CHAPTER 3

RESEARCH METHODOLOGY

3.1 General materials and methods

Section 3.1 describes the general materials and methods for Chapter 4: The effect of different crop borders on the physicochemical variation of chloroplast-rich fractions (CRFs) from sweet potato haulm and Chapter 5: The effect of heat treatments on the physicochemical variation of chloroplast-rich fractions (CRFs) from sweet potato haulm. Meanwhile, Section 3.2 describes the materials and methods for Chapter 6: The digestive stability and bioaccessibility of β-carotene and lutein of chloroplast-rich fractions (CRFs) from sweet potato haulm.

3.1.1 Chemicals and standards

Acetone, acetonitrile, chloroform, ethyl acetate, and methanol used for lipid extraction and carotenoids (β-carotene and lutein) analysis were high performance liquid chromatography (HPLC) grade, purchased from Sigma Aldrich (Merck, Darmstadt, Germany). Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) and standards of gallic acid, ascorbic acid, β-carotene, and lutein were purchased from Sigma Aldrich (Merck, Darmstadt, Germany). Other chemicals used were of analytical grade.

3.1.2 Materials

Sweet potato haulm (SPH) consists of stems, petioles (stalks), and leaves was obtained from a local farm (Perlis, Malaysia). The haulms harvested were grown in one farming area and planted at the same time with constantly implementing agriculture practices. The practices include bed size and spacing, selection of disease-resistant varieties, and selection of biological agents to control pests and diseases. The amount of water, soil type, fertiliser type, and daylight time subjected to the sweet potato plant were constant throughout the research. After harvesting the sweet potatoes, the haulm was freshly garnered and immediately brought to our laboratory facilities in Nilai, Malaysia to be further processed (within 24 hr). The SPH was put in the gunny sack and the humidity was controlled by spraying tap water on the haulm every 1 hr during transportation to prevent it from wilting. The SPH was handled with minimal light exposure after harvesting to diminish the loss of essential nutrients.

3.1.3 Isolation of chloroplast-rich fraction (CRF) from sweet potato haulm (SPH)

3.1.3.1 CRF of SPH from different crop borders

To investigate the first objective: The effect of different crop borders on the physicochemical variation of chloroplast-rich fractions (CRFs) from sweet potato haulm, the haulm was harvested from three different rows of sweet potato plants crop borders (CBs) where the plants were within the same plantation area (as illustrated in Figure 1.3). The CBs, labelled as CB 1, CB 2, and CB 3 in this research had similar agricultural practices like the use of soil, fertiliser, and pesticide type, or even water availability.

The fresh haulm from each CB (of at least 1 kg) was cleaned with running tap water to remove soil and dirt. The haulm was juiced using a cold press slow juicer (SAVTM JE-31, China). The yield of haulm juice was determined by dividing the weight of juice collected by the weight of fresh haulm. The juice was centrifuged (Thermo Jouan CR3i, France) at 10 000 rpm (revolutions per minute), 4 °C for 15 min. The pellet containing CRF (Figure 3.1) was collected while the supernatant was centrifuged again under the same conditions for more pellets.

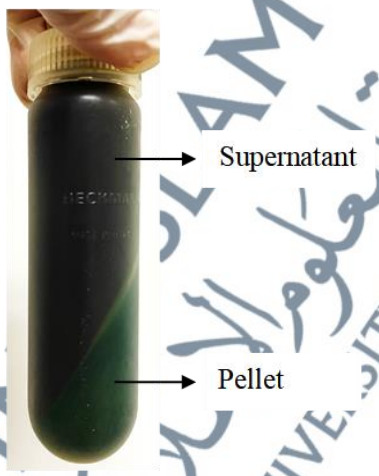

Figure 3.1: Sweet potato haulm juice (after centrifugation process)

The pellets were pooled, weighed, and frozen at – 20 °C prior to freeze-drying (Freeze Dryer FD-550 Eyela, USA) at 3 Pa, 20 °C for 24 hr. The dried CRF was ground to homogeneous powder (< 250 µm) using pestle and mortar under dim light conditions. The dried CRF was stored in a vacuum-sealed aluminium pouch at – 80 ± 1 °C until further analysis. The yield of CRF was determined by dividing the weight of CRF powder collected by the weight of fresh haulm.

3.1.3.2 CRF of SPH from different heat treatments

To investigate the second objective: The effect of heat treatments on the physicochemical variation of chloroplast-rich fractions (CRFs) from sweet potato haulm (SPH), three different heat treatments were involved in this research: Conventional pasteurisation (CP), Steam pasteurisation (SP), and Water blanching (WB). The parameter variations in each heat treatment are shown in Table 3.1. The haulm without any heat treatment was considered as a control and labelled as fresh (F). The control sample (F) was prepared as mentioned in the previous Section 3.1.3.1, where the fresh haulm was juiced using the cold press slow juicer (SAVTM JE-31, China), followed by the isolation of CRF.

Table 3.1: List of heat treatment conditions

Heat treatment	Temperature	Heating Time
a) Conventional pasteurisation (CP)	85 °C	5 min
b) Steam pasteurisation (SP)	100 °C	5 min
c) Water blanching (WB)	85 °C	3 min

The SPH for each heat treatment (CP, SP, and WB) was harvested and divided equally into three batches. The haulm from each batch (of at least 1 kg) was cleaned with running tap water to remove soil and dirt. The first batch of haulm was juiced first using a cold press slow juicer (SAVTM JE-31) before being heat-treated with conventional pasteurisation (CP) treatment at 85 °C for 5 min. Then, the haulm juice was immersed in an ice-water bath at 10 °C for 30 min to rapidly cool down the juice to room temperature before further processing to isolate the CRF.

Conversely, the second and third batches of haulm were heat-treated first before being juiced. The second batch of haulm was packed into a vacuum-sealed bag and subjected to the steam pasteurisation (SP) process in an autoclave at 100 °C, 1 bar for 5 min. Then, the bag was immersed in an ice-water bath at 10 °C for 15 min to rapidly cool down the haulm to room temperature before juicing using a cold press slow juicer (SAVTM JE-31).

The third batch of haulm was heat-treated with water blanching (WB) treatment in hot water at 85 °C for 3 min. After that, the haulm was immersed in an ice-water bath at 10 °C for 1 min to rapidly cool down the haulm to room temperature before juicing using a cold press slow juicer (SAVTM JE-31).

The yield of haulm juice was determined by dividing the weight of juice collected by the weight of fresh haulm. The heat-treated juices (CP, SP, and WB treatment) were ready for the isolation of CRF. The juice was centrifuged (Thermo Jouan CR3i, France) at 10 000 rpm, 4 °C for 15 min. The pellet containing CRF was collected while the supernatant was centrifuged again under the same conditions for more pellets. The pellets were pooled, weighed, and frozen at – 20 °C prior to freeze-drying (Freeze Dryer FD-550 Eyela, USA) at 3 Pa, 20 °C for 24 hr. The dried CRF was ground to homogeneous powder (< 250 µm) using pestle and mortar under dim light conditions. The dried powder was stored in a vacuum-sealed aluminium pouch at – 80 ± 1 °C until further analysis.

3.1.4 Total soluble solid content (°Brix)

The total soluble solid content was expressed as a percentage of fresh matter mass, which represents a strong positive correlation with sugar content. Sweet potato haulm juice was tested for total soluble solid content using a digital refractometer for each heat treatment. The value was measured in degrees Brix or °Brix, based on the number of dissolved solids in a liquid via specific gravity. A degree of Brix is equivalent to one gram of sucrose in 100 g of solution, which is equal to one percent Brix.

3.1.5 Total solid content and moisture content

The total solid content (TSC) and moisture content of chloroplast-rich fraction (CRF) were determined by the following equations:

$$Total\ solid\ content\ (\%) = \frac{Weight\ of\ wet\ sample}{Weight\ of\ dry\ sample}\ x\ 100 \quad (3.1)$$

$$Moisture\ content\ (\%) = 100 - Total\ solid\ content\ (\%) \quad (3.2)$$

3.1.6 Water activity (a_w)

The water activity of chloroplast-rich fraction (CRF) powder was measured by using Aqualab Water Activity Meter 4TE (Decagon Devices, Inc USA) based on the chilled-mirror dewpoint technique. The homogenous CRF powder was placed in the sample cup at approximately 25 °C. Detection of the condensation point, which first appears in the mirror, was observed with a photoelectric cell. A beam of light was directed onto the mirror and reflected into a photodetector cell. Any changes in

reflectance were detected by the photodetector when condensation occurs on the mirror. A thermocouple was attached to the mirror and the temperature at which the condensation occurs was recorded. Then, Aqualab gave a signal to read data by beeping and displaying the final water activity and temperature for the CRF. The length of reading times may vary depending on the difference in temperature between the chamber and the sample.

3.1.7 Bulk density

The bulk density of chloroplast-rich fraction (CRF) powder was determined manually using a measuring cylinder adapted by Beristain et al. (2001) method. Briefly, 1 g of CRF powder was weighed loosely (rest on a flat surface) into a 10 ml graduated cylinder. The volume occupied by 1 g of CRF powder was recorded. Bulk density was measured by the weight of the CRF powder divided by its volume, recorded as g/ml.

3.1.8 Dispersibility

The dispersibility of chloroplast-rich fraction (CRF) powder was determined by dissolving the CRF powder (1 g) in distilled water (10 ml) at room temperature adapted from Szulc and Lenart (2016) method. The mixture was stirred for 1 min followed by incubation at room temperature for 30 min, intended to form suspended particles. The supernatant was decanted carefully. The mass of the supernatant was obtained by transferring the supernatant into a 5 ml density bottle. The weight of the dispersed solid was calculated as double the difference in the mass of the supernatant and an equal volume (5 ml) of distilled water.

3.1.9 Microscopic imaging

The morphology of chloroplast-rich fraction (CRF) powder was observed using Field Emission Scanning Electron Microscopy (FESEM). The platinum coating was carried out in an auto fine sputter coater (JEOL, JEC-300FC, Japan). The platinum-coated sample was subsequently viewed with the FESEM (JEOL, JSM-IT800, Japan). The photomicrograph was taken at 1 kV of accelerating voltage under high pressure. FESEM was operated at several magnifications (500x, 1000x, and 3000x).

3.1.10 Water solubility index (WSI)

The warm WSI of chloroplast-rich fraction (CRF) powder was determined by the method reported by Anderson et al. (1969) with slight modification (0.1 g sample was used in our study instead of 1 g, due to sample limitation). The CRF powder (0.1 g) was mixed with distilled water (12.5 ml) in a 50 ml centrifuge tube. The mixture was incubated at 38 °C for 30 min and centrifuged (Thermo Jouan CR3i, France) at 10 000 rpm, 4 °C for 15 min to separate the pellet and supernatant. For cold WSI, the powder sample did not go into incubation, but was centrifuged right after it was vigorously mixed with water. The supernatant was collected and dried in an oven at 103 °C for overnight (16 hr). The dried suspension was weighed, and the solubility of CRF powder was calculated using equation 3.3 as follows:

$$Water\ solubility\ index\ (\%) = \frac{Weight\ of\ dried\ suspension}{Weight\ of\ initial\ CRF\ powder}\ x\ 100 \qquad (3.3)$$

3.1.11 Colour analysis (Hunter Lab)

The colour of chloroplast-rich fraction (CRF) powder was measured by colourimeter (Labscan XE, Hong Kong) using the CIELab system. The sample was spread in an optical glass with a 6.4 mm diameter diaphragm. Then, the colourimeter was calibrated with a white and black reference plate before measuring the L^* (L^* = 0 [black] and L^* = 100 [white], a^* (-a^* = greenness and +a^* = redness) and b^* (-b^* = blueness and +b^* = yellowness) for colour expression. Five replicate measurements were taken for each sample. The total colour difference was calculated using the formula in the equation 3.4:

$$Total\ colour\ difference\ (\Delta E) = [(L*_2 - L*_1)^2 + (a*_2 - a*_1)^2 + (b*_2 - b*_1)^2]^{1/2} \quad (3.4)$$

3.1.12 Proximate composition

The proximate composition consists of the determination of moisture content, crude protein content, crude fat content, crude fibre content, and ash content was applied according to AOAC (2012) methods.

Determination of moisture content (method number 952.45) was performed by drying CRF powder in an oven at 105 °C overnight (16 hr). The chloroplast-rich fraction (CRF) powder was cooled in a desiccator and was weighed until constant weight was gained. The moisture content was measured as a percentage of weight loss.

The crude protein was determined by the Kjeldahl method (method no. 981.10), whereby the percentage of nitrogen was calculated on a dry matter basis. The amount

of nitrogen was then multiplied by a nitrogen-protein conversion factor (6.25) to get the crude protein content of a sample.

The crude fat was determined by the Soxhlet continuous extraction method (method no. 945.16). It is an automatic fast extraction system, using petroleum ether as the extraction agent. Lipids showed variable solubility in various solvents due to their different polarity.

The crude fibre was obtained using dilute acid and dilute alkali hydrolysis in Fibretherm (method no. 991.43). Sample was analysed in an automated digestion and filtration process system.

The ash content was determined by charring 1 g of the sample on an electric hot plate until the smoke ceased, followed by incinerating the charred sample in a muffle furnace (method no. 945.46) at 550 °C overnight (16 hr).

Carbohydrate content was calculated by subtracting the sum of the other nutrients from 100 %.

3.1.13 Antinutrients (Oxalic and phytic acids)

3.1.13.1 Oxalic acid

Determination of oxalic content in chloroplast-rich fraction (CRF) was determined based on the method adopted by Premasiri and Ekanayake (2011). Distilled water was added to the CRF powder (0.5 g) up to 25 g in the conical flask and the mixture was homogenised (8 000 rpm, 30 °C, 3 min). The homogenised solution was purified with 6N hydrochloric acid (2.75 ml) and caprylic alcohol (2 drops). The flask

(covered with aluminium foil) was incubated in a water bath (95 °C, 15 min) and left overnight (16 hr) at room temperature.

The mixture was filtered using filter paper and the filtrate was added with tungstophosphoric acid reagent (5 ml) followed by another filtration process after 5 hr incubation at room temperature. Then, the mixture was added with ammonium hydroxide (NH_4OH) until reached pH 4.5 followed by the addition of 5 ml acetate buffer (pH 4.5). The mixture was mixed well and left overnight (16 hr) at room temperature.

The mixture was centrifuged (Thermo Jouan CR3i, France) at 1 700 rpm, 30 °C, for 15 min and the supernatant was decanted. The precipitate was washed using a filtered cold wash liquid and the decant process was repeated at least 3 times. Later, the purified precipitate was dissolved with 10 % sulphuric acid (5 ml). Sample and blank (5 ml of 10 % sulphuric acid) were incubated in a water bath (95 °C, 10 min) and subsequently titrated against 0.01N potassium permanganate ($KMnO_4$) until the visible pink colour persisted for more than 30 sec. The oxalic acid content was calculated using equation 3.5 as follows:

$$Oxalic\ acid\ (mg/100\ g\ dw) = \frac{[volume\ of\ KMnO4\ x\ 67.5\ x\ (net\ weight + 100\ g)]}{net\ weight\ x\ wet\ slurry} \quad (3.5)$$

Where 6.75 = 0.45 (mg anhydrous oxalic acid equivalent to 1 ml 0.01N $KMnO_4$) x (30/20) x (25/25) (dilution factor) x 100 (conversion to 100 g sample).

3.1.13.2 Phytic acid

Determination of phytic acid in chloroplast-rich fraction (CRF) was performed using a Megazyme kit. The phytate was measured as phosphorus released by phytase and alkaline phosphatase. The absorbance was detected at 655 nm using a UV-Vis spectrophotometer (Varian Cary 50, Australia). A calibration curve of phosphorus solution with different concentrations was prepared as standard.

3.1.14 Mineral composition

The mineral analysis of chloroplast-rich fraction (CRF) was performed using a commercial Wavelength Dispersive X-Ray Fluorescence (WDXRF) spectrometer S8 TIGER Series 2 (Bruker, Germany). The WDXRF was controlled by Spectra Plus Launcher software using the Quant Express method. The CRF powder was placed into a sample cup (thickness 15 mm, diameter 34 mm), and covered with polypropylene. The equipment was characterised by Rhodium X-ray tubes and 8 primary beam filters. Up to 8 automatic crystal changers were used, allowing for the detection of the entire range of elements from Beryllium to Uranium. The maximum power, voltage, and tube current directed were 4 kW, 60 kV and 170 mA, respectively. Analysis of the sample was carried out for 20 min individually.

3.1.15 Preparation of CRF extract

The chloroplast-rich fraction (CRF) extract was prepared as Hue et al. (2012) described. CRF powder (0.5 g) was dissolved in 50 ml methanol (1:100 ratio). The mixture was placed in a shaking incubator at 150 rpm, 25 °C for 1 hr. The mixture then was centrifuged (Thermo Jouan CR3i, France) at 2 500 rpm, 25 °C for 15 min, followed

by a filtration process using filter paper. The CRF extract was stored at – 80 ± 1 °C prior to the analysis of total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, and ferric reducing antioxidant potential (FRAP) assay.

3.1.16 Total phenolic content (TPC)

Total phenolic content (TPC) analysis was adopted from Hue et al. (2012). A 50 µl of chloroplast-rich fraction (CRF) extract (obtained in Section 3.1.15) was added to 250 µl of Folin-Ciocalteu reagent. Both solutions were mixed and incubated in the dark for 5 min at room temperature. Then, 750 µl of sodium carbonate solution (20 %) was added, mixed, and incubated in the dark for 2 hr at room temperature. The absorbance was read at 765 nm using a UV-Vis spectrophotometer (Varian Cary 50, Australia) against blank. The steps were repeated by replacing the sample extract with diluted gallic acid in a few different concentrations to plot a calibration curve for the standard. The concentration of total phenolic content in the CRF extract was calculated based on the standard curve equation and presented in gallic acid equivalent (g GAE/100 g dw).

3.1.17 DPPH radical scavenging assay

The free-radical scavenging activity of chloroplast-rich fraction (CRF) was determined using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay from Hue et al. (2012). The assay was performed using 0.1 mM DPPH solution diluted in methanol (80 %). The diluted DPPH solution (1 ml) was added to the CRF extract (500 µl), which was obtained in Section 3.1.15. The mixture was vortexed for 15 sec and incubated in a water bath (37 °C, 30 min). The absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Varian Cary 50, Australia) against a blank of methanol solution.

The steps were repeated by substituting the CRF extract with an ascorbic acid solution in different concentrations diluted in methanol as standard. The IC_{50} value was analysed based on a graph representing the concentration of the CRF required to scavenge 50 % of the DPPH free radicals. The inhibition percentage for scavenging activity was calculated using equation 3.6 as follows:

$$DPPH\ scavenging\ activity\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x\ 100 \qquad (3.6)$$

Where the $A_{control}$ is the absorbance for control and A_{sample} is the absorbance for the CRF extract.

3.1.18 Ferric reducing antioxidant potential (FRAP) assay

The reducing activity of ferric ions was measured using a method from Xu et al. (2010). The FRAP reagent was prepared by mixing 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution, 20 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) solution and 300 mM sodium acetate buffer (pH 3.6) solution with a ratio of 1:1:10 in volume. 0.2 ml of the CRF extract (obtained in Section 3.1.15) was mixed with 3.8 ml FRAP reagent followed by incubation in a water bath (37 °C, 30 min). The absorbance was read at 593 nm using a UV-Vis spectrophotometer (Varian Cary 50, Australia). The process was repeated with fresh working solutions of $FeSO_4$ to obtain a linear standard calibration curve. The antioxidant capacity was expressed as mmol equivalents per gram of sample (dw).

3.1.19 Lipid extraction

Lipids were extracted using the modified Folch et al. (1957) technique. A mixture of chloroform and methanol in a 2:1 ratio (1.2 ml) was added to 0.1 g of the CRF powder and vortexed for 1 min. A 0.9 % sodium chloride solution (0.5 ml) was added, and the mixture was vortexed again for 1 min before centrifugation (Thermo Jouan CR3i, France) at 3 000 rpm, 4 °C for 10 min to separate into 3 phases.

The lipids and chloroform, which were located at the lower phase were collected and transferred to a clean centrifuge tube. The residue was extracted twice with a 2:1 ratio of chloroform and methanol mixture (1.2 ml) followed by vortex and centrifugation steps, respectively. The process was repeated 3 times to ensure complete lipid extraction (get a clear lipid phase).

The lipid phases were collected and pooled with the original lipid layer. To separate the lipids from any residues, the pooled lipids were centrifuged again before being dried under the flow of nitrogen. The dried lipid extract was weighed to calculate the total lipid content and subsequently used for the analysis of chlorophylls, β-carotene, and lutein.

3.1.20 Chlorophyll content

The pigment content of a lipid extract was analysed using a UV-Vis spectrophotometer (Varian Cary 50, Australia). The lipid extract (obtained in Section 3.1.19) was dissolved in acetone (1 ml) and further diluted to a factor of 1:1000. The absorbance (*A*) was measured at three wavelengths: 661.6 nm (chlorophyll *a*), 644.8 nm (chlorophyll *b*), and 470 nm (total carotenoids).

The concentration of each pigment (ug/ml) was calculated using equations by Lichtenthaler and Buschmann (2001) as stated below, which was then used to determine the percentage of pigment content.

$$Chlorophyll\ a = (11.24\ A_{661.6} - 2.04\ A_{644.8}) \tag{3.7}$$

$$Chlorophyll\ b = (20.13\ A_{644.8} - 4.19\ A_{661.6}) \tag{3.8}$$

$$Total\ carotenoids = \frac{(1000\ A_{470}) - (1.90\ _{Chlorophyll\ a}) - (63.14\ _{Chlorophyll\ b})}{214} \tag{3.9}$$

$$Pigment\ content\ (\%) = \frac{Concentration\ \left(\frac{ug}{ml}\right)\ x\ Dilution\ factor}{Weight\ of\ sample\ (ug)}\ x\ 100 \tag{3.10}$$

3.1.21 β-carotene and lutein content

The carotenoid content of chloroplast-rich fractions (CRFs) was analysed by high performance liquid chromatography (HPLC) with photodiode array (PDA) detection using an Agilent 1200 Series System.

The dried lipid extract (obtained in Section 3.1.19) was dissolved in 2 ml acetone solution (containing 0.1 % butylated hydroxytoluene [BHT]). Then, the dissolved sample was syringe filtered (using 0.45 µm syringe filter) into amber HPLC vials. The flow rate of mobile phase (Acetonitrile: Methanol: Ethyl Acetate with 0.05 % Triethylamine [TEA]) was set at 0.5 ml/min. The use of TEA was to increase carotenoid recovery (Hart & Schott, 1995). Two gradient mobile phases were applied from 95:5:0 to 60:20:20 in 20 min, maintaining the proportion until the end of the run. Re-equilibration took 15 min.

The sample (10 µl) was injected through a Zorbax SB-C18 analytical guard column (5 µm, 4.6 x 12.5 mm) and separated on Nova-Pak C18 (4 µm, 4.6 x 150 mm) column, and the temperature was set at 22.5 °C. β-carotene and lutein were detected at 454 nm. The concentration of β-carotene and lutein was determined using a linear equation obtained by a calibration curve produced from a range of external standards (β-carotene and lutein) dissolved in acetone containing 0.1 % BHT. The data was presented in Chapter 6, as pre-digested carotenoid concentration of CRFs.

3.2 Materials and methods for Chapter 6

This section describes the materials and methods for Chapter 6: The digestive stability and bioaccessibility of β-carotene and lutein of chloroplast-rich fractions (CRFs) from sweet potato haulm.

3.2.1 Chemicals

The digestion enzymes including α-amylase from human saliva type XIII-A, 300 – 1 500 U/mg protein (A1031; EC 3.2.1.1), pepsin from porcine gastric mucosa (P7012; 3.4.23.1), pancreatin from porcine pancreas (8xUSP; P7545) and bile extract porcine (B8631) were purchased from Sigma Aldrich (St Louis, MO, USA).

3.2.2 Materials

The chloroplast-rich fraction (CRF) was obtained from the previous method mentioned in Section 3.1.3. The untreated (fresh) and heat-treated (conventional pasteurisation, steam pasteurisation, and water blanching treatments) CRFs were studied in this chapter. Local palm oil (Saji, Malaysia) was purchased from Lotus, Malaysia.

3.2.3 *In-vitro* digestion model

To investigate the third objective: The digestive stability and bioaccessibility of β-carotene and lutein of chloroplast-rich fractions (CRFs) from sweet potato haulm (SPH), the digestibility of nutrients *in-vitro* was subjected to the untreated (fresh) and heat-treated (conventional pasteurisation, steam pasteurisation and water blanching treatments) CRFs powder following a standardised static *in-vitro* digestion method suitable for food (Minekus et al., 2014; Wattanakul et al., 2022).

CRF powder (1 g) was dissolved in ultra-pure water up to 5 g, followed by the addition of 4 ml simulated salivary fluid (SSF) electrolyte stock solution. The mixture was vortexed for 1 min at room temperature to allow homogenisation. A 0.5 ml of salivary α-amylase solution of 75 IU/ml made up of ultra-pure water (α-amylase from human saliva Type XIII-A, 300 – 1 500 U/mg protein) was added followed by 25 µl of 0.3 M $CaCl_2$ and 0.475 ml of ultra-pure water. The digestion mixture was mixed in a shaking incubator (100 rpm, 37 °C, 2 min).

Next, 10 ml of simulated gastric fluid (SGF) electrolyte stock solution was added to the oral bolus (10 ml) followed by 0.5 ml of pepsin solution made up of ultra-pure water (pepsin from porcine gastric mucosa) and 5 µl of 0.3 M $CaCl_2$ was then added to achieve 2 000 U/ml and 0.075 mM, respectively in the final digestion mixture. Then, the pH was reduced to 3 using 1 M HCl and the volume was made up to 20 ml with ultra-pure water. The digestion mixture was put again in the shaking incubator (100 rpm, 37 °C, 2 hr). Figure 3.2 is the illustration of a simplified scheme for *in-vitro* digestion of CRF.

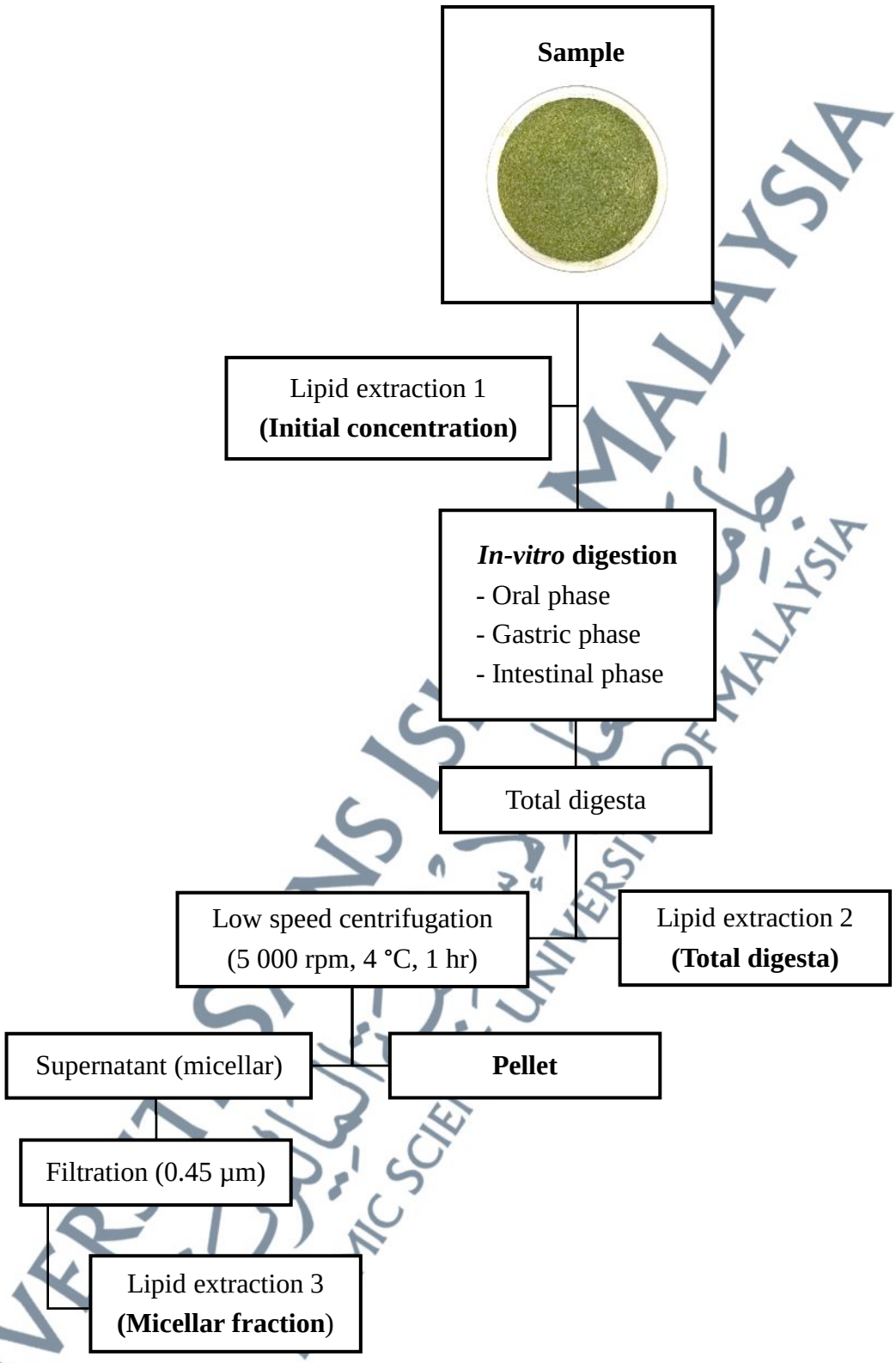

Figure 3.2: The simplified scheme for *in-vitro* digestion of CRF

Later, the digestate from gastric chyme (20 ml) was mixed with 8.5 ml of simulated intestinal fluid (SIF) electrolyte stock solution, 5 ml of pancreatin solution of 100 U/ml (based on trypsin activity) made up in SIF electrolyte stock solution (pancreatin from porcine pancreas), 2.5 ml of porcine bile extract (10 mM bile salts), and 40 µl of 0.3 M $CaCl_2$. Then, the pH was adjusted to 7 with 1 M NaOH and the volume was made up to 40 ml with ultra-pure water. The digestion mixture was incubated again in the shaking incubator (100 rpm, 37 °C, 2 hr).

Before every incubation step and at the end of the *in-vitro* digestion, samples were flushed under a blanket of nitrogen gas to protect samples from oxidation. Digestive juices were prepared for mouth (Simulated Salivary Fluid, SSF), stomach (Simulated Gastric Fluid, SGF), and duodenum (Simulated Intestinal Fluid, SIF) compartments according to Minekus et al. (2014). The digested samples (total digesta) were further processed as described in Section 3.2.4 on the same day of *in-vitro* digestion (illustrated in Figure 3.2). All samples were digested in triplicates.

3.2.4 Separation of micellar fraction

The total digesta obtained in Section 3.2.3 was centrifuged (Thermo Jouan CR3i, France) at 5 000 rpm, 4 °C, for 1 hr. The centrifuged sample was separated into two phases – see Figure 3.3 (unless oil was added to the sample, in which case, three phases could be formed, and the middle layer carries the mixed micelles). The mixed micelle located at the top was clear dark green while the sediment phase at the bottom was brownish green in colour. The top phase was assumed as the bioaccessible fraction which contained mixed micelle that entrapped the carotenoids and other nutrients.

The micellar fraction was filtered (0.45 µm PTFE filter) and washed with 1 ml of chloroform: methanol (2:1) solution to recover the nutrients trapped in the filter. Filtered (micellar fraction) and non-filtered (total digesta) samples (10 ml each) were collected into pre-weighed falcon tubes. All samples were flushed with nitrogen gas and stored at – 80 ± 1 °C until further analysis (maximum of one week). The samples were then analysed for their content of specific carotenoids (β-carotene and lutein).

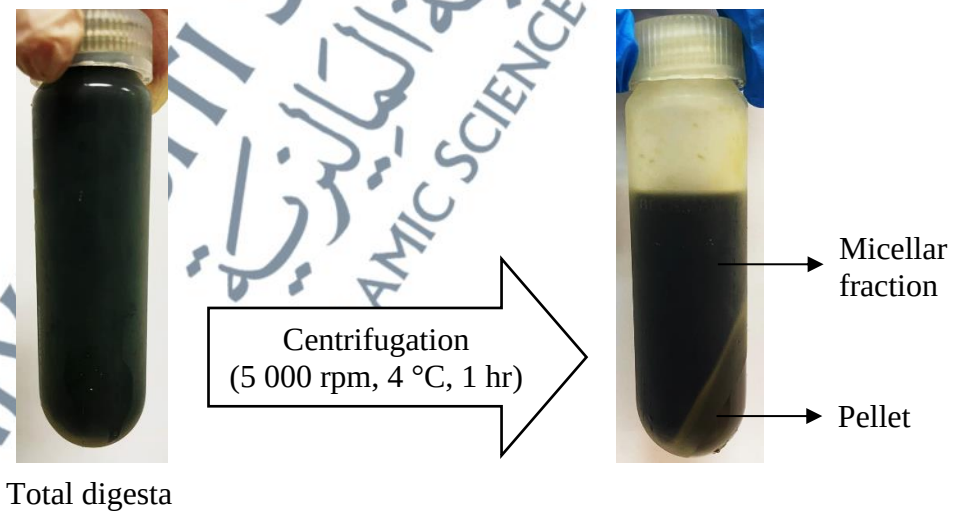

Figure 3.3: Total digesta, micellar fraction and pellet after *in-vitro* digestion

3.2.5 Retention of nutrients after digestion

The retention or stability of nutrients after digestion was determined using the following equation 3.11:

$$Retention\ of\ nutrients\ (\%) = \frac{C_{total\ digesta}}{C_{initial}}\ x\ 100 \qquad (3.11)$$

Where $C_{initial}$ is the concentration of nutrients before digestion.

3.2.6 Bioaccessibility determination

The bioaccessibility of nutrients can be determined in two ways. To avoid confusion, both ways were used and described as follows:

i) Bioaccessibility of nutrients (BA %)

The bioaccessibility of nutrients was calculated using equation 3.12 below (Garrett et al., 1999):

$$Bioaccessibility\ of nutrients\ (\%) = \frac{C_{micellar\ fraction}}{C_{total\ digesta}}\ x\ 100 \qquad (3.12)$$

ii) Nutrients accessible for uptake (NA %)

To enable comparability of the results of different experiments and matrices, the nutrients accessible for uptake were calculated using equation 3.13 below:

$$Nutrients\ accessible\ for\ uptake\ (\%) = \frac{C_{micellar\ fraction}}{C_{initial}}\ x\ 100 \qquad (3.13)$$

Where $C_{initial}$ is the concentration of nutrients before digestion.

3.2.7 Carotenoid extraction of digested materials

Extraction of lipids from the *in-vitro* digested materials was performed using the Bligh and Dyer (1959) techniques adapted from the Folch et al. (1957) method. Concisely, 5 ml chloroform: methanol (2:1) was mixed with an equal volume (5 ml) of the digested materials (either micellar fraction or total digesta) and vortexed for 1 min. Then, 1 ml of 0.9 % sodium chloride solution was added, and the mixture was vortexed again for 1 min before being centrifuged (Thermo Jouan CR3i, France) at 1 300 rpm, 4 °C for 10 min to separate the phases.

The lowest phase containing the lipids and the chloroform was transferred to a clean vessel. A further 5 ml of the chloroform: methanol (2:1) was added to the remaining extract which was then vortexed and centrifuged again with the same conditions. The lipid phase was removed and pooled with the original lipid phase before a final sequence was completed. The pooled lipid phase was centrifuged again to separate the lipids from any residual compounds.

The lipid layer was removed into a pre-weighed clean vessel and dried under a flow of nitrogen. Lipid extract was then weighed to calculate the total lipid content and to quantify the intended carotenoids (β-carotene and lutein) by high performance liquid chromatography (HPLC) analysis.

3.2.8 Carotenoids (β-carotene and lutein) analysis

The carotenoid content of digested materials was analysed by high performance liquid chromatography (HPLC) with photodiode array (PDA) detection using an Agilent 1200 Series System. The dried lipid extract obtained previously through the Folch et al. (1957) method (Section 3.2.7) was dissolved in 2 ml acetone solution (containing 0.1 % BHT) and syringe filtered (using 0.45 μm syringe filter) into amber HPLC vials. HPLC conditions were similar to the description in Section 3.1.21.

3.3 Statistical analysis

Data were analysed statistically to the analysis of variance (ANOVA) using the Minitab version 21 software (Stat Inc, USA). One way of analysis was applied according to Tukey's pairwise comparison post-hoc test with a 95 % confidence interval. The results were stated as means ± standard deviation, and the means differences at $p < 0.05$ were considered significant. Experiments were conducted in triplicates. Correlations among data obtained were determined using Pearson correlation coefficient with the Minitab software.