the daily consumption rate of food per capita, and BW is the mean weight of the human body. The date fruits consumption rate was taken from the National KSA food consumption which is the highest in the world (100 g per capita). Children's and adult body weights were assumed to be 30 kg and 60 kg, respectively (USEPA, 1989; USEPA 1996).

The health hazard index predicts the risk of long-term health hazards associated with the consumption of food contaminated with chemical residues. As shown in equation (3.3), it is expressed as a ratio of estimated daily intake (EDI) to acceptable daily intake (ADI). ADI for pesticides was from adapted FAO/WHO (2017):

$$HHI = EDI/ADI \quad (3.3)$$

where HHI is the index of health hazards, EDI is the estimated daily intake and ADI is acceptable daily pesticide residue intake.

3.6 Investigation and Optimization of Household Methods for Elimination of Pesticides Residues from Date Fruits

In this research, the contaminated date fruits were washed with tap water and various solutions such as detergent (dishwashing brand), sodium chloride, acetic acid, sodium bicarbonate, hydrogen peroxide, and potassium permanganate. The effect of temperature, contact time and concentration of the washing solutions were examined. Figure 3.2 shows the steps of elimination of pesticides residues from date fruits.

3.6.1 Instrumentation

UV spectra were recorded on Cary 50- UV-VIS spectrometer using Varians software.

3.6.2 Chemicals and Reagents

2, 2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazin-1-yl (DPPH), Folin Ciocalteu reagent, Gallic acid, Sodium carbonate and concentrated sulfuric acid 98% were purchased from Fisher.

3.6.3 Preparation of Pesticide's Solution for Contamination of Date Sample

Pesticide's solution was prepared according to the method described by Gouri Satpathy et al., (2012). A mixture of pesticide's standards solution 20 µg/mL contains of the following pesticides: Dimethoate, chlorpyrifos, and carbendazim was diluted using deionized water to prepare final concentration of 20 µg/mL. 500 g of date fruits was washed with deionized water for two minutes to remove the adhered dust and then immersed in the pesticide solution (20 µg/mL) for two minutes to allow for pesticide deposition on them followed by air drying for 2 hrs. The contaminated samples were put in plastic case and kept in the refrigerator for analysis.

3.6.4 Preparation of Washing Solutions

In addition to tap water, different washing solutions were prepared as the following: Dishwashing brand detergent solution (1% w/v in deionized water), sodium chloride, acetic acid, sodium bicarbonate hydrogen peroxide, and potassium

permanganate were prepared in three concentrations each (1 %, 2 % and 3 % w/v) in deionized water according to the methods described by Satpathy et al., (2012) and Yang, et al., (2017).

3.6.5 Washing of Contaminated Samples

The contaminated samples prepared in Section 3.2.3 were divided into two groups A and B. i) group one (A). Group A was divided in two groups, A1 and A2 which were kept as controls for both pesticide residue analysis and nutrition value determination respectively. ii) Group B were washed in the washing solutions for intervals of (2, 5, and 10 min), after an initial 15 sec gentle rotation by hand to imitate the real procedure of washing in the households. Date samples were air-dried for 1 hr. Then seeds were removed and the flesh was chopped and divided into two groups B1 and B2. Group B1 was extracted for pesticide residue analyses and group B2 was kept for determination of washing effect on nutrition values. The activity of washing on pesticide % removal and its effect on nutrition value was evaluated by comparison of the data collected from the analysis of the washed and unwashed samples as shown in Figure 3.2 and is explained in the following sections.

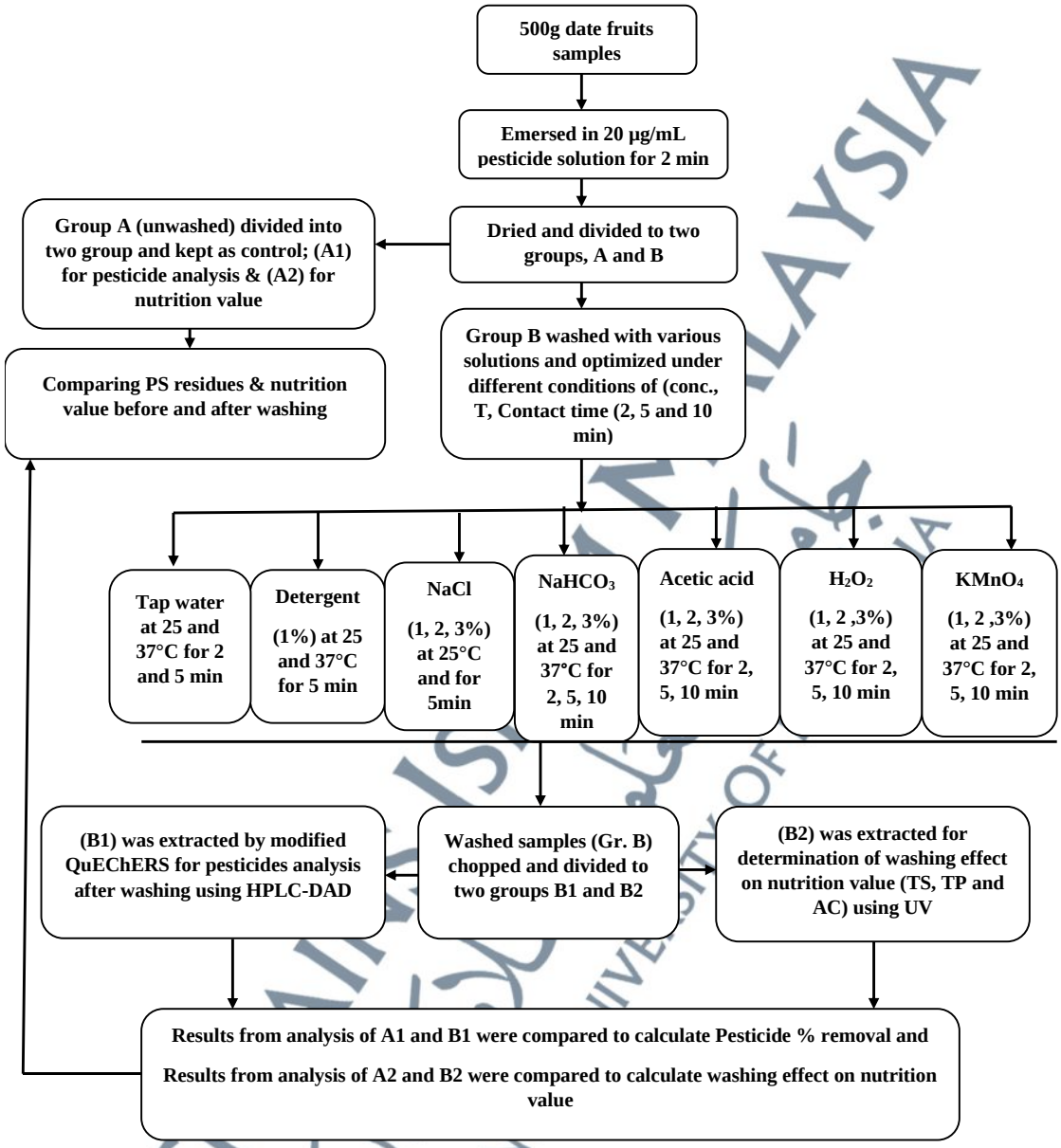

Figure 3.2: Scheme of Washing Process Steps for Elimination of Pesticide Residues from Date Fruits and Its Effect on Nutrition Value

3.7 Determination of Washing Effect on Date Fruit's Nutrition Value

The effect of washing on date fruit's nutrition value was studied through the following factors: Total sugar, total phenolics and the antioxidant activity (scavenging activity of DPPH free radicals).

3.7.1 Preparation of Water Extract of Date (Flesh) Samples

The water extraction of date fruits was done according to the method described by Biglari et al. (2008). Washed (B2) and unwashed (A2) dates samples were chopped into small pieces. One gram of the small pieces from each sample was extracted with 10 mL of water at 25°C for 24 hours. The dates samples were filtered, and the filtrates were kept in the refrigerator for determination and comparison the nutrition value of the washed and unwashed date samples.

3.7.2 Total Sugars Determination in the Date Samples

The Sulfuric Acid–UV method described by Albalasmeh et al., (2013) was used with some modification as follows. One mL of concentrated sulfuric acid is rapidly mixed with 1 mL of date water extract filtrate (100 mg/mL) or glucose standard solution in a test tube and vortexed for 30 s. Due to the rapid rise in temperature within 10–15 seconds after the addition of sulfuric acid, the solution was rapidly cooled in an ice bath for 2 minutes to bring it down to room temperature. The UV absorbance at 315 nm was read using UV spectrophotometer (Fig 3.3). Blank solution was prepared using the same method as above, except that the dates extract was replaced with deionized water. The standard curve was prepared using 0, 20, 40, 60, 80, 100 mg/mL solutions of glucose in water. The total sugars value was calculated from the equation of calibration curve and

expressed as glucose equivalent mg/g fresh weight, which is a common reference compound.

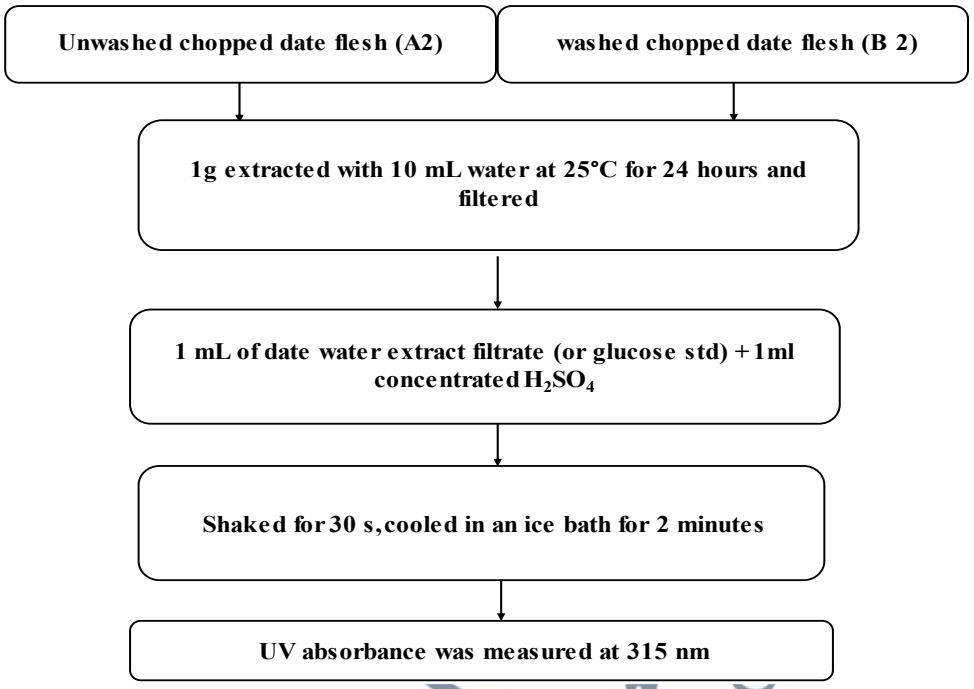

Figure 3.3: Determination of Total Sugars Content in Date Fruit Samples

3.7.3 Determination of Total Phenolic Contents in the Washed and Unwashed Date Fruits Samples (A2, B2)

Determination of total phenolics was done according to the method reported by McDonald et al. (2001) using Folin Ciocalteu reagent. Folin Ciocalteu reagent was prepared by dilution the concentrated reagent by the ratio of 1:10 with deionized water. Diluted date fruits extract prepared in section 3.3.1 (0.1 mL of 1mg/mL) or phenolic standards was mixed with 3mL Folin Ciocalteu reagent and (3mL, 1M) of sodium carbonate Na_2CO_3. Solutions were heated in water bath at 45°C for 15 min. The total phenolic content was determined calorimetrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 250, 300 mg/L solutions of gallic acid in methanol. The

total phenolic value was calculated from the equation of calibration curve and expressed as gallic acid equivalent (mg/g) of fresh weight).

3.7.4 Determination of Antioxidants Activity (Free Radical Scavenging Activity) of the Washed Date Samples

The scavenging activity of DPPH free radical of date fruits were done according to the method reported by Oktay et al. (2003). Date fruit extracts prepared in section 3.3.1 were used in each reaction. 200 µL date extract (1mg/mL) in methanol was mixed with 4 mL of 0.1 mM DPPH-methanol solution. Methanol was used as blank sample for this experiment. After 60 min of incubation at room temperature, the reduction of DPPH free radicals measured by reading the UV absorbance at 715 nm. Unwashed sample extract was used as positive controls. Each experiment was triplicated. The DPPH inhibition % was calculated using the following equation:

DPPH Inhibition % = {(absorbance of control – absorbance of test sample)/ absorbance of control} *100

(3.4)

3.8 Assessment of Heavy Metals Content and Washing Effect on Heavy Metal Contents

3.8.1 Instruments

Atomic absorption spectrometer Agilent AA200 with Zeeman background correction system equipped with graphite furnace (GTA 120) and an auto sampler (PSD 120) was used. The purity of argon gas was 99.99%. Hollow cathode lamps were used for Cd (228.8 nm and slit 0.5 nm), Pb (283.3 nm and slit 0.5 nm), Ni (352.5 nm slit 0.2 nm), Al (257.4 nm slit 0.5 nm), Sn (286.3 nm slit 0.5 nm) Cu (327.4 nm slit 0.5 nm), As (189.0 nm slit 1.0 nm) and (Fe 248.3 nm, slit 0.2). The instrument was operated

according to the conditions recommended by the manufacturer. Atomic signals were measured in peak area mode.

3.8.2 Preparation of Heavy metals Standard Solutions

The working standard solutions (100 ppm) were prepared by using 1000 ppm stock standard solutions from (Sigma-Aldrich) using 0.05 M analytical grade nitric acid (Merck, KGaA, Germany) as recommended by manufacturer. Moreover, palladium (II) nitrate (Merck, KGaA Germany) and ascorbic acid (Sigma-Aldrich) were used as modifiers during the sample analysis. Solutions were prepared using deionized water. All containers and glassware were cleaned by sonicating in nitric acid solution (20%) and rinsed with DDW water prior to use.

3.8.3 Washing and Collection of the Dust

To determine the concentration of heavy metals in the adhered dust on fruits, about 20 g from each sample were washed thrice with about 15 mL in three portions (30 seconds each) with deionized water (DIW) and the washing residues were collected in beakers. All beakers were put on hot plates until near dryness. The washing residues were kept in refrigerator for digestion and analysis of heavy metals in dust. The washed samples were dried, cut in to slices and kept for digestion and analysis of heavy metals in the tissues (Aldjain et al., 2011).

3.8.4 Digestion Procedure

Sample digestion was done according to the wet digestion method described by Aldjain et al., (2011) which modified from that of Hseu (2004) and Zheljazkov and Nielson (1996). 1g of each date flesh sample or dried washing residue (dust) prepared

in experiment described in section 3.8.3 was added into conical flask contains 15 mL of HNO_3 65 % and heated on hotplate in fume cupboard for 30 min. 20 mL H_2O_2 33 % were added in portions (5 ml each) to get clear solution. The mixture was heated at 110°C until a clear solution achieved. After complete digestion of the sample, the flasks were allowed to cool and then diluted by 10 mL acidified deionized water to avoid precipitation. Then solution was filtered using Whatman No 0.42 filter paper. The filtrate was made up to 50 mL with DIW and kept for GFAAS analysis.

3.8.5 Optimization of Digestion

To optimize the adapted method described in section 3.8.4 for determination of heavy metals in date fruits matrix, the following parameters including composition of HNO_3/H_2O_2 mixture, digestion temperature and dissolution time were optimized for mineralization of date sample. Four elements were chosen for optimization according to their melting points, Sn (lowest melting point), Pb (medium melting point), Fe (highest melting point) and As which is sublimate at 615°C. The temperature was adjusted at 70, 100, 110, 120 and 130°C during digestion of 1 g of date flesh sample with 35 ml of 15:20 HNO_3/H_2O_2 acid mixture for wet digestion. Obtaining the clear colorless solution was considered as an indicator on the mineralization completion.

The digestion time was varied from 15 to 90 min, at intervals of 15 min, during the mineralization of 1 g of date sample heated at 110°C with 35 ml HNO_3/H_2O_2 (15:20) acid mixture. The results obtained indicate that a minimum of 60 min digestion time is sufficient to complete mineralization and getting acceptable recoveries for the tested elements (Fe, Sn, As, and Pb). The composition of HNO_3/H_2O_2 mixture varied during the mineralization by heating at 110°C for 60 min. The best results were obtained with

the acid mixture ratio of 15:20 HNO_3/H_2O_2. Increase of ratio from 15:20 to 20:20 and 25:20 did not influence the mineralization of date fruits sample for wet digestion.

From the optimization procedure it was concluded that the following method should be applied for digestion of the dust residues samples described in section 3.8.3 and date flesh samples. 1g from each sample was added into conical flask contains 15 mL of HNO_3 65 % and heated on hotplate in fume cupboard for 30 min. 20 mL H_2O_2 were added in portions (5ml each) to get clear solution. The mixture was heated at 110 °C for 1 hour until a clear solution was achieved. After complete digestion of the sample, the flasks were allowed to cool and then diluted by 10 mL acidified deionized water to avoid precipitation. The solutions then filtered using Whatman No 0.42 filter paper. The filtrate was made up to 50 mL with acidified deionized and kept for GFAAS analysis as explained in Figure 3.4.

3.8.6 Validation of the Modified Digestion Method

The accuracy of the modified method used for digesting the date fruit samples was evaluated by recovery study using the method described by Deribachew et al., (2015) by spiking the samples with standard solutions of known concentration of each metal regarding the LOQ of each element. The spiked date fruit samples were digested following the same procedure applied in the digestion of the examined samples. Therefore, a 0.5 g from each dates sample was spiked by two concentrations from each element as the following: 20 and 100 ppb of (Ni, Cu, Sn and Pb) and 2 and 10 ppb of (Cd), 100 and 200 ppb of (Al), 5 and 100 ppb of (As) and 1 ppm of (Fe) solution. The recoveries were performed in triplicates and recovery >75 % was considered satisfied. The mean recoveries of the analyzed heavy metals were calculated from the following equation.

$$\text{Recovery \%} = \left[\frac{\text{HM content in spiked sample - HM content in unspiked sample}}{\text{HM content in spiked sample}} \right] * 100$$

(3.5)

3.8.7 Risk Assessment of Heavy Metals

The joint FAO/WHO group established standards for the investigation of dietary exposure to chemical pollutants, including heavy metals. National authorities must ensure that food products do not include harmful chemical compounds (heavy metals, pesticide residues, aflatoxins) in amounts that are likely to adversely affect the health of the consumer, according to the FAO/World Health Organization report. An ongoing system of monitoring food pollutants is therefore necessary to protect consumers while also facilitating international trade in food products. (Nasreddine & Parent-Massin, 2002). A risk assessment procedure that is constantly evolving as new knowledge on pollutants, their linked health consequences, and their prevalence in food are all aspects that should be investigated and monitored on a continuous basis (Kuhnlein & Chan, 2000). The Estimated daily intake per meal (ED_{ml}) for washed sample (100 mg dates/day) for each element were calculated according to the following equation.

$$ED_{ml} = Ms * C$$

(3.6)

where Ms is the meal size (g), and C is the mean metal concentration (µg/g). Estimated daily intake EDI (µg/kg bw/ day) for (70 kg bw) were calculated from the equation below (Nasreddine & Parent, (2002):

Estimated daily Intake EDI (µg/kg bw/day) = EDI = EDml / 70kg

(3.7)

where 70 kg is the average body weight.

Hazard quotients of the tested heavy metals was calculated from the equation:

HQ = EDI/ PTDI, where PTDI is provisional tolerable daily intake (µg/kg bw/day) (3.8)

Pearson correlation coefficients of the studied elements in flesh and dust were done using Minitab software.

3.8.8 Determination of Water Washing Effect on Heavy Metals Content

After determination of heavy metals content in all date samples using GFAAS, the washing effect on heavy metals contents was calculated as follows:

$$\text{washing effect} = [C_D / (C_D + C_F)] * 100 \quad (3.9)$$

where CD is concentration of metal in the adhered dust and CF is the concentration of metal in the flesh of the fruit result.

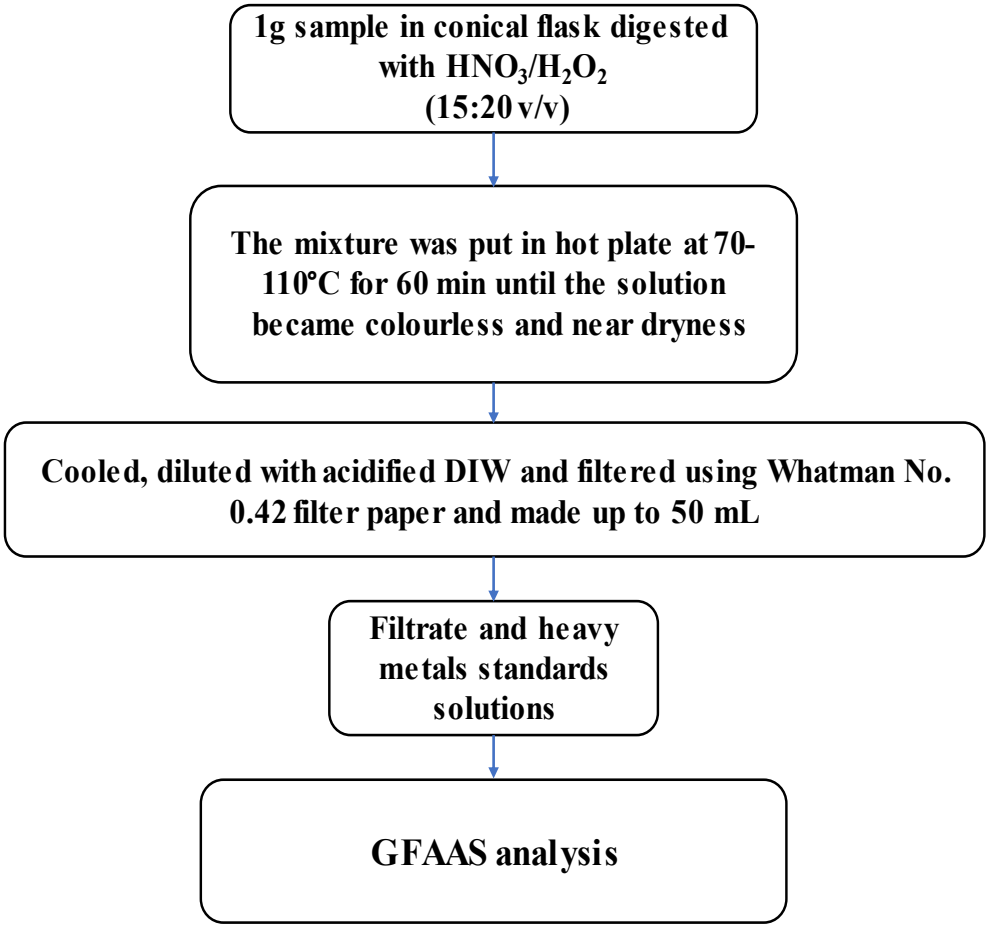

Figure 3.4: Flowchart of Wet Digestion and Heavy Metals Quantification