Improving the extraction of hemp proteins from hemp seed meals

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Abstract

The global population growth leads to an increasing demand for proteins. This increasing demand creates pressure on natural resources and the environment, of which the impact of animal protein sources is more significant than that from plant proteins. Besides that, the use of animal proteins might cause some health concerns to consumers. Therefore, alternative sources of proteins, such as plant proteins, are necessary. Oilseed meals (also known as oilseed cakes) are becoming increasingly promising alternative protein sources; they have high protein content and can be a sustainable source. Oilseed cakes are considered a by-product of the oil industry and their use as a protein source would create less impact on the environment. Hemp seeds have a significant content of protein highly digestible and with great nutritional value. However, the application of hemp protein into food products has not been fully exploited because of several technological challenges, such as the poor solubility in water-based foods - a key challenge in the adoption of novel sources of proteins. The aim of this work was to explore different approaches for improving the extractability of hemp protein from hemp seed meals and investigate the effect of processing conditions on the yield of extraction and the characteristics of resulting proteins.

Four commercially available hemp seed meals (HSMs) in this study were chosen based on the availability in the New Zealand markets and investigated for physicochemical characteristics to understand the properties of the starting material for the protein extraction process. The microstructure, proximate compositions, particle size, and protein solubility of four HSMs was measured. The effect of reduced particle size by using nitrogen grinding on the solubility of hemp seed protein was studied. The compositions and particle size distributions of solubilised hemp proteins in water were characterised by using SDS-PAGE and dynamic light scattering, respectively.

To improve the extractability of hemp seed proteins from HSMs, two approaches were taken in this work. Firstly, the effect of different process variables was investigated; these included heating, pH adjustment, the use of salts, pH cycling and ultrasound. The effect of heating conditions on extractability was investigated using three different temperatures (20, 65 and 95 ^{o}C). The pH 7 and pH 9 were used to investigate the effect of pH on protein extractability. Four types of salt (KCl, NaCl, $CaCl_2$ and $MgCl_2$) were added at different concentrations (0 to 0.8 M) to determine the effect of salts on the protein extraction yield. Two pH cycles (pH-9-2-9) and (pH 9-12-9) were employed to investigate whether pH cycling improves the yield. In addition to the above, ultrasound was used at different amplitudes (20 and 80%), different temperatures (20, 65 ^{o}C), and in different ultrasonic duration times and the impact of these conditions on extraction yield was investigated. These

treatment combinations were studied further in the second approach. To investigate the combined effect of ultrasound with pH cycling and the addition of salt, the condition of amplitude 80%, 65 ^{o}C, and 15 min was used. The extractability of proteins was derived from the estimation of protein contents on the supernatants obtained after centrifugation of samples (protein extraction yield), and this was analysed to determine the effectiveness of treatments. In addition, the protein compositions, total phenolic and available lysine content, and microstructure of hemp seed meals after protein extraction were analysed to understand the impact of treatment on resulting protein characteristics and food matrix.

The commercial HSMs powder contained 48 to 65% protein. However, their corresponding aqueous mixtures (10g powder/90 mL water) had low protein solubility, from 6 to 13%. Reducing the particle size of HSMs by nitrogen grinding improved the solubility of hemp proteins (22% higher than non-grinded samples). The compositions of hemp protein solubilised in the water were not significantly different between the four samples investigated and included two main fractions albumin and globulin.

The results of this study reveal that heating at 65 ^{o}C and pH 9 are the optimal conditions for hemp protein extraction. The combination of these two optimal conditions led to a yield of approximately 31%. Under these conditions (65 ^{o}C, pH 9), the additional treatments showed a further increase in the extraction of hemp proteins. For example, the addition of salt (NaCl) up to 0.8 M resulted in a significant increase in the extraction yield (up to 81.2 %). In comparison, the alkaline pH cycling (pH 9-12-9) increased the yield to approximately 47%. Furthermore, the application of ultrasound treatment (amplitude 80%, 15 min) showed an extraction yield of 37 %.

The combination of ultrasound and addition of salt showed a synergistic effect at a low salt concentration (NaCl 0.25 M), but not at higher concentrations. Similarly, ultrasound treatment combined with alkaline pH cycling (pH 9-12-9) increased the yield of extraction by 81%. The SDS-PAGE analysis of extracts showed that samples from all investigated treatments had largely similar protein compositions. The albumin and two globulin fractions (globulins 7s and 11s) were contained in the extracts.

Almost all conditions resulted in co-extraction of phenolic compounds but the total phenolic content in the extracts varied as per extraction conditions. The ultrasound treatment extracted most efficiently the phenolic compound in hemp seed meals. Furthermore, the use of alkaline pH cycling reduced the available lysine content per gram extracted protein, while the ultrasound treatment increased this value. Overall, the results of this study have compared the different methods of extraction on the

extractability of hemp proteins. This might provide the basis for the design protocols of hemp protein extraction and extend research on the functional properties and applications of hemp proteins.

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List of Abbreviations

AS	Acidic subunit
BS	Basic subunit
CLSM	Confocal laser scanning microscopy
EAAs	Essential amino acids
FAO	Food and Agriculture Organization
GAE	Gallic acid equivalent
GLA	Gamma-linolenic acid
HSM	Hemp seed meal
HSMs	Hemp seed meals
MW	Molecular weight
PDCAAS	Protein Digestibility-Corrected Amino Acid Scores
p*I*	Isoelectric point
SDA	Stearidonic acid
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis
SEM	Scanning electron microscopy
TPC	Total phenolic content
V/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

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Chapter 1

1. Introduction

The global population is rising and will increase to approximately 9.7 billion by 2050 (United Nations, 2019). The increasing population is driving demand for food and nutrients upwards. The need for good quality macronutrients such as protein, a crucial part of the diet, is rising accordingly. The Food Agriculture Organization (FAO) (2017) asserts that the global protein demand would increase by 40% in 20 years from 2010 to 2030. In addition, the climate is changing and has an impact on the food production systems. Coupled with this, the high demand for protein creates pressure on natural resources and the environment (Kumar et al., 2015); in which, the impact from animal protein is more significant than that from the plant proteins. An estimated 12% of greenhouse gas emissions are caused by livestock production (Henchion et al., 2017). The water consumption to produce 1 kg of grain feed beef is 4-5 times more than that used to create 1 kg of cereal grain (Malik et al., 2017). Plant proteins, therefore, are comparatively more sustainable in terms of water and land use compared to animal proteins.

Moreover, there is an increase in health concerns related to consuming animal-derived proteins. Evidence has shown a strong relationship between red or processed red meat consumption and a rising risk of stroke, esophageal cancer, or early death (Bernstein et al., 2012; Choi et al., 2013). Ethical issues in animal production have also created a trend towards flexitarianism, which aims to reduce the consumption of meat and other animal protein sources and use alternative protein sources, such as plant proteins (Henchion et al., 2017). Therefore, the need for alternative sources of protein, such as plant-based protein is increasing rapidly.

Among plant protein sources, oilseed cake proteins are becoming very promising resources for the extraction of biomaterials. Oilseed cakes are by-products of oil processing, which contain a significant content of storage proteins. Of these oilseeds, rapeseed is the most important crop in New Zealand, in which protein occupies about 24% to 27% by weight of the seeds (Nosenko et al., 2014). Linseed, another important crop in New Zealand, contains about 20% to 25% crude protein by weight (TrevinAo et al., 2000). However, oilseed cakes have a high amount of anti-nutritional components that limit the application of those proteins in food formulations (Sutton et al., May 2018).

Hemp is an eco-friendly plant; it can grow organically without fertilizers, herbicides, and pesticides, and it has a natural resistance to several pests. Compared to other plants, hemp absorbs five times

more carbon dioxide, which helps reduce global warming (Aluko, 2017). Compared to other plant proteins, such as peanuts, soybeans, and different kinds of legumes, hemp protein is considered less allergic. In New Zealand, the use of hemp seeds in food formulations was permitted from 2018. Currently, dehulled hemp seeds are the only part of the hemp plant allowed to be sold as food or be used in food formulations (MPI, 2020).

Nutritional values of hemp seed have been recognized in Canada and some Asian and European countries. Hemp has been an industrial crop in these countries for decades and has been used in traditional food preparations. For example, hemp seed has been sold as a street snack in China and porridge made from oat and hemp has been a popular nutritional meal in the Czech Republic (Callaway, 2004).

However, the application of this nutritional protein source has not been fully exploited due to its low functionality and low extractability (Ajibola, 2020; Chardigny et al., 2016; Potin et al., 2019). Hemp seed meals (HSMs) are a rich source of proteins that are obtained after pressing the oil out of the hemp seeds. Chapter 4 investigates the physicochemical properties of commercial hemp seed protein products (hemp seed meals) used in this study. HSMs from different suppliers in New Zealand were procured and the extractability of proteins from these HSMs was investigated.

Extraction of hemp protein out of the non-protein components is the way to broaden the application of hemp proteins in the food industry. To improve the extractability of protein, different methods including saline extractions and isoelectric precipitation singly or in combination have been investigated, however, the reported yields and the quality of proteins in extracts resulting from these methods has been variable. A systematic study investigating the effect of temperature, pH and type of salt has not yet been done. Furthermore, whether a combinational approach would improve extraction has not yet been explored on protein-rich hemp seed meals.

Apart from the conventional methods of protein extraction, novel methods such as ultrasound have been shown to improve protein extraction from plant materials. These materials include wheat germ (Xue et al., 2009), sunflower meal (Dabbour et al., 2018), rice brain (Muhoza et al., 2017), rapeseed (Yagoub et al., 2017), and rice dreg flour (Li et al., 2017). Chapter 5 and 6 explore the different strategies to improve the yield of hemp protein extraction. Finally, Chapter 7 presents the overall discussion, limitations and applications of this work and explores avenues for future work.

To achieve the above-mentioned objectives, two approaches were proposed in this study and are listed below.

1. To investigate the effect of different extraction condition on the yield of protein extraction:
   - Temperature
   - pH
   - pH cycling
   - Adding salts
   - Ultrasound
2. To evaluate the effect of the combination of treatments in approach 1 on protein extraction:
   - Adding salts and ultrasound
   - pH cycling and ultrasound

A brief overview of the thesis chapters is presented in Figure 1.1

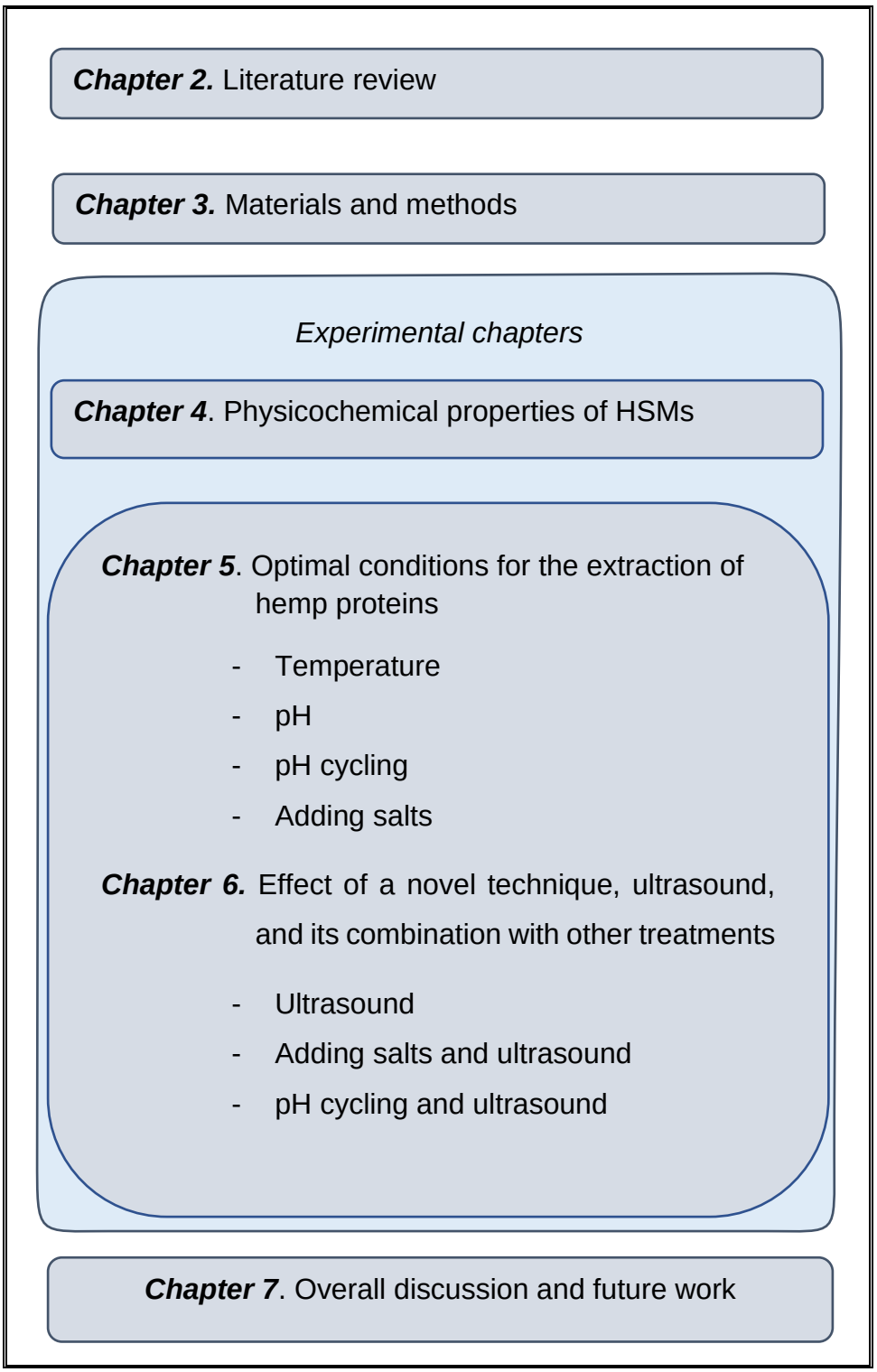

Figure 1.1: Overview of thesis chapters

Chapter 2

2. Literature review

2.1. Hemp seed compositions

Hemp seed contains about 35% (w/w, wet basis) fat; 25% (w/w, wet basis) protein, considerable content of dietary fibre (especially non-digestible), vitamins and minerals (Callaway, 2004; Deferne et al., 1996). In addition, hemp seed has a low moisture content (only 6.5%) and high ash content (5.6%) (Callaway, 2004). Protein and fat from hemp seed are considered of high nutritional value (Aluko, 2017). After removing the hull, the percentages of protein and fat increase to about 36% and 47%, respectively (Wang et al., 2019).

Recently, the addition of hemp seed products (flour, protein isolates, oil, etc.) into food formulation has been studied, and their use in foods is developing fast and becoming more common (House et al., 2010). Hemp flour has been researched and incorporated into different products (Ajibola, 2020). In the bakery, hemp flour has been used to replace other flours to improve the nutritional value (Pojic et al., 2015) and the quality attributes of finished products (Hruskova et al., 2016; Korus et al., 2017). Hemp flour and hemp protein isolate has been used in beverages, dairy products, and processed meats (Dabija et al., 2018; Naumova et al., 2017).

The most commercially valuable component of hemp seed is hemp oil which is extracted from seeds using different methods, such as cold-pressing and solvent extraction (Leonard et al., 2019). After the oil is removed, the remaining residual material is known as hemp seed cake or meal, a by-product of the oil production in which the proteins and carbohydrates are dominant. The typical nutritional content of hemp seed and hemp seed meal (HSM) is presented in Table 2.1.

Table 2.1: Typical nutritional content (%) of hemp seed and hemp seed meal. Compiled from Callaway (2004).

Composition (%)	Whole hemp seed	Hemp seed meal
Oil	35.5	11.1
Protein	24.8	33.5
Carbohydrate	27.6	42.6
Moisture	6.5	5.6
Ash	5.6	7.2
Total dietary fibre	27.6	42.6
Digestible fibre	5.4	16.4
Non-digestible fibre	22.2	26.2

2.2. Minor components of hemp seed

Hemp seed is also abundant in tocopherols, which is represented by vitamin E, as shown in Table 2.2. Specifically, alpha-tocopherol (5 mg/100 g) and gamma-tocopherol (85 mg/100 g) make up a total of 90 mg/100 g (Callaway, 2004). In addition to the vitamin E, the vitamin B group, including around 400 µg of B_1 and 100 µg of B_2, is also found in hemp seed.

Besides the vitamins, considerable minerals content is also present in hemp seed. The minerals profile of hemp seed includes macro-minerals (phosphorus, potassium, sodium, magnesium, calcium, sulphur) and micro-minerals (manganese, iron, zinc) (Callaway, 2004; Leyva et al., 2010). The content of phosphorous in hemp seed is higher than that found in other kinds of oilseed (Farinon et al., 2020). Hemp seed is considered one of the optimal sources of phosphorous. Interestingly, the high potassium content and the low sodium content result in a high ratio of K/Na, which is believed to brings cardioprotective effects. Details of minerals composition available in hemp seed are shown in Table 2.3.

Table 2.2: Typical vitamin content (mg/100g) in hemp seed. Compiled from Callaway (2004)

Vitamins	Content (mg/ 100 g)
Vitamin E	90
Alpha-tocopherol	*5*
Gamma-tocopherol	*85*
Thiamine (B_1)	0.4
Riboflavin (B_2)	0.1

Table 2.3: Typical mineral content in hemp seed. Compiled from Callaway (2004)

Minerals	Content (mg/100 g)
Phosphorous (P)	1160
Potassium (K)	859
Magnesium (Mg)	483
Calcium	145
Iron (Fe)	14
Sodium (Na)	12
Manganese (Mn)	7
Zinc (Zn)	7
Copper (Cu)	2

2.3. Hemp seed oil

The properties of hemp seed oil are affected by the extraction methods (Leonard et al., 2019). The unrefined hemp seed oil has a dark green colour due to the presence of chlorophyll (Leonard et al., 2019). The oil is liquid at room temperature and is contained within spherical organelles in hemp seeds called oil bodies. Other physicochemical properties of hemp seed oil are presented in Table 2.4.

Compared to other vegetable oils, the hemp seed oil has a lower iodine value, which reflects the high degree of unsaturation, of which unsaturated fatty acid accounts for about 90% of total fatty acids (Leonard et al., 2019). Hemp seed oil is dominated by essential fatty acids, such as linoleic acid and alpha-linolenic acid. It has the highest proportion of polyunsaturated fatty acid (PUFA) among the other vegetable oils (Callaway, 2004) (Table 2.5). Most of the health benefits of hemp seeds are associated with the high content of PUFAs and their fatty acids profile. Remarkably, linoleic (ω-6) and α-linolenic (ω-3) acids occupy about 80% of total fatty acid content, and they are present at an optimal ratio for health, (ω-6):(ω-3) between 2:1 and 3:1 (Aluko, 2017; Callaway, 2004). Also, hemp seed oil contains both gamma-linolenic acid (GLA) and stearidonic acid (SDA), which are associated with anti-inflammatory effects, which prevents infections and oxidative stress (Nandakumar et al., 2008).

Table 2.4: Common physicochemical characteristics of hemp seed oil.

Properties	Specification
Colour	Dark green
Flavour	Nutty
Taste	Bland
Congealing point	15 – 72 ^{o}C
Refractive index (40^{o}C)	1.457 to 1.478
Density (24^{o}C, mg/mL)	0.918 to 0.927
Iodine value (g/ 100g oil)	153.6 to 169.1
Acid value (mg KOH/g of oil)	1.76 ± 0.05

Compiled from Borhade (2013) and Leonard et al. (2019)

Table 2.5: Typical fatty acid profile of hemp seed oil and other vegetable oils. Compiled from Callaway (2004)

Type of oil	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	α-Linolenic acid	GLA	SDA	% PUFA	n6/n3 ratio
	C16: 0	C18: 0	C18 : 1	C18 : 2	C18 : 3	C18 : 3	C18 : 4	-	-
Hemp seed	5	2	9	56	22	4	2	84	2.5
Linseed	6	3	15	15	61	0	0	76	0.2
Sunflower	5	11	22	63	< 1	0	0	63	>100
Rape seed	4	<1	60	23	13	0	0	36	1.8
Soy	10	4	23	55	8	0	0	63	6.9
Corn	12	2	25	60	1	0	0	60	60
Olive	15	0	76	8	< 1	0	0	8	>100

2.4. Hemp seed proteins

In hemp seed, the protein is stored inside the aleurone grains, so-called protein bodies (Angelo et al., 1969). The microstructure of the protein bodies was recently revealed by Schultz et al. (2020). Figure 2.1 shows protein bodies and cellulose of the cell walls stained by Coomassie and calcofluor white, respectively. Micrographs show that each cell contained numerous rounded-shape protein bodies, and they can be found in most cell types (Schultz et al., 2020). Furthermore, images obtained by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) proved that each cotyledon cell contains numerous lipid and protein bodies in which the lipid bodies are present in different diameters and located around the protein bodies (Figure 2.2) (Lee et al., 2011).

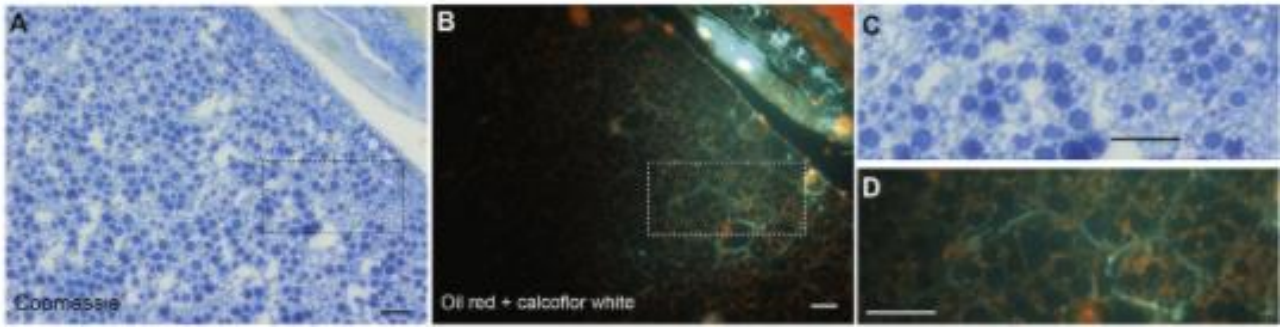

Figure 2.1: Hemp heart tissue highlighting protein bodies (Coomassie staining) and cellulose in plant cell walls (calcofluor white staining) (A - D). Hemp cotyledon stained with Coomassie and imaged in colour (A, C) and an overlay image of the same region showing autofluorescence and calcofluor white staining (B, D). The dashed boxed regions in A and B are magnified in C and D respectively and show that protein is packaged in spherical protein bodies. Reproduced with permission from Schultz et al. (2020).

The hemp proteins are extracted or enriched from the raw material (hemp seed, HSM) to broaden the application of hemp seed protein in food formulation. Hemp proteins start to denature at 86 ^{o}C and reach maximum peak at 95 ^{o}C (Tang et al., 2006). The isoelectric point (p*I*) of hemp protein isolate ranges from pH 4-5 (Malomo, 2015). Fat is one of the components that limit the extraction of hemp protein out of plant material by forming cross-link with protein. Therefore, the input material of protein extraction process is typically defatted.

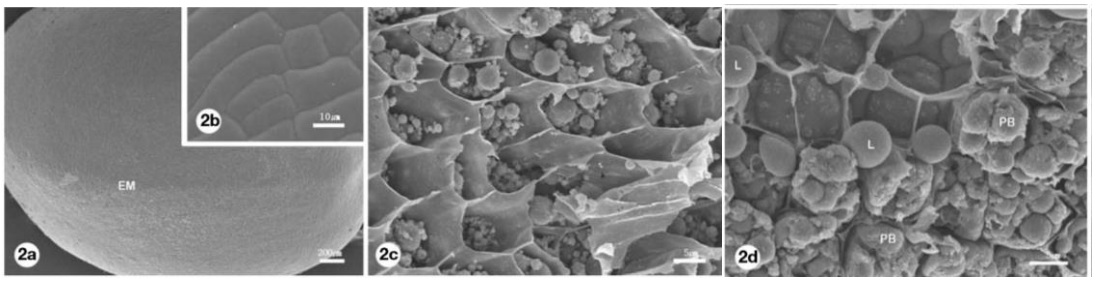

Figure 2.2: SEM image of hemp seed structure, (2a) part of cotyledon surface, (2b) close-up of cotyledon surface showing long and short rectangular cells. Cotyledon cell with numerous protein and lipid bodies (2c), and at higher magnification (2d). Reproduced with permission from Lee et al. (2011).

2.4.1. Hemp seed protein products and the corresponding extraction methods

The plant protein products can be classified as three types depending on protein content, including flour/ meal, concentrates, and isolates.

2.4.1.1. *Hemp seed meal*

For oilseed meals, i.e., flour, the protein content is not higher than 65% (Chardigny et al., 2016; Malomo et al., 2014). The HSM is obtained after the hemp oil is removed by cold-pressing or solvent extraction (Leonard et al., 2019). Depending on the oil extraction technique employed, the protein content of an HSM may vary, i.e., the more oil is extracted, the higher protein is obtained in the HSM (Malomo et al., 2014).

2.4.1.2. *Hemp seed protein concentrate*

Hemp seed protein concentrate contains at least 65% protein (Wang et al., 2019). This type of hemp protein product is commonly obtained after removing the non-protein components of the HSM. An enzymatic method has been reported by Malomo et al. (2015b) for hemp protein extraction in which, a mixture of enzymes (cellulase, hemicellulase, xylanase, phytase) was used to hydrolyse the non-protein components. The authors combined enzymatic hydrolysis with membrane ultrafiltration to transform a low protein HSM into a hemp protein concentrate, with a final protein content of about 70%. Given the high protein content of hemp seed meals, the HSMs have been sometimes commercially described as hemp seed protein concentrates.

2.4.1.3. *Hemp seed protein isolate*

The protein content in hemp seed protein isolates is a minimum of 90% (Chardigny et al., 2016; Malomo et al., 2014). An isolation process is applied to the defatted hemp seed or HSM to create hemp seed isolate (Wang et al., 2019). The most common process used in the manufacture of hemp seed protein isolate is isoelectric precipitation. This involves alkaline solubilisation of proteins at pH 8-10, followed by isoelectric precipitation of solubilised proteins at pH 4.5-5.0 (Potin et al., 2020). This method has been previously reported by Tang et al. (2006) and Kim et al. (2011b). Although similar pH values of solubilisation (pH 10) and precipitation (pH 5) have been used in these studies, the extraction yield reported was different (73% and 49.1%, respectively). The difference in yields of extraction might be related to variations in the protocols used in the recovery steps. However, the alkaline condition extraction might result in a detrimental effect on the final extract e.g., undesirable

reactions of proteins, decreased protein digestibility, loss of certain amino acids, difficulties to control the final product properties and process conditions (Gencdag et al., 2020).

The extraction yield reported from the above-mentioned studies shows a wide variation. While one study reported about 40% of extraction yield, the other showed only 6.33% for albumins and 6.07% for globulins. These studies do not report the effect of adding salts to the extraction media on the protein extraction yield. In a recent study, Potin et al. (2019) have shown the effect of varying the concentration of sodium chloride (0.5 M) on protein extraction, and it is not known whether a higher concentration of salt will improve the extractability of proteins. The combination of isoelectric precipitation and micellisation methods enhance the extraction yield (Hadnadjev et al., 2017). This combination has been applied to other plant materials, such as *Rosa rubiginosa* seeds (Mourea et al., 2001), and cowpea (Mune et al., 2008). So far, combinational extraction methods have not been explored on protein-rich hemp products.

The pH shift method has mostly been used for modification of the functional properties of plant proteins, such as soy protein isolate (Jiang et al., 2009; Jiang et al., 2010; Liu et al., 2015) or peanut protein (Li et al., 2020a; Wang et al., 2020). This technique also has been applied to hemp material by Wang et al. (2018b) to modify the structure, cross-link and emulsifying properties of hemp protein isolate. In another study, Wang et al. (2018a) also employed pH shift to improve the stability of hemp milk. Furthermore, the combination of pH shift and ultrasounds has been used in rapeseed protein (Li et al., 2020b) and pea protein (Jiang et al., 2017) to modify the protein functionality. However, the application of pH shifts alone or in combination with ultrasounds has not been studied in the extraction of hemp protein.

2.4.2. Hemp protein composition

A total of 181 proteins have been identified in hemp seed, of which two protein fractions, globulins and albumins, are found in the majority (Leonard et al., 2019). These two significant fractions have different amino acid profiles and functional properties that will be discussed below.

2.4.2.1. *Albumins*

The albumin fraction accounts for about 15% of hemp proteins (Tang et al., 2006). This fraction is water-soluble. Malomo et al. (2015a) reported that this protein fraction comprises seven polypeptides molecules with molecular weight (MW) ranging from 6 to 35 kDa. Of these, most of this fraction are proteins with MW lower than 18 kDa (Figure 2.3). However, another study revealed that the albumin

fraction is composed of 2s albumin of 10 kDa (Odani et al., 1998; Ponzoni et al., 2018). And consists of two subunits of molecular weights 7 and 3 kDa linked by two disulfide bridges. This protein fraction is rich in sulphur-containing amino acids occupying about 18% of the total amino acids of albumin. The 3 kDa subunit has two cysteine and three methionine residues, while the 7 kDa subunit contains six cysteine and five methionine residues. The largest subunit forms two intra-chain disulfide bonds (Potin et al., 2020).

According to Malomo et al. (2015a), the albumin fraction has lower aromatic amino acids (Tyr, Phe, Trp) and hydrophobic amino acids (Ala, Cys, Val, Met, Ile, Leu) than the globulin fraction. Therefore, albumins have a more flexible structure with higher water solubility and do not show dramatic changes with pH. Albumins are less soluble at pH 3, but this improves with the increase of pH, which probably confers albumins higher foaming capacity but lower foaming stability than globulins (Malomo et al., 2015a).

2.4.2.2. ***Globulins***

Globulins account for about 85% of total hemp proteins (Tang et al., 2006). This fraction is soluble in salty solutions and very insoluble in water. It exhibits the lowest solubility at pH 5 (Malomo et al., 2015a). Based on the sedimentation coefficient, the globulin fraction is made of 93% of 11s legumin-type globulins and is also called edestin, and 7% of 7s vicilin-type globulins (Hadnađev et al., 2020; Potin et al., 2019; Tang et al., 2006). These two components, globulins 7s and 11s, can be separated by using pH shifts. They can be obtained by adjusting the pH to pH 6.4 and pH 4.6 to precipitate globulin 11s, and globulin 7s, respectively (Aluko, 2017). Edestin is a hexamer, MW of about 300 kDa, composed of six identical subunits (Potin et al., 2019; Tang et al., 2006) linked by non-covalent interactions (Hadnađev et al., 2020). Each subunit has five cysteine residues. Two of five cysteine residues are linked together by a disulphide bond through basic subunit (BS), MW about 18 to 20 kDa; and acidic subunit (AS), MW about 34 kDa. The band at 52 kDa under non-reducing conditions corresponds to the AS-BS units (Figure 2.3). Two other cysteine residues form an intrachain disulphide bond, and the rest of the cysteine residues keep the thiol group free (Chuang et al., 2019). The disassociation or reassociation of disulphide bonds is considered as a reason for the poor solubility of hemp protein (Wang et al., 2008b). For the 7s-globulin the MW is approximately 48 kDa (Wang et al., 2008b).

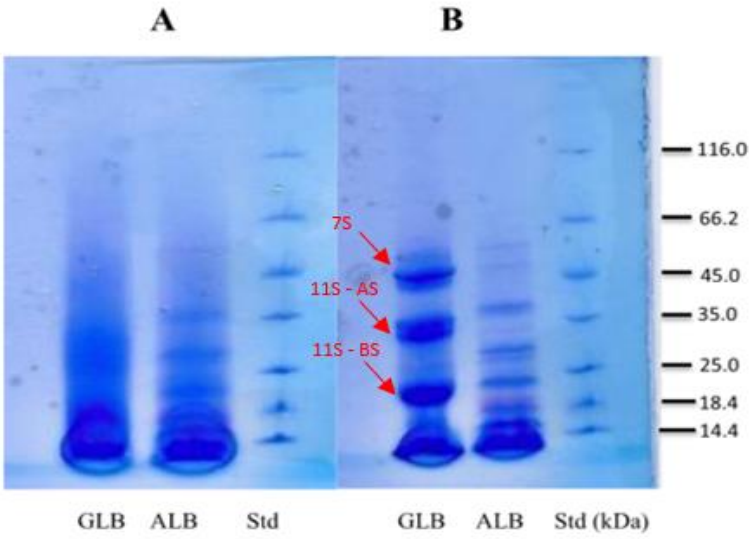

Figure 2.3: SDS-PAGE of hemp seed protein fractions, albumin (ALB) and globulin (GLB) under non-reducing (A) and reducing condition (B). Adapted from Malomo et al. (2015a).

2.4.3. The quality of hemp seed proteins

2.4.3.1. *Nutritional quality*

Hemp seed proteins are rich in essential amino acids (EAAs) and contain all of the EAAs required by humans (Farinon et al., 2020). The amino acid composition of hemp seed proteins is shown in Table 2.6. They are considered a good source of sulphur-containing amino acids, i.e., methionine and cysteine, which are commonly absent in other plant proteins (Aluko, 2017). Hemp proteins contain a very high amount of arginine, a precursor of nitric oxide that contributes to increased blood flow. Therefore, hemp proteins may have cardio-protective properties (House et al., 2010). Except for lysine and sulphur-containing amino acids, EAAs of hemp protein meets the amino acid requirement of FAO/WHO for infants from 2 to 5 years old (Tang et al., 2006; Wang et al., 2008b).

Table 2.6: Amino acid composition of hemp protein isolate and soy protein isolate. Adapted from Tang et al. (2006).

Amino acids	Content (mg/g of protein)	
	Hemp protein isolate	Soy protein isolate
Asp	98.0	118.1
Glu	168.1	212.9
Ser	54.0	54.8
Gly	41.7	38.6
His	29.3	29.0
Arg	103.2	75.7
Thr	47.6	41.0
Ala	47.0	38.3
Pro	47.2	52.9
Tyr	38.2	37.1
Val	51.8	44.1
Ile	41.5	44.8
Leu	69.0	70.0
Met	14.5	9.3
Cys	1.7	0.6
Phe	49.6	53.0
Lys	43.3	53.9

Compared to soy protein isolate, which is considered a good nutritional source of plant proteins for infants, hemp protein isolate has a higher proportion of EAAs (Wang et al., 2008b). Except for lysine, other essential amino acids are present in similar or higher content than in soy protein isolate. (Tang et al., 2006). Hemp protein isolate has a higher content of arginine, methionine, cysteine, and lower content of aspartic acid, glutamic acid, and lysine than soy protein isolate. Other amino acids are present at similar content in hemp and soy proteins isolate (Tang et al., 2006).

2.4.3.2. ***Digestibility of hemp proteins***

Compared to soy protein, hemp proteins have very low amounts of trypsin inhibitor, which is considered an anti-nutritional substance that can be inactivated by heat treatment. The presence of a very low content of this anti-nutritional substance makes hemp protein more digestible (Mamone et al., 2019; Park et al., 2012). The molecular structure of proteins defines the accessibility of enzymes, therefore, regulating their digestibility. Undertaking *in vitro* digestion experiments, Wang et al. (2008b) showed that the digestibility of hemp protein isolate is significantly higher than that of soy protein isolate after digestion with pepsin and trypsin. The digestibility of dehulled hemp seed may range from 90.8% to 97.5% due to variations in the hemp sources, whereas casein exhibits digestibility values of 97.6%.

In both native form and HSMs (whole seed and hulled see meals), the hemp proteins exhibit high digestibility. However, the removal of the hull helps to improve the digestibility of hemp protein and its bioavailability. This is because the presence of fibre limits protein digestion (House et al., 2010). The details of *in vivo* and *in vitro* digestibility of hemp and other kinds of protein are presented in Table 2.7.

Table 2.7: In vivo and in vitro percentage of digestibility of hemp proteins and other plant proteins. Adapted from Potin et al. (2020).

Plant material		*In vivo*	*In vitro*
Hemp	Whole seed	85	-
	Hulled seed	95	-
	Meal	87	85
	Hem protein isolate	-	88
	11S	-	91
	7S	-	88
Soy	Bean	93	-
	Flour	86	-
	Meal	80	-
	Soy protein isolate	95	80
Pea	Seed	86	-
	Pea protein concentrate	99	-
Sunflower	Seed	91	-
	Flour	90	-
Cotton	Seed	90	-
	Meal	85	81
Chickpea flour		84	72

2.4.3.3. *Bioavailability of hemp proteins*

Digestibility-Corrected Amino Acid Scores (PDCAAS) of hemp protein range from 0.48 to 0.61. The lower lysine and tryptophan contents make hemp protein have lower PDCAAS than soy protein isolated (0.92) (House et al., 2010; Wang et al., 2019). However, the PDCAAS based on fecal singly nitrogen digestibility might not reflect the digestibility of all amino acids (Rutherfurd et al., 2012). Another limitation of using the PDCAAS method is that it primarily focuses on the relative ability of a given protein to meet the amino acid needs of the host and that it uses as a reference the protein needs of children who require a larger proportion of lysine. The method does not indicate other potential attributes of the protein for hemp protein, which has a high content of arginine compared to other foods. Therefore, the potential exists to position hemp proteins as a source of digestible arginine. The process conditions impact protein digestibility (House et al., 2010).

2.4.4. Phytochemicals and antinutritional compounds in hemp seed

2.4.4.1. *Phytic acid*

The beneficial effect of phytic acid in reducing colon cancer risk and lowering serum cholesterol and triglycerides (Mattila et al., 2018; Pap et al., 2020). However, the presence of this anti-nutritive compound may result in the reduction of hemp protein bioavailability and may cause the undesirable colour or other organoleptic properties of end-products as well as inhibit the nutrient absorption (Pap et al., 2020).

According to Pap et al. (2020), about 25% of the phytic acid in defatted-hemp seed cake is insoluble form. However, this ratio is still lower than that of soybean and rapeseed meal (about 60% and 50% respectively) as reported by Han (1988). The concentration of this compound in different hemp seed protein products is not known; however, it is can be predicted that the content of phytic acid in hemp protein isolate and concentrate is lower than that in hemp seed meal. It is supposed that the water-soluble phytic acid might be solubilized in the water (Pap et al., 2020); and therefore being removed during the protein purifying process to produce hemp protein isolate and concentrate.

2.4.4.2. *Phenolic compounds*

Phenolic, a natural antioxidant is found in hemp seed and can help to reduce the risk of chronic diseases (Irakli et al., 2019). According to Irakli et al. (2019), phenolic compounds found in hemp seed are classified into two main groups, namely phenolic amides and lignanamides. Fourteen different lignanamides have been identified by Yan et al. (2015) in hemp seed and many of them have

good antioxidant activity (Mattila et al., 2018). However, the presence of the phenolic compound in the extracts together with protein might cause the interaction of these two bio-components which may impact the protein functionality, i.e., foaming capacity, emulsifying capacity, solubility. This interaction might be strong in some condition, such as low pH (Ozdal et al., 2013). It might also be reversible or irreversible depending on the phenolic and protein structure (Martin et al., 2014; Ozdal et al., 2013).

The presence of phenolic compounds in the hemp seed oil as presented by Smeriglio et al. (2016) reveals that the phenolic compounds are taken out of the seeds together with the hemp seed oil during the oil pressing process. It can be said that the phenolic content is different among the different hemp seed meals. This is due to the remaining phenolic content in the hemp seed meal depends on the oil processing conditions and how the oil has been removed from the seeds.

The data to compare the phenolic compound concentration in hem seed meal to other hemp protein products (hemp protein isolate and concentrate) is not available. Furthermore, the evidence of co-extraction of phenolic compounds during protein extraction, as confirmed by Potin et al. (2019), supports that the content of phenolic compounds in protein isolate and concentrate highly depends on the protein purifying conditions and how the hemp protein isolate and concentrate was produced from hemp seed meal.

2.5. Factors affecting plant proteins extractability

Plant proteins are located inside the matrix of plant materials. Any factors that cause an impact on protein and the food matrix might result in an effect on protein extractability. The impact of factors will render changes in the protein structure and therefore, regulate the interaction of the protein with other components occurring around the protein molecule. Modifications on the food matrix cause the loosing or tightening of the matrix that contains the proteins. This affects the release of protein out of the matrix. This section provides an overview of the impact of different factors on the food matrix and the protein structure, including pH, temperature or heating, salt, and shear.

2.5.1. Effect of pH

At high pH, the terminal amino groups of amino acids are protonated, increasing the total negative charge and solubility of proteins (Gao et al., 2020). This also increases the net charge of the protein, which enhances the electrostatic repulsion of protein molecules (Novak et al., 2016). Besides, the protein structure in this state may lose some intermolecular interactions causing the protein to have a

dynamic structure (Jiang et al., 2017) and become less compact. The increased repulsive forces between protein molecules weaken the interactions between protein molecules, i.e., strengthen the interaction of protein and water. As a result, proteins are solubilized more easily in the aqueous medium at high pH, increasing their solubility (Makinen et al., 2016; Malomo et al., 2015a; Mundi et al., 2013). The increase in solubility in an alkaline aqueous medium leads to the improvement of extractability of plant proteins. According to Kim et al. (2011a), the dissociation of edestin (the major component of the hemp globulin fraction) at alkaline pH (pH > 8) is the main mechanism by which hemp proteins are solubilised at these conditions.

Apart from the effect on the protein molecule structure, an alkaline pH can dissolve the non-protein components which interfere with the protein extraction (Liu et al., 2013). An alkaline pH can also make the cell wall partially permeable, simplifying the extraction of intracellular components, such as proteins (Safi et al., 2012). The effect of alkaline pH conditions on the cell walls and the protein structure facilitates the release of proteins from the matrix. Therefore, alkaline pH conditions have been favourably used for protein extraction processes of plant proteins, as highlighted in Table 2.8.

In terms of protein quality, the alkaline extraction also minimizes phytate content (known as an anti-nutritional component) in the protein retain. Phytate is less soluble at high pH and therefore, limits the forming of unexpected phytate-protein complex (Rodrigues et al., 2012). However, an alkaline pH might result in the degradation of protein by forming lysinoalanine compounds that reduce the nutritional value of protein (Liu et al., 2013). The balance between extraction yield and protein quality needs to be considered when the pH of extraction is chosen.

At the p*I*, the negative and positive charges of proteins are equal, making the net charge of protein zero. The electrostatic repulsion between protein molecules is reduced while the attractive forces are predominant. As a result, proteins tend to associate and likely precipitate (Novak et al., 2016), decreasing their solubility in solution. Therefore, to separate the protein from the non-protein component, the pH normally is adjusted close to p*I*, 4 to pH 5 for hemp proteins (Malomo, 2015), to precipitate protein, as shown in Table 2.8.

Table 2.8: Extraction conditions for different plant proteins

Plant materials	pH extraction	pH precipitation	Recovery/ extraction yield	References
Defatted hemp meal	10	5	40.17 ± 1.05	*(Hadnađev et al., 2018; Mamone et al., 2019; Tang et al., 2006; Yin et al., 2008)*
Ground hemp seed press-cakes	12	N/A	67.1%	*(Potin et al., 2019)*
Defatted chickpea, lupine and lentil flour	11 -12	4.5	80 – 87%	*(El-Sohaimy et al., 2007)*
Pea flour	8.5 – 9.5	4.5	49 – 58%	*(Gao et al., 2020)*
Defatted peanut flour	10	4.5	45 %	*(Jamdar et al., 2010)*
Canola meals	12	4.5-6	> 60%	*(Ghodsvali et al., 2005)*
Defatted sour cherry kernel flour	12	4.5	76.9%	*(Celik et al., 2019)*

Acidic pH conditions might hydrolyse the links between elements of the matrix, resulting in partial disruption of the cell wall (Kot et al., 2020). When a protein is exposed to acidic pH (lower than p*I*), the functional groups on the proteins are protonated, which increases the density of positively charged functional groups and provides a net positive charge to the protein. The resulting repulsive forces between protein molecules may lead to the weakening of protein-protein interactions (Novak et al., 2016) and hence aid in extraction. However, when the hemp proteins are released from the seed

matrix, the acidic medium may limit the solubility of protein causing loss of protein solubility. Nevertheless, the lower yield may also be explained by the poor effects of the acid on the seed matrix. The acidic conditions used in the study were not sufficient to disrupt the matrix effectively. According to Malomo et al. (2015a), albumins exhibit the lowest solubility at pH 3 while globulins show less solubility at pH 5. Furthermore, it has been reported that the edestin protein, a major component of hemp protein, tends to aggregate at pH < 7 (Kim et al., 2011a; Leonard et al., 2019).

2.5.2. Effect of temperature

Heating is a physical treatment that affects the structure of the plant cell wall (Sterlikg, 1955). Two mechanisms have been reported in the literature. The first describes the heating effect on softening the cell walls, which facilitates the separation of proteins (Mason et al., 2017). The effect of heat on the rupture of the cell wall has been revealed by many previous authors (Choi et al., 2006; Saberian et al., 2017; Sterlikg, 1955). An SEM image of plant material with and without heat treatment was reported by Saberian et al. (2017). The authors confirmed physical changes of the plant cells after heat treatment particularly, the rupture of the cell wall and separation of cells from each other (Figure 2.4).

The second mechanism proposed that an increased temperature also contributes to swelling of the plant cell membrane. Therefore, these effects accelerate water diffusion into the plant material and improve the mass transfer during the extraction process, leading to an increase in solubility and extractability (Mason et al., 2017). According to Shirsath et al. (2017), an increase in temperature might open the matrix, and as a result, more protein is available for extraction. Also, the temperature can reduce the viscosity of the medium increasing diffusivity; therefore, the efficiency of extraction improves (Shirsath et al., 2017). The higher temperature has been reported to benefit the extraction yield of tea protein (Shen et al., 2008; Zhang et al., 2014), *Rosa rubiginosa* seed protein (Mourea et al., 2001). Similarly, Wang et al. (2018b) showed an increase in hemp protein solubility with an increase in temperature from 20 ^{o}C to 80 ^{o}C.

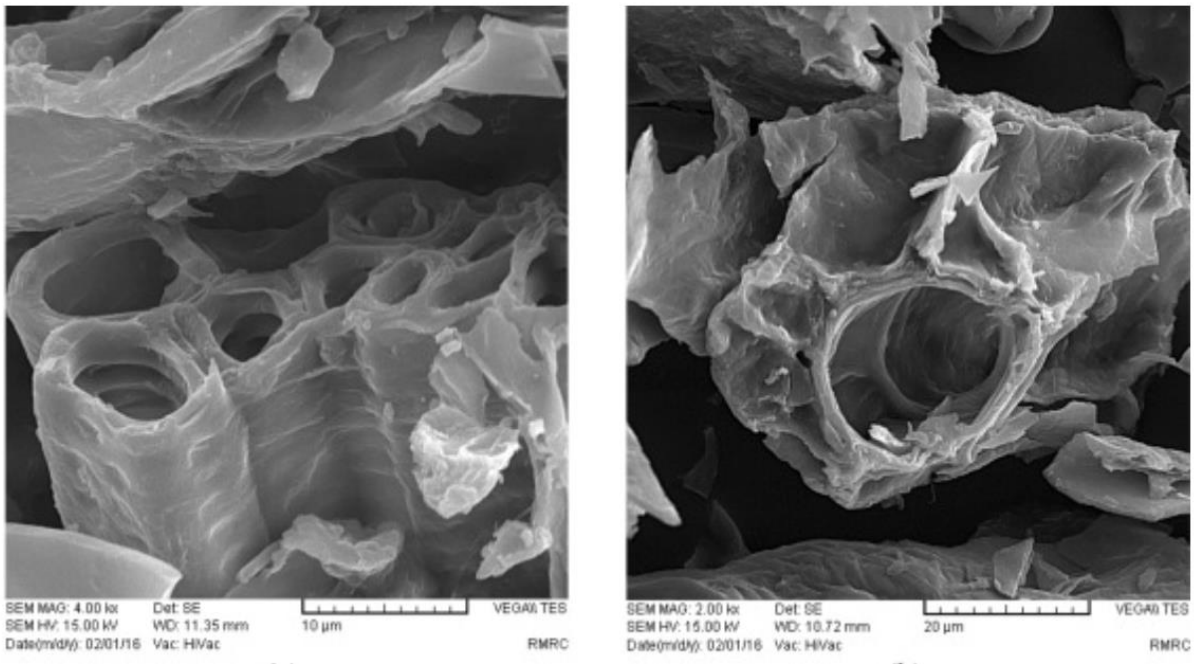

Figure 2.4: Scanning electron micrographs of the orange juice waste solutions without heating (left) and with heating (right). Reproduced with permission from Saberian et al. (2017).

Temperature also affects the protein structure. Thus, the temperature of the process is one of the most important factors for proteins extraction due to their sensitivity and tendency to denature at high temperature (Selling et al., 2007). Heat treatment unfolding of globular proteins (Raikos et al., 2015) and depending on the thermal treatment conditions, the changes might lead to a partial or entire unfolding of native tertiary structure, dissociation, or aggregation (Sirtori et al., 2012). The unfolding exposes hydrophobic groups of the proteins, which promotes hydrophobic interactions that cause aggregation and decreases the yield of protein extraction (Raikos et al., 2015). In addition, heating favours protein-lipid interactions (Ndlela et al., 2012) that may result in an adverse effect on the extraction of protein.

Hemp proteins are rich in sulphur-containing amino acids; therefore, under heat treatment conditions, the thiol-disulphide exchange reactions are promoted between two adjacent protein monomers, which may lead to thermal aggregation (Chuang et al., 2019). The effect of these reactions is expected to increase the molecular size of the protein aggregates and, perhaps, to 'lock' the unfolded proteins in the denatured state (Schofield et al., 1983). Consequently, the heating at a specific temperature might cause a reverse effect on the protein extraction yield. Therefore, the temperature should be controlled under that denature point. For hemp protein isolate, the heat treatment should be restricted to below

80 ^{o}C to inhibit protein denaturation and aggregation (Raikos et al., 2015). Differently, according to Chuang et al. (2019), hemp globulin starts to denature at a temperature of 90 ^{o}C.

2.5.3. Effect of salts

Adding salts to the extraction medium creates a system of 3 components: protein, water, and salt (Arakawa et al., 1984). Understanding the preferential interactions of the proteins and water in that system provides insights and benefits for the protein extraction process. The presence of salts in an aqueous medium can greatly modulate the solubility of proteins by "salting-in" or "salting-out" effects.

The "salting-in" phenomena occur at the beginning when the salt concentration in the aqueous medium increases gradually wherein the solubility of protein increases until it reaches the maximal salting-out phenomena that happen following which reduces the solubility (Zayas, 1997). In the presence of excessive salt, there is a competition between salt and proteins to bind to water which reduces protein solubility (Beauchamp et al., 2012; Zayas, 1997). Therefore, in the protein extraction method using salt, the concentration of salt needs to be controlled to maintain the salting-in stage.

Besides the effect of salt on screening charge-charge interactions, the presence of cations in the medium can help to break the salt bridges in protein and then enhance the extractability (Guzman et al., 2020). The effect of salt varies with the type of salt used (Jenkins, 1998). They are governed by the order of anion and cation in the Hofmeister series. The salting-out effectiveness decrease in the order $SO_4^{-2} > HPO_4^{2-} > Cl^- > Br^- > NO_3^- > I^-$ for anions, and in the order $NH_4^+ > K^+ > Na^+ > Mg^{2+} > Ca^{2+}$ for cations (Roberts et al., 2015). Thus, at a given concentration of salts, the effect of divalent cations extractability of proteins is greater than that of monovalent cations and hence a higher concentration of monovalent ions is required (Jenkins, 1998).

The degree of solubilization of proteins increases according to an increase in salt concentration until it gets to a maximum value. The concentration to get the maximum values of protein solubilization depends on the type of salt, usually less than 1.0 M is effective (Schweizer et al., 2014). Schweizer et al. (2014) suggested that hemp proteins are solubilized by calcium chloride.

In the food industry, sodium chloride is a food-grade salt widely used (Hussain et al., 2012) and is known as a weak salting-out agent (Dumetz et al., 2007). Many authors have used NaCl in the extraction of different plant materials, as shown in Table 2.9.

Table 2.9: Types and concentrations of salt have been used in plant proteins extraction.

Material	Concentration & types of salt	Extraction/ recovery yield	References
Defatted hemp meal	0.8 M NaCl	40.17 ± 1.05%	*(Hadnađev et al., 2018)*
Hemp seed protein meal	0.5 M NaCl	6.33% for albumins and 6.07% for globulins	*(Malomo et al., 2015a)*
Soybean Meal Proteins	0.3 N calcium chloride or 0.7 N sodium chloride.	65%	*(Anderson et al., 1973)*
Coconut meal	0.5 M NaCl	80%	*(Kwon et al., 1996)*
Defatted *Gevuina avellana*	0.5 M NaCl	56%	*(Moure et al., 2002)*
Pumpkin seed	4.26% NaCl	7.84 g protein/100 g raw material	*(Quanhong et al., 2005)*
Defatted sunflower meal	2.8 M NaCl	80%	*(Pickardt et al., 2009)*
Defatted macadamia nut kernel flour	0.5 M NaCl	69.4%	*(Bora et al., 2016)*

2.5.4. Effect of shear

The shear treatment has been employed for years in food processing of protein to improve the functionalities of proteins and assist the extraction of protein (Bogahawaththa et al., 2019; Kong et

al., 2019; Zou et al., 2019). Shearing might be carried out by traditional methods, such as magnetic stirring, or by other new technologies, e.g., ultrasound, high-speed mechanical shearing (Zou et al., 2019). In this section, the effect of shear on protein extraction will be restricted to those of ultrasound.

During ultrasound processing, the sound wave causes an implosive collapse of gas-filled cavitation bubbles, resulting in intense local shock waves causing the physical disruption (Zheng et al., 2011). Therefore, ultrasounds can disrupt plant cell walls, which make proteins more easily extracted from the matrix to the medium (Ashokkumar, 2015; Shirsath et al., 2017; Sun et al., 2011; Wang et al., 2006). It has been reported that ultrasound can create greater penetration into the plant cell material resulting in mass transfer enhancement. The effect of loosening the matrix by plant material swelling and hydration enhancement allows the intra-particle diffusivity of water (Ashokkumar, 2015; Shirsath et al., 2017; Sun et al., 2011; Wang et al., 2006). Xiea et al. (2015) used SEM images to illustrate the ability of ultrasounds to destroy the surface of the material. The surface of the material after ultrasound treatment showed more damage and less thickness than that without treatment (Figure 2.5). Therefore, ultrasound has been used widely for assisting the extraction of proteins in different plant materials, as observed in Table 2.10.

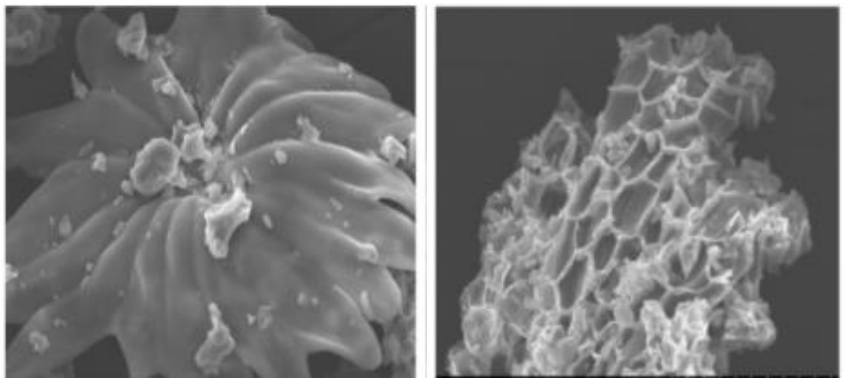

Figure 2.5: SEM images of the surface of plant material (olive leaves), without ultrasound treatment (left) and with ultrasound treatment (right). Reproduced with permission from Xiea et al. (2015).

However, besides the positive effect on the extraction by disruption of the cell wall, the ultrasound might create an impact on the protein structure itself. The shearing can pose the unfolding or even aggregation of protein under a specific mechanical effect (Sirtori et al., 2012). This point is considered a limitation of ultrasounds; therefore, the control of the processing conditions is necessary.

Table 2.10: Ultrasound treatment for protein extraction of plant proteins

Plant material	Ultrasound conditions	Protein extraction efficiency	References
Defatted wheat germ	Output power 363 W, ultrasonic time 24 min	Yield extraction increased from 37 to 57%	*(Xue et al., 2009)*
Sunflower meal	220 W/L, 45 ^{o}C and 15 min	The yield extraction increased by 20.7% compared to the traditional extraction	*(Dabbour et al., 2018)*
Defatted rice bran	Frequency 20 kHz, sonication power 15 W/g, and ultrasound time 2 min	Extraction yield 1.67 times higher than that in the conventional method	*(Muhoza et al., 2017)*
Rapeseed meal	Frequency 28 kHz, Power intensity of 0.228 W/cm^2, pH 11.71, time 41.48 min, and ultrasound power 40%	The yield of protein extracted increased by 43.3% over the conventional extraction (control).	*(Yagoub et al., 2017)*
Rice Dreg Flour	Frequency of 20 kHz, NaOH concentration 0.08 mol/L, treating time 40 min, temperature 40 °C, power 448 W	Extraction yield increases from 43.2% in the absence of ultrasound, whereas it reached up to 88.5% under ultrasonic condition	*(Li et al., 2017)*

Ultrasound is considered an innovative method used for protein extraction from plant material (Gencdag et al., 2020). However, this method has not been studied for hemp proteins so far. Therefore, employing this novel technique in the extraction of hemp protein is one promising approach to improve the extractability of hemp proteins.

In conclusion, the extraction of hemp protein out of the non-protein components is the way to broaden the application of hemp proteins in the food industry. Currently, studies on how to improve the extractability of hemp proteins from hemp seed materials are still limited. In particular, a systematic study investigating the effect of various factors on the extraction yield has not been done. The application of novel technique ultrasound in hemp protein extraction has not been explored. Furthermore, the combined effect of different variables to improve the extractability of hemp proteins has not been fully explored. These are the gaps that should be filled to improve and broaden the application of hemp seed protein.

2.6. Objectives

The main objectives of this work were:

(i) to investigate the extractability of hemp protein under different process conditions;
(ii) to understand the effect of treatment conditions on the microstructure of plant-based matrix.

Chapter 3

3. Materials and Methods

This chapter presents the materials and methods used in this study. Modifications of the methods are described below, and specific methods have been reported at the beginning of individual chapters. All experiments were performed three times unless otherwise specified.

3.1. Materials

3.1.1. Commercial hemp seed meals

The commercial hemp seed meal powders were purchased from local markets (Davis Trading Palmerston North, New Zealand). Four samples were chosen due to the availability on markets. These four samples will be referred to as Sample 1- 4 which are used in Chapter 4 to investigate their physicochemical properties. The details of four samples and claimed nutritional information is presented in Table 3.1.

Table 3.1: Commercial hemp seed meals and nutritional information claimed on labels

No.	Sample 1	Sample 2	Sample 3	Sample 4
Package image				
Manufacturer	Hempfarm	Nzprotein Ltd	MyProtein	Essente
Origin	Canada	New Zealand	China	New Zealand
Labelling nutrition information per 100 g	Protein 50 g Total fat 11.6 g Carbohydrate 26.6 g *Sugar 3.3 g* *Fibre 23.3 g*	Protein 64.6 g Total fat 15.9 g Carbohydrate 13.7 g *Sugar 5.7 g* *Fibre 7.8 g*	Protein 54 g Total fat 12 g - *Sugar 6 g* *Fibre 25 g*	Protein 70 g Total fat 2.85g Carbohydrate 19 g *Sugar 7.25 g* -

3.1.2. Chemicals

All reagents and chemicals used in the experiments were of analytical grade and were made up in Milli-Q water (Milli-Q apparatus; Millipore Corp., Bedford, MA, USA) unless otherwise stated.

Table 3.2: List of chemicals and reagents used and their product codes

No.	Chemicals/ reagents	Product code
1	Boric acid	BSPBL952.500 [a]
2	Molecule weight markers for SDS-PAGE	1610377 [b]
3	10–20% Criterion™ Tris-HCl Protein Gel, 26 well, 15 µl	3450044 [b]
4	Sodium Chloride (NaCl)	106404 [c]
5	Potassium Chloride (KCl)	BSPPL402.500 [a]
6	Magnesium Chloride ($MgCl_2$)	105833 [c]
7	Calcium Chloride ($CaCl_2$)	102378 [c]
8	Glycerol	G8773 [d]
9	Tris (hydroxymethy) aminomethane (Tris)	252859 [d]
10	Sodium dodecyl sulphate (SDS)	BP166500 [a]
11	Bromophenol Blue	B0126 [d]
12	β-Mercaptoethanol	B0149 [d]
13	Tris Base	BP152-5 [a]
14	Glycine	G8898 [d]
15	Coomassie Brilliant Blue	B0149 [d]
16	Isopropanol	109634 [c]
17	Glacial acetic acid	A38-212 [a]

a: Fisher Scientific Pittsburgh, PA, USA *b: Bio-Rad Laboratories, AC, USA*

c: Merck KGaA, Darmstadt, Germany *d: Sigma Aldrich Ltd, St. Louis, MO, USA*

3.2. Methods

3.2.1. Characterisation of commercial hemp seed meal

3.2.1.1. Proximate nutritional analysis

The proximate nutritional compositions including total dietary fibre, fat, and protein content of four commercial hemp seed meals were analysed using different methods as described in the following sections.

3.2.1.1.1. Total dietary fibre content

The total dietary fibre (TDF) content of four commercial hemp seed meal powders was determined by the method of Megazyme, AOAC 991.43. Samples were analysed by an external certified laboratory (The Nutrition Laboratory of Massey University has been International Accreditation New Zealand (IANZ) accredited to ISO 17025). The determination procedure is presented in Figure 3.1.

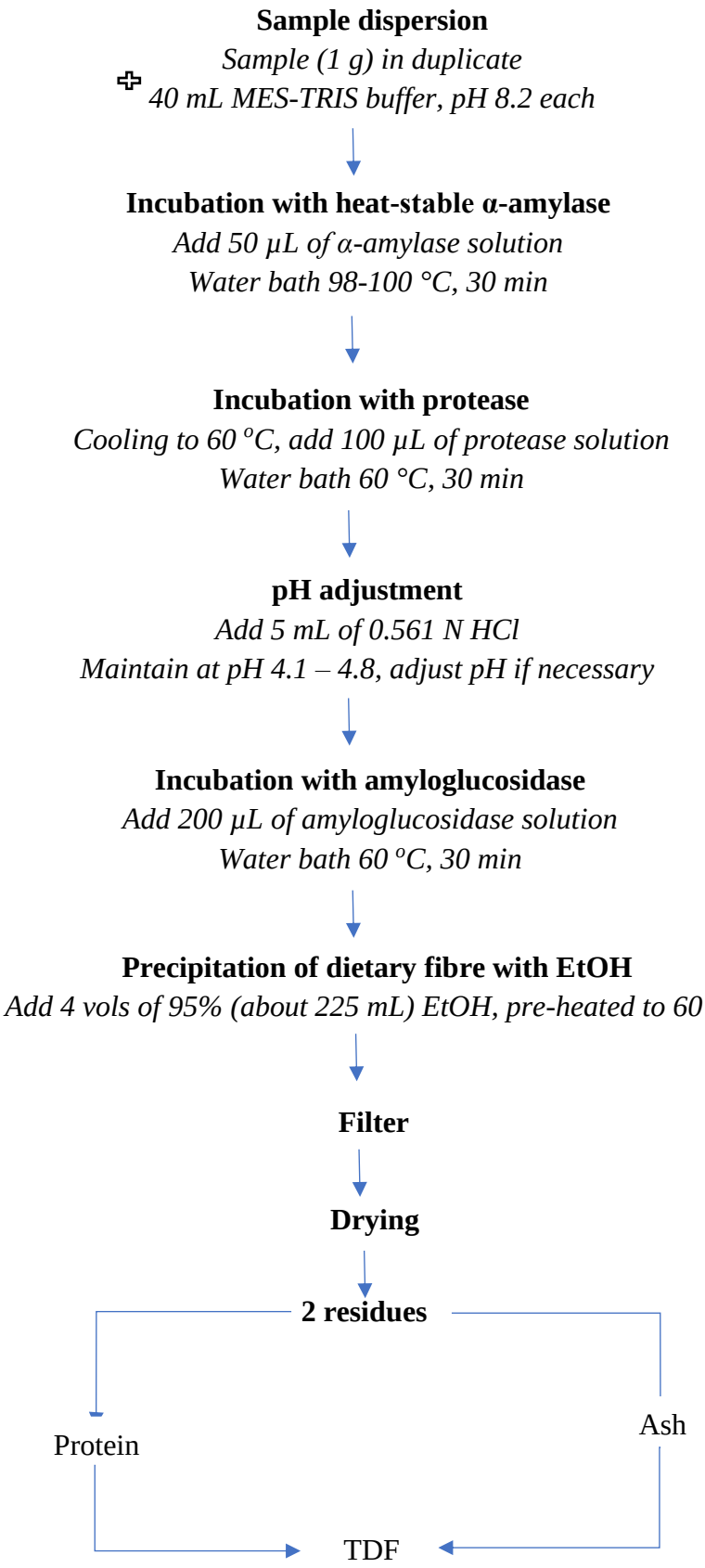

Figure 3.1: Analytical scheme for the total dietary fibre determination procedure.

The total dietary fibre content was calculated as follows:

$$Total\ fiber\ content\ (\%) = \frac{\frac{R1+R2}{2} - p - A - B}{\frac{m1+m2}{2}} \times 100 \qquad \text{(Eq. 3.1)}$$

where:

R1 = residue weight 1 from m1

R2 = residue weight 2 from m2

m1 = sample weight 1

m2 = sample weight 2

A = ash weight from R1

p = protein weight from R2

and, B = blank

$= \frac{BR1+BR2}{2} - BP - BA$

where:

BR = blank residue

BP = blank protein from BR1

BA = blank ash from BR2.

3.2.1.1.2. Fat content

Sample preparation

The fat content of samples was analysed by Mojonnier method, AACC 30 -10/ AOAC 922.06, at an external test laboratory (The Nutrition Laboratory, Massey University).

In brief, two g of a hemp seed meal was placed in a 50 mL beaker. 2 mL of ethanol was added to prevent particle lumping before adding 10 mL of HCl. The beaker was put in a boiling water bath for 30 minutes and then cooled down before mixing 10 mL of ethanol. The mixture in the beaker was transferred to a Mojonnier tube. The beaker was rinsed with 25 mL of diethyl ether. The tube then was stoppered and rocked gently for 1 minute. Twenty-five mL of petroleum ether was added to the mixture and the tube was rocked gently again for 30 seconds followed by centrifugation at 600 rpm for 2 minutes. After centrifugation, the solvent layer was decanted into a pre-weighed flask. The flask

then was placed on a hot plate set at mild temperature (below 40 ^{o}C) to evaporate the solvent. 5mL ethanol was added into the Mojonnier tube and mixed before adding 15 mL of diethyl ether. The extractions were repeated as above but with 15 mL of petroleum ether. The second fat extraction was decanted into the same fat flask used above and heated to evaporate the solvent. Then the flask was put into an oven set at 100 ^{o}C for 10 minutes to dry completely.

The flask was cooled down to room temperature before being weighed.

The fat content was calculated using the following equation:

$$\%\ fat\ in\ food = \frac{W2-W1}{W3} \times 100 \qquad \text{(Eq. 3.2)}$$

where:

W1: Weight of empty flask (g)

W2: Weight of flask and fat (g)

W3: weight of the sample was taken (g)

3.2.1.1.3. Protein content

The protein content in the sample was analysed by Kjeldahl method (AOAC 991.20). In this method, the protein content in the sample was determined by measuring the total nitrogen content in the sample and then converting it to protein content by multiplying with a conversion factor of 6.25.

A small amount of the sample (approximately 1.5 g in powder form and 2.5 g in liquid form) was placed into a Kjeldahl digestion tube. 16 mL concentrated H_2SO_4 and 2 Kjeltabs tablets (catalytic agent, each tablet includes 3.5 g K_2SO_4 /3.5 mg Se) was added into the tube. The digestion tube was heated in a digestion block (Tecator digestion block DS20, Tecator, Sweden) at unchanged 420 °C until the liquid inside turned colourless. After being cooling down, approximately 30 mL of reverse osmosis treated (RO) water was added into the tube. The tube then was set in a distillation unit (Kjeltec™ 8200). An amount of 80 mL of concentrated alkali (40%, w/v NaOH) was automatically added into the tube to convert the $(NH_4)_2SO_4$ into free ammonia (NH_3). During distillation, the free ammonia escaped with steam into a flask containing 25 mL of 4% (w/v) boric acid prepared with indicators (Methyl red and Bromocresol green). The ammonia reacted with boric acid resulting in an ammonium borate complex which changed the receiving solution from reddish to greenish-blue

colour. The amount of ammonia present in the receiving solution was determined by titration with 0.1 M HCl until the colour of the solution turned back to reddish or grey mauve.

The volume of used HCl was recorded and used to calculate protein concentration using the following equations. A blank tube without any sample was also treated in the same way as above.

$$\text{Nitrogen (\%)} = \frac{(A-B) \text{ x } 1.4 \text{ x } 100}{C \text{ x } 1000} \quad \text{(Eq. 3.3)}$$

$$\text{Protein content (\%)} = \text{Nitrogen content x } 6.25 \quad \text{(Eq. 3.4)}$$

where:

A: HCl used (ml)

B: exact molarity HCl

C: weight of the original sample (g)

3.2.1.2. Particle size by sieving method

The particle size distribution of four commercial hemp seed meals was measured by sieve analysis. Hemp seed meal powders, including the sample after nitrogen grinding, were passed through a stack of sieves (Fritsch GmbH, Germany) with decreasing grades of sieve pore sizes from top to bottom, ranging from 45 µm to 600 µm. Hemp seed meal powder (100 g) was put on the top sieve and the lid was put on. Then the sieve stack was placed in a sieve shaker and sieved for 15 minutes. Thereafter, the amount of sample retained on each of the sieves was weighed. The particle size distribution by intensity was calculated by the percentage of the sample amount retained in each sieve relative to the total amount of sample retained on all sieves.

3.2.1.3. Particle size reduction of hemp seed meal by using nitrogen grinding method

To test the effect of particle size of hemp seed meal particles on extraction, hemp seed meal powder (100 g) was placed in a pestle. Approximately 1L of liquid nitrogen was gently poured into the pestle and the mixture was held for 3 minutes with gentle mixing using the mortar until no liquid nitrogen remained. The powder was then placed into a blender and mixed at high speed for 30 seconds to further reduce the particle size. The reduced- size hemp seed meal powder was subjected to particle size measurement as described in section 3.2.1.2 and protein solubility determination as described in section 3.2.2.2.

3.2.2. Characteristic analyses on hemp seed meal dispersions

The supernatant of four commercial hemp seed meal powders gained by dispersion and centrifugation were subjected to the Dynamic light scattering, protein solubility and Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE) analyses, as described in the following sections.

The pellet gained after centrifugate was freeze-dried and used for SEM analysis as described in section 3.2.5.

3.2.2.1. Dynamic light scattering

This method was employed to measure the size distribution of hemp seed protein solubilised in Milli-Q water. To prepare the samples, 10 g of each sample powder and 90 g of Milli-Q water were added to a beaker. The dispersion was stirred continuously at 20 ^{o}C for 2 h without pH adjustment. Dispersions were centrifuged (10,000 x *g*, 20 min, 20 ^{o}C).

The hydrodynamic diameter of hemp seed meal proteins in the supernatant was measured using a Zetasizer Nano ZS device (Model ZEN 3600, Malvern, Worcestershire, UK). The supernatant was placed into a 2 ml quartz cuvette (1 cm path length). The measurement was carried out after equilibrating at 25 °C for 80 s. Three measurements were taken with an automatic setting of measurement duration, measuring position, and attenuator. The particle size of soluble proteins was considered the average diameter based on volume-based particle size distributions.

3.2.2.2. Protein solubility

The protein solubility of commercial hemp seed meal powders, including the sample after nitrogen grinding, was determined following the method described by Adebiyi et al. (2011), with slight modifications. Briefly, 10 g of hemp seed meal powder was dispersed in 90 mL of Milli-Q water at room temperature. The resulting mixture was stirred for 2 h at 20 ^{o}C, and then centrifugated (10,000 x *g*, 20 ^{o}C, and 20 min). The protein content in the supernatant was considered as soluble proteins (PS).

Total soluble protein content was determined by dissolving the 10 g of hemp seed meal powder in 90 g of NaOH 0.1 M solution under stirring for 2 h at room temperature. Centrifugation was performed as described above. The protein content of the supernatant was considered as total soluble protein content (PT).

The protein solubility was expressed as the percentage of soluble protein content ratio to the total soluble protein content using the following equation.

$$\text{Protein solubility (\%)} = \frac{\text{PS}}{\text{PT}} \times 100 \qquad \text{(Eq. 3.5)}$$

where:

PS: soluble protein content (%)

PT: total soluble protein content in NaOH 0.1M (%).

3.2.2.3. SDS-PAGE

The SDS-PAGE method was used to characterize the polypeptide compositions of hemp proteins solubilised in Milli-Q water. The SDS-PAGE was carried out under reducing condition.

Sample preparation

To prepare the samples, 10 g of each hemp seed meal powder and 90 g of Milli-Q water were added to a beaker. The dispersion was stirred continuously at 20 ^{o}C for 2 h without pH adjustment. Dispersions were centrifuged (10,000 x *g*, 20 min, 20 ^{o}C).

The supernatant obtained after centrifuge which contains soluble hemp seed proteins was diluted with SDS-PAGE sample buffer to get the final protein concentration at 1.0 mg/mL. The compositions of the sample buffer are shown in Table 3.3 below. The sample in reducing buffer was heated at 95 °C in 5 minutes to allow a reduction of disulphide bonds to happen.

Table 3.3: Composition of SDS-PAGE sample buffer (Pavlovic et al., 2019)

Buffer components	Volume
Glycerol	2.5 mL
0.5 M Tris-HCl, pH 6.8	3.125 mL
10% SDS (w/v)	10 mL
0.1 % Bromophenol Blue (w/v)	625 µL
Milli-Q water	20 mL
β-Mercaptoethanol	1.908 mL

All the samples, after being diluted in the sample buffer, were deposited in the wells of the precast gels. Molecular weight markers from Bio-Rad (Precision Plus Protein Unstained Standards, Mw 2 to 250 kDa) were used. The gels were run in an Electrode Buffer (composition as shown in Table 3.4) at a constant voltage of 150 mV for about 90 minutes until the dye reached the bottom of the plate. Thereafter, the gels were stained by Coomassie brilliant blue solution (0.3% w/v), in 20% (v/v) isopropanol and 10% (v/v) glacial acetic acid for 1 hour. The gels were then destained by a destaining solution containing 10% (v/v) isopropanol and 10% (v/v) glacial acetic. A flat-bed scanner (Scan maker 900, Microtek, Carson, CA, USA) was used to scan the gels.

Table 3.4: Composition of Electrode buffer (pH 8.3 ± 0.2) (Pavlovic et al., 2019)

Components	Quantity
Tris Base	7.5 g
Glycine	36 g
SDS	2.5 g
Milli-Q water	Bring total volume to 500mL (concentrate solution). Then dilute the concentrate 5-folds with Milli-Q water

3.2.3. Extraction of protein from hemp seed meal powder

3.2.3.1. Effect of temperature at different pH

Hemp seed meal (HSM) powder was dispersed in Milli-Q water (10% w/w). The pH of the dispersions was adjusted to pH 7 or pH 9 by using a 2 M NaOH solution. Then, the dispersions were placed in a shaking water bath set at examined temperatures (20 ^{o}C, 65 ^{o}C, and 95 ^{o}C) for 2 h. Afterwards, the dispersions were cooled down to room temperature and then subjected to centrifugation (10,000 x *g*, 20 °C, and 20 min). The supernatant and/or the pellet were recovered for further analysis (SDS-PAGE, available lysine, total phenolic content, SEM).

3.2.3.2. Effect of pH cycling

Hemp seed meal powder was suspended in Milli-Q water (10% w/w) at room temperature (20 ^{o}C). The pH of the dispersion was adjusted to pH 9 with a NaOH 2M solution. After continuous stirring

at pH 9 for 30 min, the pH was adjusted to pH 12 (alkaline pH cycling) or pH 2 (acidic pH cycling) with a 2 M NaOH or HCl solution, respectively. The dispersion was placed in a shaking water bath set at 20 ^{o}C or 65 ^{o}C for 1 h. Thereafter, the dispersion was cooled down to room temperature, adjusted back to pH 9, and then placed under continuous stirring for half an hour. The alkaline pH cycling process terminated at pH 7 or pH 9. Afterwards, centrifugation was performed (10,000 x *g*, 20 °C, and 20 min) and the supernatant and the pellet were recovered for further analysis (SDS-PAGE, available lysine, total phenolic content, SEM).

For this experiment, the ambient temperature (20 ^{o}C) was used as a controlled temperature. The 65 ^{o}C was used is high enough to cause an effect on treatment but not too high enough to denature the hemp proteins (Raikos et al., 2015). According to Tang et al. (2006), hemp seed proteins are alkaline-soluble proteins where the hemp proteins showed a significant solubility increase at pH 8 and above. Therefore, pH 7 was used as a control value and the alkaline pH 9 was used as the alkaline condition to favour the solubility of hemp proteins. A higher pH was not considered due to the risk to the loss of the protein quality.

3.2.3.3. Effect of adding salts

The extraction was prepared by suspending the HSM (10% w/w) in salt solutions (NaCl, KCl, $CaCl_2$, $MgCl_2$) at different concentration (0 M, 0.25 M, 0.5 M, and 0.8 M NaCl). The pH of the dispersion was adjusted to pH 7 or pH 9 by using a 2 M NaOH solution. Then, the dispersion was placed in a shaking water bath set at examined temperatures (20 ^{o}C, 65 ^{o}C, 95 ^{o}C) for 2 h. Afterwards, centrifugation was performed (10,000 x *g*, 20 min, and 20°C) after the suspension was cooled down to 20 ^{o}C. The supernatant was recovered for SDS-PAGE analysis.

3.2.3.4. Effect of ultrasound and its combination with other treatments

3.2.3.4.1. Effect of ultrasound singly

A volume of 150 mL of the dispersions of 10% w/w hemp seed meal powder in Milli-Q water was prepared. The dispersion then was adjusted to examined pH (pH 7; pH 9). The dispersion was placed in a water bath set at 20 ^{o}C or 65 ^{o}C under continuous shaking for one hour. After that, the dispersion was subjected immediately to ultrasound treatment. Ultrasonic laboratory equipment was used (UIP 1000hd, Hielscher, Germany). The set-up provided a constant frequency of 20 kHz and different amplitudes adjustable by a booster. The temperature of the ultrasound system was not controlled during operation. The ultrasound duration was set to a certain time (30 s; 120 s; 600 s; 900 s; and 1200

s) with investigated amplitude (20%; 80%). After ultrasound treatment, the dispersions were cooled down to room temperature (20 ^{o}C) and kept under stirring for 1 h. Finally, centrifugation was performed (10,000 x *g*, 20 ^{o}C, and 20 min) to collect the supernatant and pellet for further analysis.

3.2.3.4.2. Effect of ultrasound combination with adding salts

A dispersion of 10% (w/w) of hemp seed meal powder in a salt solution (NaCl, $CaCl_2$) at concentrations of 0.25 M; 0.5 M and 0.8 M was prepared. The pH of the dispersion was adjusted to pH 9 and then the dispersion was placed in a water bath set at 20 ^{o}C or 65 ^{o}C under continuous shaking for one hour. Afterwards, the dispersion was treated by the ultrasound at 80% amplitude for 15 min and then cooled down to room temperature (20 ^{o}C) and kept under stirring for 1 h. Finally, centrifugation was performed (10,000 x *g*, 20 ^{o}C, and 20 min) to collect the supernatant for further analysis.

3.2.3.4.3. Effect of ultrasound in combination with pH cycling

HSM powder was suspended in Milli Q water (10% w/w) at room temperature (20 ^{o}C). The pH of the dispersion was adjusted to pH 9 with a NaOH 2M solution. After continuous stirring at pH 9 for 30 min, the pH was adjusted to 12 (alkaline pH cycling) or pH 2 (acidic pH cycling). After that, the dispersion was placed in a shaking water bath set at 65 ^{o}C for 1 h, then cooled down immediately to room temperature before adjusting back to pH 9. After that, the dispersion was heated to 65 ^{o}C right before the ultrasound treatment was applied. The ultrasound condition of 80% amplitude in 15 min was used. The brief order of the pH cycling process and ultrasound treatment is shown in Figure 3.2. Afterwards, centrifugation was performed (10,000 x *g*, 20 °C, and 20 min) and the supernatant and the pellet were collected for further analysis.

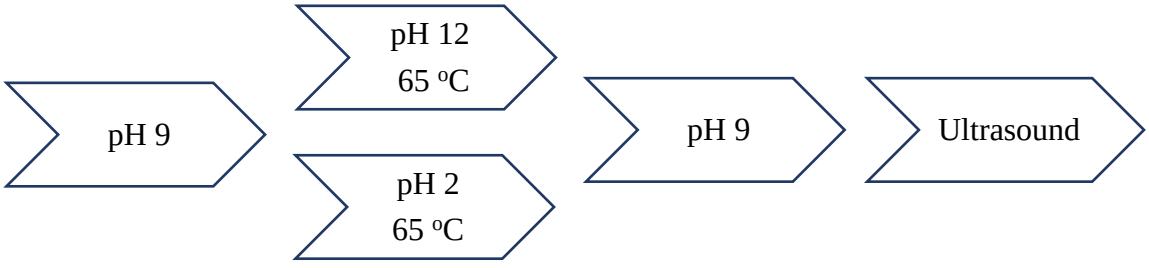

Figure 3.2: pH cycling process combined with ultrasound treatment

3.2.4. Physicochemical characteristics of the HSM extracts

The supernatant obtained after centrifugation as mentioned in section 3.2.2 was used for measurements of extraction yield; SDS-PAGE method described in section 3.2.1.3.2; and total phenolic content. Additionally, the supernatant was freeze-dried and then used for the analysis of available lysine content. All methods were described in the following section

3.2.4.1. Protein extraction yield measurement

The protein extraction yield was determined as described by Potin et al. (2019), with slight modifications. The HSM dispersion was prepared as described in section 3.2.2. The protein content in the dispersion before centrifugation, considered as an initial total protein content (PI), and in the supernatant obtained after centrifugation, so-called extracted protein content (PE), was determined using the Kjeldahl method (described in section 3.2.1.1.3).

The protein extraction yield (PY) was calculated using the following equation:

$$\text{PY}\ (\%) = \frac{\text{PE}}{\text{PI}} \times 100 \qquad \text{(Eq. 3.6)}$$

where

PE: extracted protein content (%)

PI: initial total protein content in hemp seed meal (%).

This method was used to determine the yield of hemp protein extraction under different conditions as presented in section 3.2.2.

3.2.4.2. Total phenolic content determination

The total phenolic content (TPC) in the extracts was measured following the Folin–Ciocalteu method (Malik et al., 2017). The analyses were performed by an external laboratory (The Nutrition Laboratory, Massey University). The concentration mentioned below was the final concentration after the addition of all reagents. The supernatant obtained after centrifugation was mixed with Folin–Ciocalteu reagent and Milli-Q water to get the mixture with a final concentration of 0.002% (w/w) final total nitrogen, and 0.1 M Folin–Ciocalteu. The mixture was left in the dark for 8 min, then 3% (w/w) of sodium carbonate was added. The mixture was vortexed for 5s and then left in the dark for 1 h. Absorbance measurements were performed at 725 nm wavelength with a Perkin Elmer 2030

Victor X plate reader. The total phenolic content was calculated based on a standard curve of gallic acid.

This method was used to analyse the phenolic content in the extract under the effect of temperature at different pH, pH cycling, ultrasound singly, and the combination of ultrasound with pH cycling.

3.2.4.3. Available Lysine content determination

The supernatant obtained after centrifugation was freeze-dried and then sent for analysis at an external laboratory (The Nutrition Laboratory, Massey University). In brief, for analysis, approximately 1.0 g of protein was mixed with 0.6 M O-methylisourea solution. The protein to reagent ratio was 1: 8 (w/w). The pH was adjusted to pH 10.6 The mixture was incubated at 4 °C for 6 days. The pH was checked at 12 hours intervals and adjusted, as necessary.

After incubation, the pH was gradually reduced to 3.0 using 4 N HCl to precipitate the protein. The solution was allowed to stand for 3 hours and then centrifuged at 1600 *g* for 15 minutes to separate the precipitate. The precipitate then was washed with Milli-Q water at pH 3.0 and recentrifuged; this step was repeated twice. Then the precipitation was then lyophilized, ground by a laboratory grinder.

The samples were then hydrolysed by 6 M glass-distilled HCl containing 0.1% phenol for 24 h at 110 ± 2 °C in evacuated sealed tubes then the homoarginine is detected by Waters ion-exchange HPLC system, utilizing post-column ninhydrin derivatization and detection using absorbance at 570 and 440 nm. Then converted into the amount of useful lysine.

This method was used to analyse the available lysine content in the extract under the effect of temperature at different pH, pH cycling, ultrasound singly, and the combination of ultrasound with pH cycling.

3.2.5. Microstructure of hemp seed and hemp seed meals

3.2.5.1. Microstructure of hemp seed

The microstructure of hemp seeds was examined by cryo-SEM imaging. Dehulled hemp seeds (brand Pure Hemp Hearts, HempFarm, origin Canada were purchased from Davis Trading, Palmerston North, New Zealand) were placed in the sample holder and flash frozen. The frozen sample was then transferred to the cryo-unit sectioning chamber and placed under a vacuum. The temperature of the sample was lowered to -120 °C and the was fractured using a cold knife. After which the temperature was gradually raised to -100°C for 20 minutes for sublimation. The fractured surface of the seeds was

then coated with a thin platinum coating (10 mA for 240s) and the samples were then transferred to the imaging chamber. SEM images of fractured sections were recorded at 6 to 20 kV on a Joel JSM 6500F Field Emission Scanning Microscope.

3.2.5.2. Microstructure of hemp seed meals

The SEM method was used to analyse the microstructure of the hemp seed meal powder and the hemp seed meal after the extraction, so-called spent hemp seed meals.

- ***Sample preparation**:*

Hemp seed meals: To prepare the sample for SEM, 20 g Milli-Q water was added to 10 g of the hemp seed meal powder and the mixture was stayed for 30 minutes to soften the powder and then subjected to freeze-drying to form the powder for SEM analysis.

Spent hemp seed meals: The pellet obtained after centrifugation was collected as described in section 3.2.3, washed twice with Milli-Q water and freeze-dried before being subjected to SEM analysis. The pellet of extraction affected by the temperature at different pH, pH cycling, ultrasound singly, and the combination of ultrasound with pH cycling were used for this analysis.

To conduct the SEM, a small amount of the freeze-dried sample was mounted on the holder using double-sided conductive tapes. A low-vacuum sputter coating was then performed in 200 seconds to deposit an ultra-thin layer of gold, an electrically - conducting metal. The photomicrographs of the coated sample were taken with an FEI Quanta 200 Environmental Scanning Electron Microscope. The digital images were captured with different magnifications at a high voltage of 20 kV.

3.3. Statistical analyses

The statistical analyses were performed with Minitab 17 Statistical Software, by analysis of variance (ANOVA). Three replications were done per assay. A Tukey post hoc test was used to determine significant differences between group means. The significance threshold was set at $p < 0.05$.

Chapter 4

4. Physicochemical properties of commercial hemp seed meals

4.1. Introduction

Hemp seed meal is a by-product of the hemp seed oil extraction process. Oil manufacturers further use the oil extraction process to increase the protein content and commercialise the hemp seed meals. The content of high nutritional and digestible protein in hemp seed meals available in the market ranges from 46.2% to 65.1%. In this study, the hemp protein was extracted from hemp seed meals to broaden its application in food formulation. To optimise the protein extractability, the physical and chemical properties of hemp seed meals used in the protein extraction process need to be well understood. This chapter presents the results of the analysis of proximate compositions and physicochemical properties of four commercially available hemp seed meals.

4.2. Results

4.2.1. Comparison of the microstructure of hemp seed and hemp seed meal

The microstructure of hemp seed and the hemp seed meal used in this study was investigated using Scanning Electron Microscopy (SEM). The use of high magnification SEM allowed the identification of specific organelles, e.g., cells or protein bodies contained within the cells. The images are shown in Figure 4.1

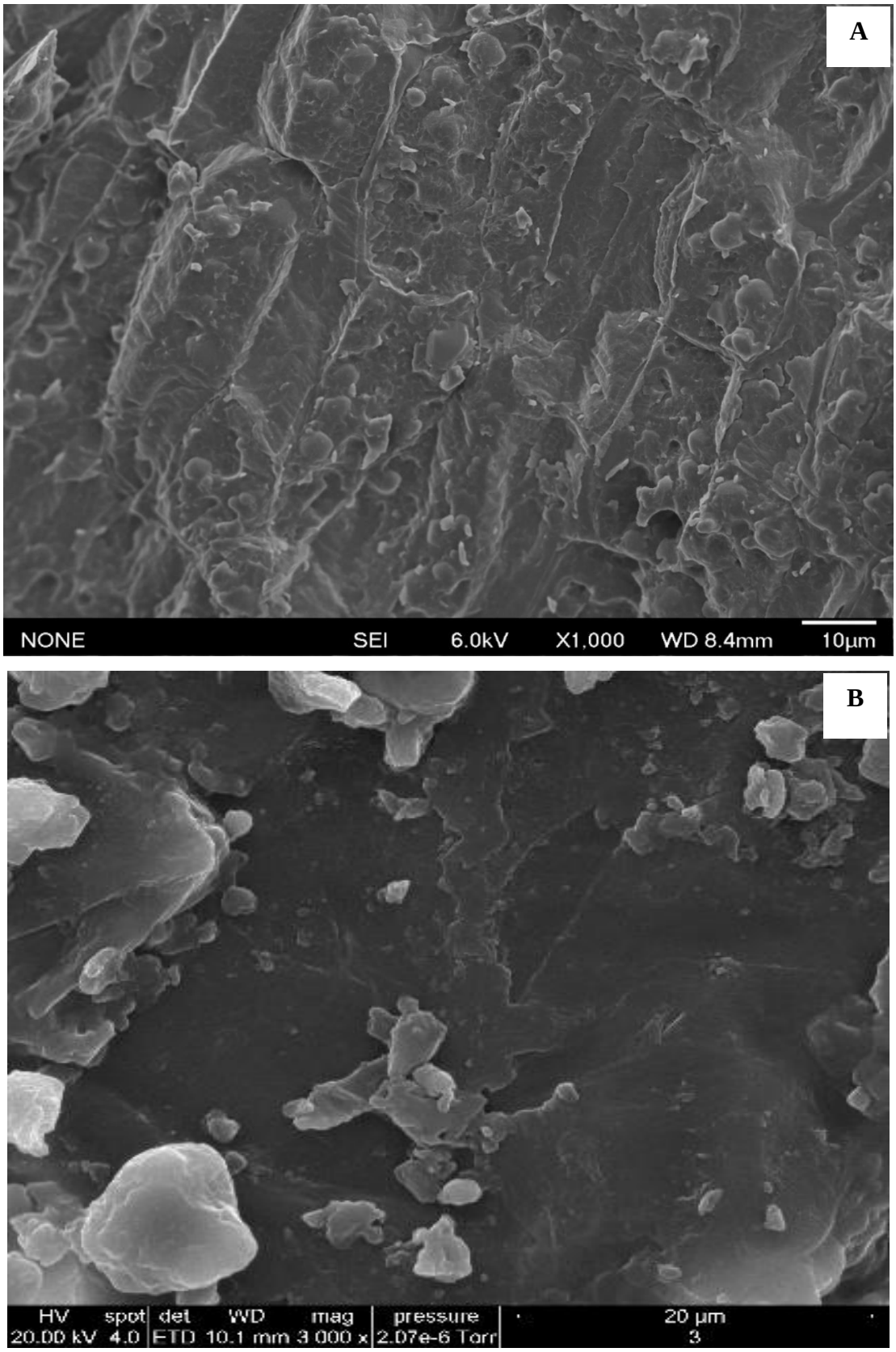

Figure 4.1: Microstructure of hemp seed (A) and hemp seed meal (B)

As can be seen in Figure 4.1A, the microstructure of hemp seed shows layers of elongated ellipsoid cells of different sizes. The structure appeared to be densely packed without any finer details of either the cells or the matrix surrounding the cells. In comparison, the microstructure of hemp seed meals showed the cells or the matrix after milling and oil extraction (Figure 4.1 B). The image showed the typical structure of broken seed in which, the matrix appeared dense without any well-defined structure.

4.2.2. Proximate compositions of commercial hemp seed meal

The four commercial hemp seed meal powders were analysed for the proximate compositions, as shown in Table 4.1. The proximate compositions of dehulled and whole hemp seed meals are also presented for comparison purpose.

Table 4.1: Typical proximate compositions of different hemp seed meal powders sourced from NZ supermarkets

Parameters	Sample 1	Sample 2	Sample 3	Sample 4	Dehulled hemp seed meal [a]	Whole hemp seed meal [a]
Fibre (%)	22.6	5.8	17.8	8.7	39.0*	51.4*
Fat (%)	12.1	20.1	14.6	5.1	8.77	5.6
Protein (%)	48	56.2	46.2	65.1	41.8	32.8

[a] *Values compiled from (Gao et al., 2020)*

(*): Carbohydrate content

From Table 4.1, sample 2 had the highest fat content, with 20.1%. Samples 1 and 3 had lower fat content, with 12.1% and 14.6%, respectively. However, the lowest was observed in sample 4 with 5.1%. Regarding fibre content, sample 2 showed the lowest content (5.8%), whereas sample 1 had the highest (22.6%). Compared to sample 1, the fibre content of sample 3 was lower (17.8%) but still relatively higher than in sample 4 (8.7%).

In terms of protein content, sample 4 had the highest content (65.1%); the protein content was 10% higher than in sample 2 and almost 20% higher than in sample 1 and sample 3. The significantly low fibre and fat content found in sample 4 probably led to the highest residual protein component content of sample 4 (65.1%).

When comparing the proximate compositions of the four samples with the dehulled and whole hemp seed meals (Table 4.1), significant differences were observed in terms of fibre and protein content. Specifically, the fibre content of the four samples was much lower than dehulled and whole hemp seed meals, whereas the protein content was higher than both reference samples.

4.2.3. Particle size distribution of commercial hemp seed meal

4.2.3.1. Untreated with nitrogen grinding

Particle size analysis of the four hemp seed meal powders was carried out by sieve analysis. The hemp seed meal powder (100 g) was passed through a series of sieves with different pore sizes ranging from 45 µm to 600 µm. The particle size distributions of the hemp seed meal powders are presented in Table 4.2.

Table 4.2: Comparison of particle size of four hemp seed meals

Mesh size (µm)	Percentage (w/w)			
	Sample 1	Sample 2	Sample 3	Sample 4
≥ 600	4.8	81.5	47.3	0.2
425 - 600	22.7	14.5	21.4	17.7
180 - 425	61.9	3.9	27.4	48.9
150 - 180	8.4	0.0	3.9	23.6
90 - 150	2.1	0.0	0.0	9.2
75 - 90	0.0	0.0	0.0	0.3
63 - 75	0.0	0.0	0.0	0.0
45 - 63	0.0	0.0	0.0	0.0

From Table 4.2, the particle size of all four commercial samples showed wide variation; however, all samples had particle sizes >150 µm. Samples 1 and 4, with most particles between 180 µm and 600 µm showed relatively smaller particles compared to the other two samples. In contrast, samples 2 and 3 presented coarse particles with most particles size >600 µm. Specifically, 81.5% of sample 2 and 47.3% of sample 3 were >600 µm (remained on the sieve 600 µm).

4.2.3.2. Treatment with nitrogen grinding

Liquid nitrogen grinding was employed to reduce the particle size of HSM and it was expected that reducing the particle size may increase solubility and improve protein extractability. Sample 4 was dipped in nitrogen and subjected to grinding to reduce the particle size.

Table 4.3 below compares the particle size before and after treatment with grinding in liquid nitrogen. As can be seen, the treatment resulted in a reduction of the particle size of hemp seed protein powder. The range of particle size decreased from 90 µm - 600 µm to 75 µm - 180 µm.

Table 4.3: Particle size distribution of hemp seed meal with and without liquid nitrogen grinding

Mesh size (um)	Percentage (w/w)	
	Before Liquid Nitrogen Grinding	After Liquid Nitrogen Grinding
≥600	0.2	0.0
425 - 600	17.7	0.0
180 - 425	48.9	7.3
150 - 180	23.6	35.3
90 - 150	9.2	43.5
75 - 90	0.3	10.4
63 - 75	0.0	3.5

4.2.4. Properties of soluble hemp seed protein in water

4.2.4.1. Untreated with nitrogen grinding

4.2.4.1.1. Protein solubility

The low extractability of hemp protein was mostly attributed to its low solubility (Potin et al., 2019). This section investigates the solubility of hemp protein in four commercial products. The solubilities of four commercial hemp seed meal samples were analysed and shown in Table 4.4. The solubility of sample 4 was the highest, with 12.9%. The solubility of samples 2 and 3 was not significantly different. However, sample 1 was significantly lower than the other samples, only 5.9% was recorded.

Table 4.4: Protein solubilities of hemp seed meals

Sample	Protein solubility (%)
1	5.9 ± 0.21
2	10.2 ± 0.11
3	10.5 ± 0.09
4	12.9 ± 0.15

4.2.4.1.2. Relationship between protein solubility and proximate compositions of hemp seed meals

Figure 4.2 presents the relationship between proximate compositions of four samples and their protein solubility. The results show that the percentage of soluble protein increased as the protein content of hemp seed meal increased. Sample 4, with the highest protein content, showed the highest percentage of solubility. In contrast, sample 1 with relatively high fibre and fat content showed the lowest protein solubility. It seems that the presence of fat and fibre inhibited the protein solubility in water. When comparing sample 1 and sample 3, which were similar in their proximate compositions, the solubility of sample 3 was two times higher than in sample 1.

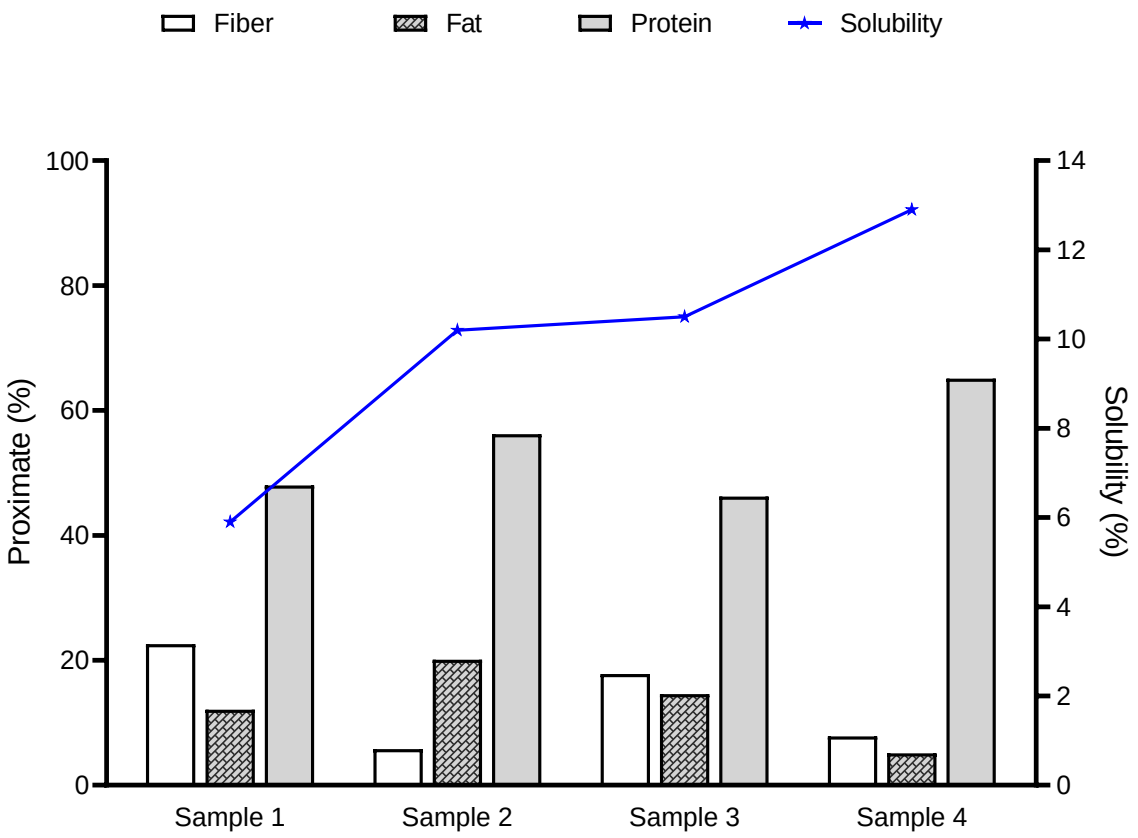

Figure 4.2: The relationship of typical proximate composition and protein solubility of 4 commercial samples.

4.2.4.1.3. Composition of solubilised hemp proteins in water

The supernatant obtained after centrifugation of the four samples was subjected to reducing SDS-PAGE to identify the major soluble protein fractions present in HSMs. The protein profiles are shown in Figure 4.3.

The four samples exhibited a similar protein profile, which included four bands. The three distinct bands were band D at an MW less than 18 kDa, band C at an MW of 18-20 kDa, and band A at 48 kDa. Band B at 34 kDa was intense at the lands of samples 1 and 3 but appeared with pale shade at the lands of samples 2 and 4.

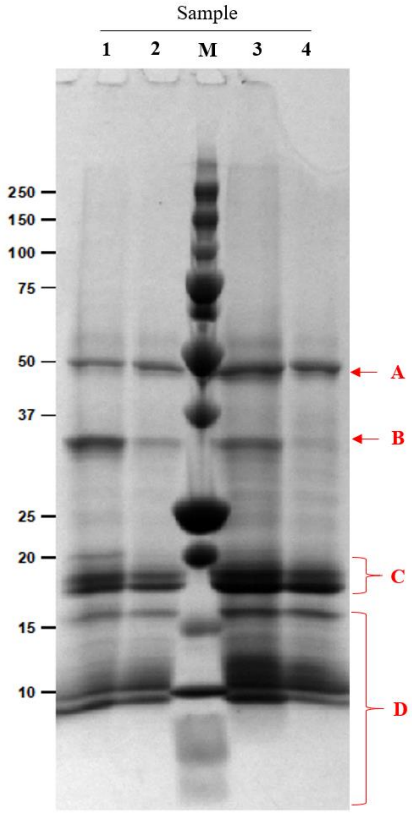

Figure 4.3: Composition of soluble hemp proteins of four commercial samples. M, molecular mass marker in kDa. Bands A-D indicate likely globulin albumin fractions of hemp proteins, see text for more details. The protein concentration for all samples in the SDS-PAGE loading buffer was adjusted to 1 mg/mL before loading.

4.2.4.1.4. Particle size distribution of solubilised hemp proteins in water

The particle size distribution of soluble hemp proteins in Milli Q water was analysed. The supernatants of the four samples obtained after centrifugation were measured by dynamic light scattering. The particle size distribution by intensity is presented in Figure 4.4.

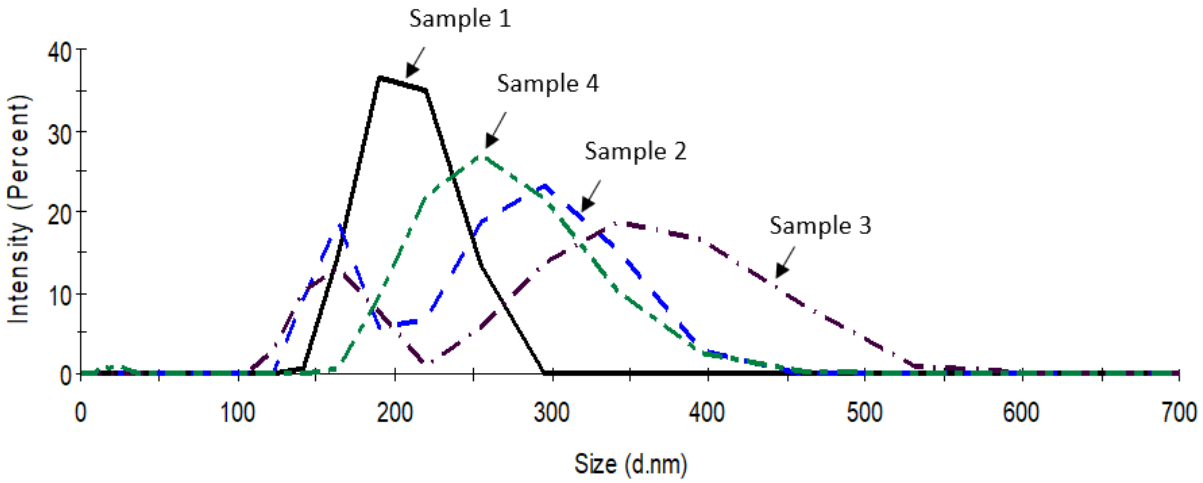

Figure 4.4: Particle size distribution of soluble hemp proteins in distilled water present in four commercial samples

In general, the four samples showed different particle size distributions. Sample 1 and sample 4 are monomodal, whereas sample 2 and sample 3 are bimodal. The particle size range of hemp seed proteins dissolved in distilled water without pH adjustment was from about 110 nm to 600 nm, most of which ranged from 150 to 400 nm. Out of the four samples, sample 1 was different from others as it showed only one peak at 190 nm. Sample 2 and sample 3 presented close particle size distributions, with the same minor peaks at 164 nm. Sample 3 presented a larger particle size distribution because of its major peak (342 nm), whereas the major peak of sample 2 was at 295 nm. In comparison, sample 4 also had a minor peak at 13 nm besides having a major peak at 255 nm.

4.2.4.2. Hemp seed meal treatment with nitrogen

The effect of grinding on the solubility of hemp proteins after nitrogen treatment was studied. The solubility of sample 4 was measured before and after treatment with nitrogen grinding, as shown in Table 4.5. The results revealed that treatment with nitrogen grinding increased the amount of protein released from the material by 14%. An improvement of protein solubility from 19.4% to 22.3% was also observed.

Table 4.5: Protein solubilities of hemp seed meal powders before and after liquid nitrogen grinding

	Before liquid nitrogen grinding	After liquid nitrogen grinding
Soluble protein* (%)	12.7 ± 0.05	14.5 ± 0.08
Protein solubility (%)	19.6 ± 0.12	22.3 ± 0.09

** Determined as total protein in the supernatants obtained after centrifugation at 10,000* x *g for 20 minutes at 20 °C. Protein solubilisation condition at pH 9, 20 °C.*

4.3. Discussion

The hemp proteins are located as rounded protein bodies and distributed throughout the hemp seed (Schultz et al., 2020) along with the embedded oil bodies (Lee et al., 2011). Edestin, a major component of hemp proteins, stayed in a crystalline structure with a diameter of about 4.5 nm packed into large crystalloids (∼1–3μm) covered inside the protein bodies (Chuang et al., 2019). The break-down of the cell structure facilitates the extraction of protein out of the food matrix. Milling the hemp seeds provides effective mechanical forces to disrupt cell structure removing lipids from the oil bodies. Likely, these mechanical forces may also disrupt the protein bodies. Nevertheless, the microstructure of hemp seed meals appeared to be dense, as observed in Figure 4.1 A.

Comparing with the proximate composition of whole hemp seed meals (dehulled and no-dehulled, Table 4.1), all four commercial samples presented significantly high protein content and low fibre content. It could be assumed that there was a purification process applied for those four products before commercialising them, which led to increased protein content and partial removal of non-protein components, such as fibre. The difference in proximate compositions between the four HSM powders might be due to the differences in the processing of the manufacturer, resulting in different purity levels that are unknown.

The high residual fibre and fat levels hinder the dissolution of protein, represented by low solubility. The presence of fat in the food matrix might contribute to the aggregation, in which lipids are oxidised and the resulting lipid-protein complexes prevent the solubilisation of protein (Potin et al., 2019). From this perspective, sample 4 was expected to show the highest protein solubility because it had low-fat content. In addition, protein might have crosslinked to fibre (Jodayree et al., 2012), causing

fibre-protein interactions that inhibited the release of protein from the food matrix (Gao et al., 2020). Therefore, a high fibre content could have caused a negative impact on the solubility of proteins. This may also explain the low protein solubility of sample 1 due to its high fat and fibre contents.

Dehulling reduces the fibre content in hemp seed meals and, therefore; is considered one potential method for increasing protein solubility (Gao et al., 2020). This statement is consistent with a previous study by Oshodi (1992) who presented the inhibitory effect of the hull on the release of *denopus breviflorus benth* seed protein. On the contrary, Arogundade et al. (2006) showed that the hull and lipid had no significant effect on broad beans protein solubility. These contradictory findings might be explained by differences in the materials used in the studies.

The protein solubility of hemp seed meals is generally considered low (Dapcevic-Hadnađev et al., 2019; Tang et al., 2006). In the current study, the protein solubility of hemp seed meals ranged from 5.9% to 12.9% (pH 7, 20 ^{o}C). This result was quite similar to that found by Hadnađev et al. (2018), who showed the protein solubility of hemp seed protein to range from 4% to 12%. However, another study by Malomo et al. (2015b) showed that protein solubility of hemp seed meals at pH 7 was higher (about 20%). The different results might come from different protocols for sample preparation and analytical protocols used to determine protein solubility (Wang et al., 2019).

The particle size of the powder also appeared to affect the solubility of hemp protein. Sample 4 showed the smallest particle size but had the highest protein extraction yield. The effect of particle size on protein releasing from the material has previously presented by Vishwanathan et al. (2011). The authors showed that the smaller the particle size the higher the percentage of protein released. Small particle size increased the surface area contacting with water (Balasubramanian et al., 2012); therefore, contributing to the improvement of protein solubility. It is possible that the small particle size of the HSM in sample 4 could have facilitated the release of protein out of the matrix.

In this study, the differences in the solubility of hemp proteins in the four samples may come from the different methods or techniques that manufacturers used to purify HSMs (Hadnađev et al., 2018; Krause et al., 2002; Sun et al., 2011). A positive correlation between protein content and solubility is also mentioned by Banavara et al. (2003). However, the authors pointed out that this correlation was not always consistent, attributing the differences to variable processing conditions (Banavara et al., 2003). This observation could explain why sample 2, which had high protein content compared to sample 3, showed low solubility.

Nitrogen grinding has been used to reduce the particle size of plant materials (Balasubramanian et al., 2012; Balbino et al., 2019; Hemery et al., 2011) and it has been efficiently used to release more protein into an aqueous environment. Sample 4 that was subjected to nitrogen grinding showed increased protein solubility, which in agreement with the findings of Balasubramanian et al. (2012). In their study, the cryogenic grinding method was proved to be an effective process for fast particle size reduction, and subsequently, enhanced the extractability of substances from the food matrix. Besides its effect on reducing particle size, liquid nitrogen grinding also contributed to disrupt the cell wall and release more components inside, such as protein (Balasubramanian et al., 2012; Balbino et al., 2019). The effect of the mechanical grinding process in the disruption of cells of plant material was also presented by Vishwanathan et al. (2011).

The particle size distributions of soluble hemp proteins in distilled water found in the current study were different in the four samples. In general, the particle size range was bigger than that found in previous studies (Teh et al., 2016). Teh et al. (2016) showed that the particle size of soluble protein in hemp protein isolate was smaller than 177 nm. Another study showed different particle sizes of hemp globulin, with a significant peak at 17 nm, and a minor peak at 248 nm (Chuang et al., 2019). However, in the study, the protein was extracted in the presence of salt (0.5 M), which might have caused the dissociation of large globulin complexes resulting in small sizes of the protein (Chuang et al., 2019). Chuang et al. (2019) used polyvinylpolypyrrolidone and sodium metabisulfite to prevent the formation of protein-phenolic crosslinking during the extraction. These differences in sample preparation might explain the variable results in particle size of soluble hemp proteins. Another possibility that cannot be excluded is that the hemp seed proteins might have partially aggregated after extraction, causing larger particle size proteins. The tiny size of the minor peak (13 nm) of sample 4, might have appeared because of protein hydrolysed during previous isolation techniques employed to purify the sample.

At neutral pH, two main fractions of hemp proteins (albumin and globulin) are partially solubilised (Malomo et al., 2015a). Therefore, the protein profile analysed under reducing SDS-PAGE reflected those two components in the supernatant. The most distinct bands were bands D (less than 18 kDa), corresponding to the albumin fraction. The presence of the globulin fraction was also observed. The globulin 11s split into two components under reducing conditions (acidic and basic subunits) at an MW of 18-20 kDa (band C) and 33 kDa (band B). The globulin 7s fraction had a pale shade at 48 kDa. This protein profile was consistent with many previous studies that investigated hemp seed proteins from different sources of hemp protein, including defatted hemp seed meal (Malomo et al.,

2015a), press cake (Potin et al., 2019), and hemp protein isolate (Hadnađev et al., 2018; Tang et al., 2006).

The results from this chapter indicate that different commercial HSMs exhibit different physicochemical properties. These properties cause an effect on the solubility, and hence, the extractability of hemp proteins. Only sample 4, which showed the highest solubility compared to the other samples, was used for further investigation about the protein extraction process. The next chapter discusses these results in detail.

Chapter 5

5. Optimal conditions for extraction of hemp proteins

5.1. Introduction

From chapter 4, the sample that exhibited the highest protein solubility was used as an ingredient for further experiments. To extract as much protein as possible contained in the hemp seed meals (HSM, about 65% w/w, as is), this chapter investigated the different processing approaches to improve the yield of hemp proteins extraction from the HSM.

This chapter investigates the protein extractability under different conditions such as three different temperatures (20˚, 65˚ and 95 ºC), two different pH values (pH 7 and pH 9), pH cycling, and addition of different salts to extract hemp proteins from HSM. The impact of these treatments on protein quality, as represented by available lysine content, and the co-extraction of phenolic compounds were also studied.

5.2. Results

5.2.1. Effect of temperature on the extraction of hemp proteins at different pH values

5.2.1.1. Extraction yield of hemp proteins

Figure 5.1 shows the effects of temperature and pH on the extractability of proteins from HSM. At pH 7, the control sample at 20 ºC had a protein extraction yield of approximately 13%. At the same pH, the yield was 20% when the temperature of extraction was increased to 65 ºC, which means that increasing the temperature from 20 ºC to 65 ºC resulted in a 1.5-fold increase in the yield. However, a further increase in the temperature to 95 ºC caused a significant decrease ($p < 0.05$) in the extraction yield. The extraction yield was however still higher than in the control sample.

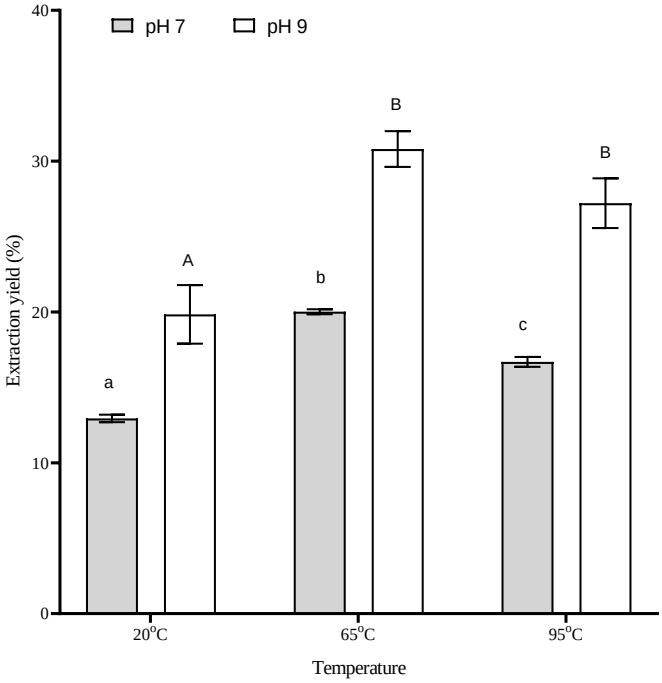

Figure 5.1: Protein extraction yield as a function of different temperature and pH values. Different letters indicate a significant difference ($p<0.05$) at each pH value. Error bars represent the standard deviation of three replications.

At pH 9 and 20 °C, the protein extraction yield was approximately 20%. However, at 65 °C the yield increased to 30.8% (i.e. 55% higher). Increasing the temperature to 95 °C did not significantly increase the yield ($p<0.05$), even though it was still remarkably higher than that obtained at 20 °C. Noticeably, there was no significant difference ($p<0.05$) in the extraction yield values obtained at 65 °C and 95 °C, as seen at pH 7.

5.2.1.2. Composition of proteins in the extracts

The soluble hemp seed proteins after heating at different temperatures (20 °C , 65 °C , and 95 °C) and pH values (pH 7 and pH 9) were analysed by SDS-PAGE under reducing conditions (Figure 5.2). The composition of all samples was largely similar irrespective of the extraction conditions.

From the SDS-PAGE profiles, it is possible to identify different fractions of hemp proteins in the extracts. The intensity of band A, with an approximate molecular weight of 48 kDa, representing the 7s-globulin component, decreased at both the pH values at temperature 95 °C.

The bands B and C, with approximate molecular weights 34 kDa and 18-20 kDa respectively, most likely correspond to at 11s-edestin acidic and 11s-edestin basic units (Malomo et al., 2015a). At pH

7 the intensity of these bands was lower at pH 7 (20°C and 65°C) as compared to corresponding temperatures at pH 9. Remarkably, bands A, B and those in region C were absent at temperature 95 °C at both pH values. There were some prominent bands in all lanes in the region D with molecular weights <18 kDa and these most likely corresponded to the albumin fraction.

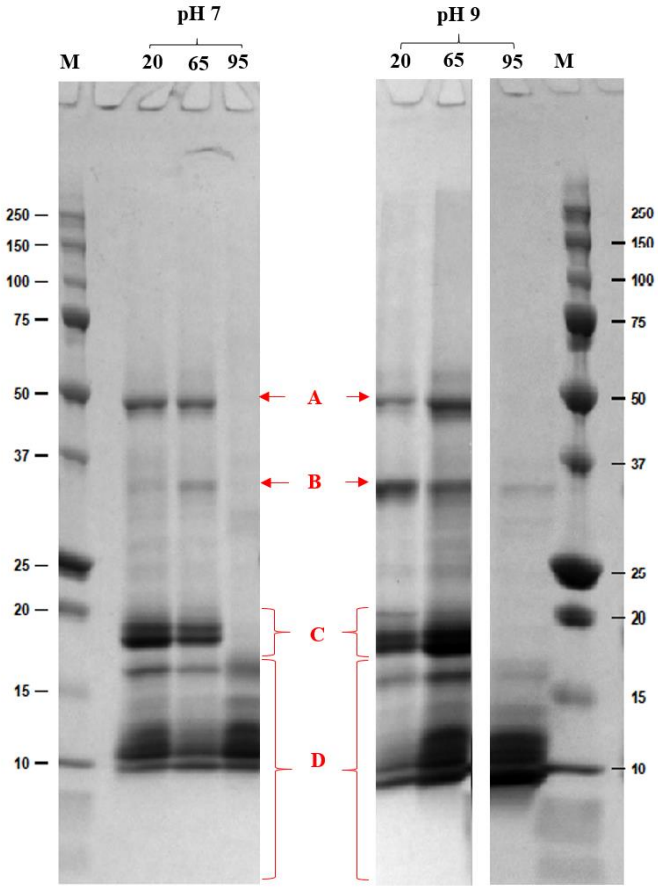

Figure 5.2: SDS-PAGE of soluble hemp proteins under different heating temperatures at pH 7 and pH 9; M, molecular mass marker in kDa. Numbers above the lands indicate the heating temperature (°C). Bands A-D indicate likely globulin albumin fractions of hemp proteins, see text for more details. The protein concentration for all samples in the SDS-PAGE loading buffer was adjusted to 1 mg/mL before loading.

5.2.1.3. Phenolic content in extracts

From the results shown in Figure 5.1, the optimal conditions for hemp protein extraction were found to be 65 °C temperature and pH 9, which resulted in the highest extraction yield compared to other conditions. Therefore, the total phenolic content of the extracts obtained by the combined conditions was analysed. Results obtained using lower temperatures and pH conditions were also shown for

comparison. The phenolic compounds were co-extracted with the proteins during the extraction process. The relative concentrations of the extracted phenolic compound were expressed as mg of Gallic acid equivalent (GAE) per g of protein in the extract. The results are presented in Figure 5.3.

At pH 7 and 20 °C, the concentration of total phenolic compounds in the extracts was 0.231 ± 0.004 mg GAE/g supernatant. Increasing the temperature of the extraction to 65 ^{o}C resulted in a significant increase ($p < 0.05$) in TPC in the extracts (0.290 ± 0.006 mg GAE/g supernatant). At pH 9 and 20 ^{o}C, the TPC was 0.359 ± 0.014 mg GAE/g supernatant. Increasing the extraction temperature to 65 ^{o}C at the same pH 9 extracted an additional amount of approximately 18% of the phenolic compounds from the hemp seed meal to the aqueous medium.

When the TPC was expressed as mg GAE/g protein in the extracts, the effect of temperature on the extraction of phenolic compounds was insignificant at pH 7. Increasing the pH of extraction to pH 9 increased the TPC /g of protein in the extracts, but the effect of temperature on the TPC was insignificant.

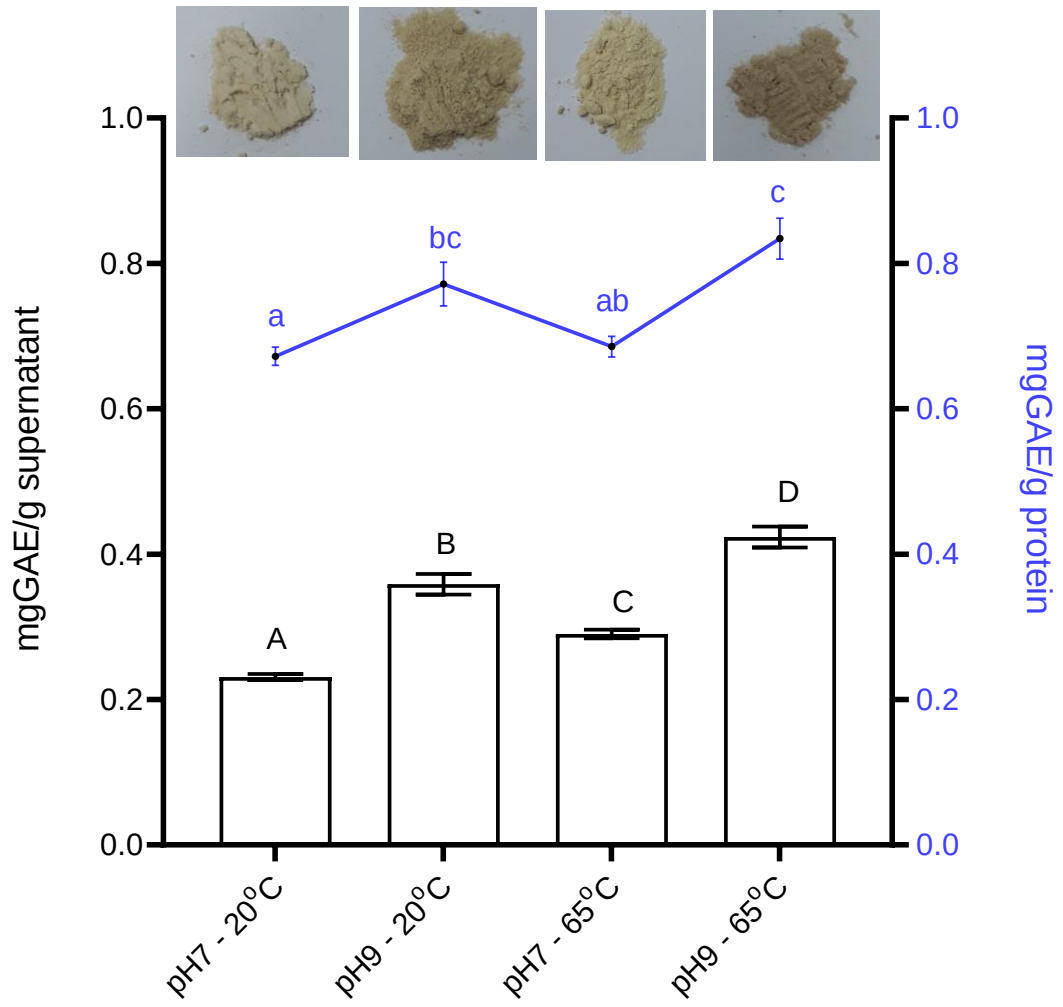

Figure 5.3: Total phenolic content (TPC) contained in the extracted protein under different treatments (temperature and pH). GAE: gallic acid equivalent. Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of two replications. The images show the extracted protein powder obtained after centrifugation and freeze-drying of the supernatants.

5.2.1.4. Available lysine content of extracts

The available lysine content in the extracts obtained after different treatments was analysed and expressed per gram of extracted hemp protein (Figure 5.4). Any detrimental effect of the extraction process was expected to result in a loss of available lysine.

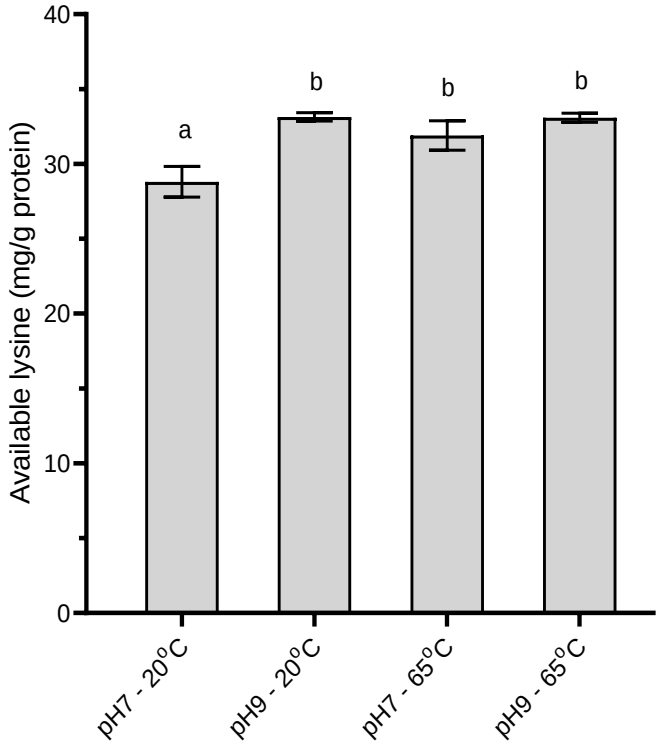

Figure 5.4: Available lysine content of extracted protein under different conditions (temperature and pH). Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of two replications.

At pH 7 and 20 °C, the available lysine content was about 28.8 ± 1.03 mg/g protein. Increasing the temperature of extraction to 65 °C at the same pH resulted in a very small but significant increase ($p < 0.05$) in the available lysine content to 31.9 ± 0.98 mg/g protein. Comparing with pH 9, the increase in the temperature of extraction from 20 °C to 65 °C did not result in any significant difference in available lysine content.

Between pH 7 and pH 9 at 20 °C, the higher available lysine content was recorded at the alkaline pH. Specifically, a significant increase of 12% more ($p < 0.05$) of the available lysine content was observed when pH increased from 7 to 9 at 20 °C. At a higher temperature (65 °C), a 4% increase in the available lysine was recorded, but this increase was not significant ($p < 0.05$).

5.2.1.5. Microstructure of the spent hemp seed meals

To investigate the effect of temperature and pH on the matrix, the hemp protein meal after extraction, i.e, spent hemp seed meal, was observed under SEM and is shown in Figure 5.5. It was difficult to

conclude from the SEM images. Therefore, a further investigation relating to the effect of these treatments on the microstructure of hemp seed meals are necessary.

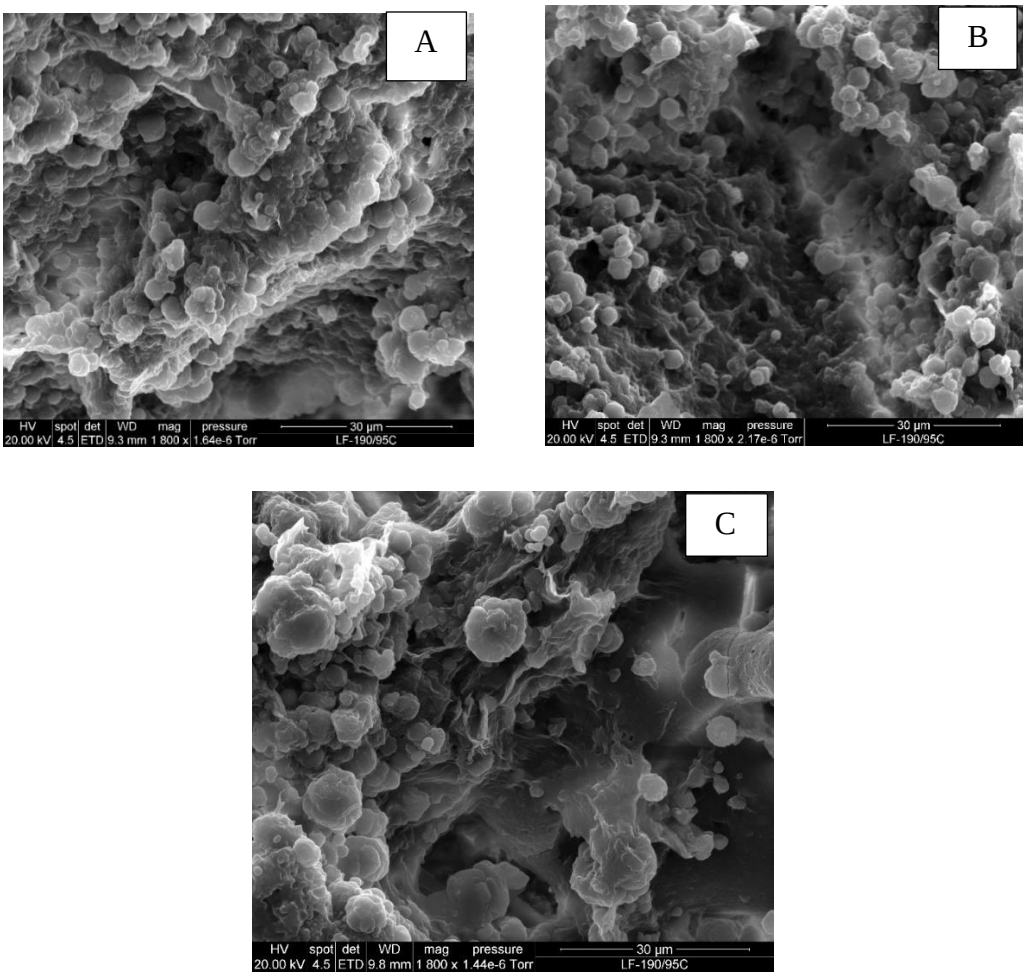

Figure 5.5: Scanning electron micrographs of spent hemp seed meals; (A) Untreated sample (20 ^{o}C, pH 7); (B) heating at 65 ^{o}C and pH 7; (C) heating at 65 ^{o}C and pH 9.

5.2.2. Effect of pH cycling on the extraction of hemp proteins

5.2.2.1. Extraction yield of hemp proteins

The effect of pH cycling on the extractability of proteins was investigated. The samples were exposed to pH 9 followed by pH 12 at 20 ^{o}C for 1h, and then again returned to pH 9 or 7. The results of protein extraction yield are shown in Figure 5.6.

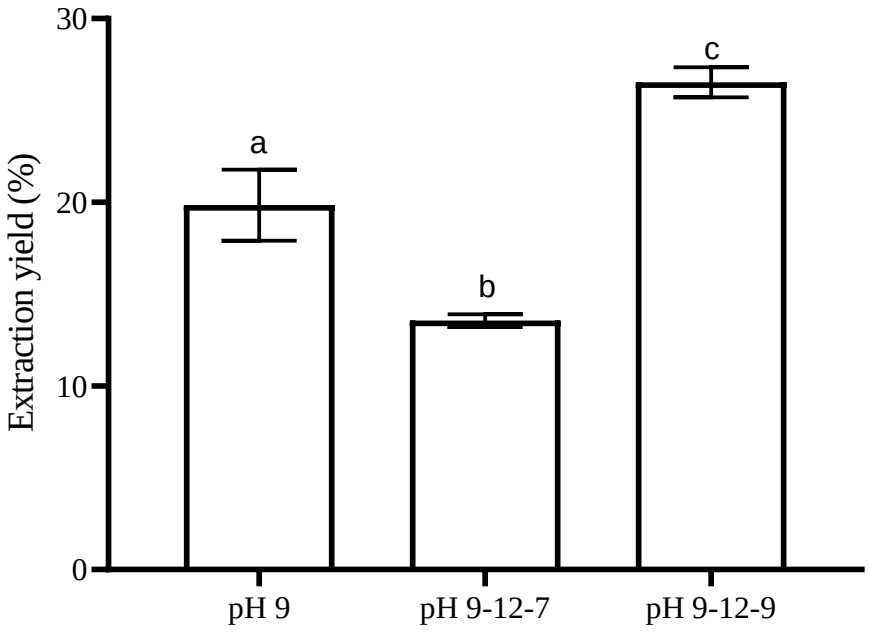

Figure 5.6: Effect of pH cycling at room temperature on the extraction yield of hemp protein. From pH 9 subjected to pH 12 at room temperature for 1 h, then back to pH 9 (pH 9-12-9) or pH 7 (pH 9-12-7); control sample (pH 9) at room temperature without pH cycling. Different letters indicate a significant difference at $p<0.05$. Error bars represent the standard deviation of three replications.

At 20 ^{o}C, the effect of pH cycling depended on the final pH (7 or 9) at the end of pH cycling. When the process ended at pH 7, a significant decrease ($p < 0.05$) of approximately 32% less in yield compared to the control sample was observed i.e., the absolute yield decreased from around 20% to about 14%. In contrast, when the process ended at pH 9, a significant increase in yield (about 34% higher) was recorded compared with the control sample. The yield after pH cycling (pH 9-12-9) treatment reached almost 27%. Remarkably, this yield was 2-fold greater than the yield created by cycling (pH 9-12-7).

The pH cycling (pH 9-12-9) was found to have a better protein extraction yield than the (pH 9-12-7). Therefore, the best condition was used to investigate the combined effect of pH cycling and heating conditions. The results are shown in Figure 5.7.

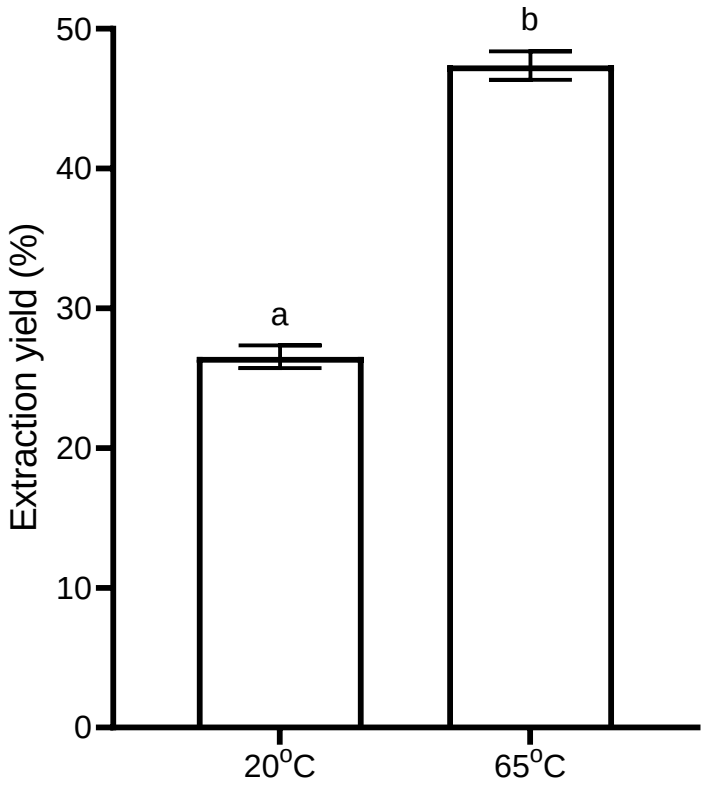

Figure 5.7: Effect of pH cycling (pH 9-12-9) at different temperatures (20 °C, 65°C) on the yield of hemp protein extraction. Different letters indicate a significant difference ($p < 0.05$). Error bars represent the standard deviation of three replications.

There was a significant difference in the yield between the two investigated temperatures. The yield was about 47% and 27% at 65 °C and 20 °C, respectively. Under heating condition at 65 °C, the yield was higher (approximately 74% more) than that of the sample extract 20 °C. The results revealed that the pH cycling at 65 °C was even more effective to improve the yield of hemp protein extraction.

Figure 5.8 below shows a comparison of yields after an acidic pH cycling (pH 9-2-9) and alkaline pH cycling (pH 9-12-9) at 65 °C.

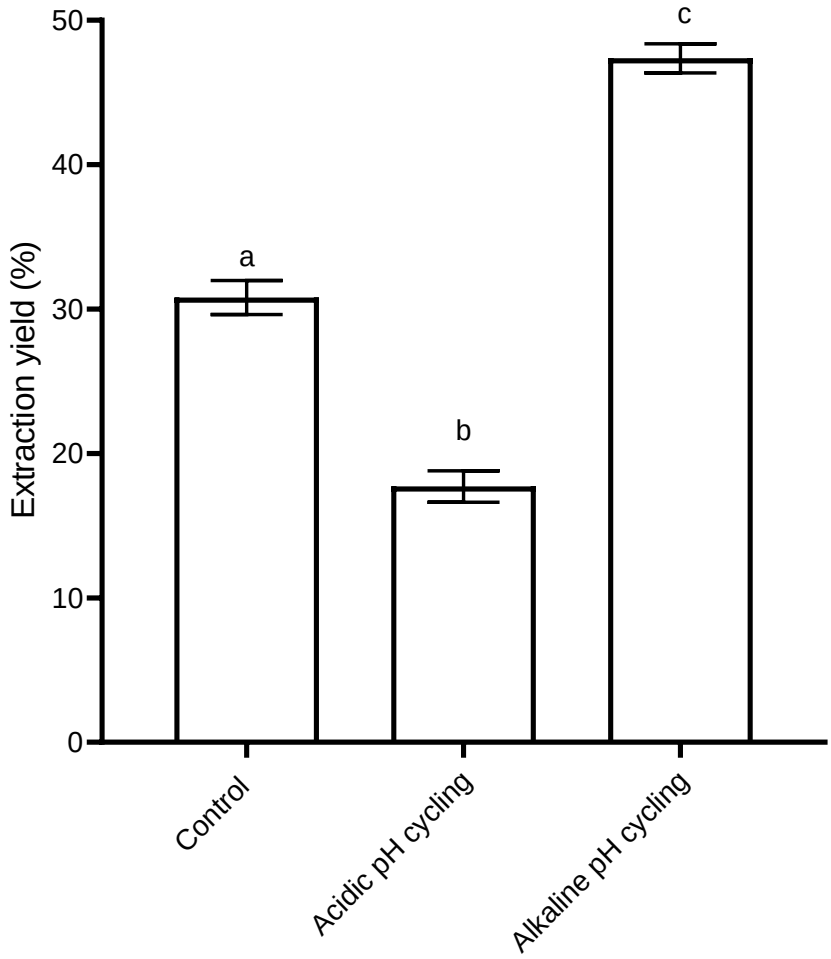

Figure 5.8: Effect of acidic and alkaline pH cycling at 65 ^{o}C on the yield of hemp protein extraction. Sample at pH 9, 65 ^{o}C marked as the control sample. Different letters indicate significant different ($p < 0.05$). Error bars represent the standard deviation of three replications.

The protein yield resulting was significantly higher (almost 3-fold) at alkaline pH cycling than at acidic pH cycling (47.4% and 17.7%, respectively). Comparing to the control sample, the yield obtained at acidic pH cycling was 42% lower, whereas the yield obtained at alkaline pH cycling was 54% higher.

5.2.2.2. Composition of proteins in the extracts

The differences in protein profiles under alkaline and acidic pH cycling extraction were presented in Figure 5.9. Also, the profile of the sample without pH cycling was shown for comparison. From Figure 5.9, the main groups of proteins extracted with alkaline pH cycling and without pH cycling were similar. Three main distinct bands were observed including bands B, C and D corresponding to

the molecular weight about 33 kDa, 18-20 kDa and less than 18 kDa, respectively. They represented the presence of two globulin 11s subunits (acidic and basic subunits) and albumin fractions in the extracted hemp proteins. Furthermore, the band A corresponding to the globulin 7s at the molecular weight of about 48 kDa appeared faint in these two samples. The lane with acid pH cycling sample showed a more intense band of albumin fraction (band D) but very faint at globulin fraction bands, i.e., globulin 7s (band A) and the two subunits of globulin 11s (bands B and C).

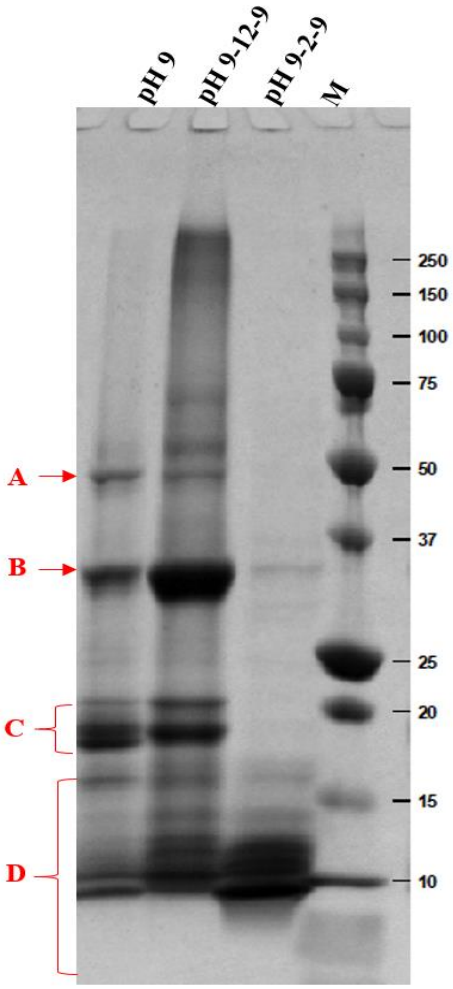

Figure 5.9: SDS-PAGE comparison of hemp proteins extracted by acidic (pH 9-2-9), alkaline pH cycling (pH 9-12-9) and without pH cycling (pH 9) at 65°C. M, molecular mass marker in kDa. Bands A-D indicate likely globulin albumin fractions of hemp proteins, see text for more details. The protein concentration for all samples in the SDS-PAGE loading buffer was adjusted to 1 mg/mL before loading.

5.2.2.3. Phenolic content in the extracts

The appearance of extracts and their total phenolic contents obtained after alkaline pH extraction is shown in Figure 5.10. Alkaline pH cycling led to a significantly higher content (p <0.05) of phenolic compounds extracted from the material. Without pH cycling, there was about 0.42 mg GAE per gram of supernatant. A ~19% increase in the phenolic compound content was observed after alkaline pH cycling extraction. When values were corrected to GAE per gram of extracted protein, an increase of about 32% was recorded in alkaline pH cycling compared to treatment without pH cycling. The difference in this amount was consistent with the difference in the visible colour observed in the hemp protein powder after centrifugation and freeze-drying, in which the pH cycling produced a darker colour compared to without pH cycling treatment.

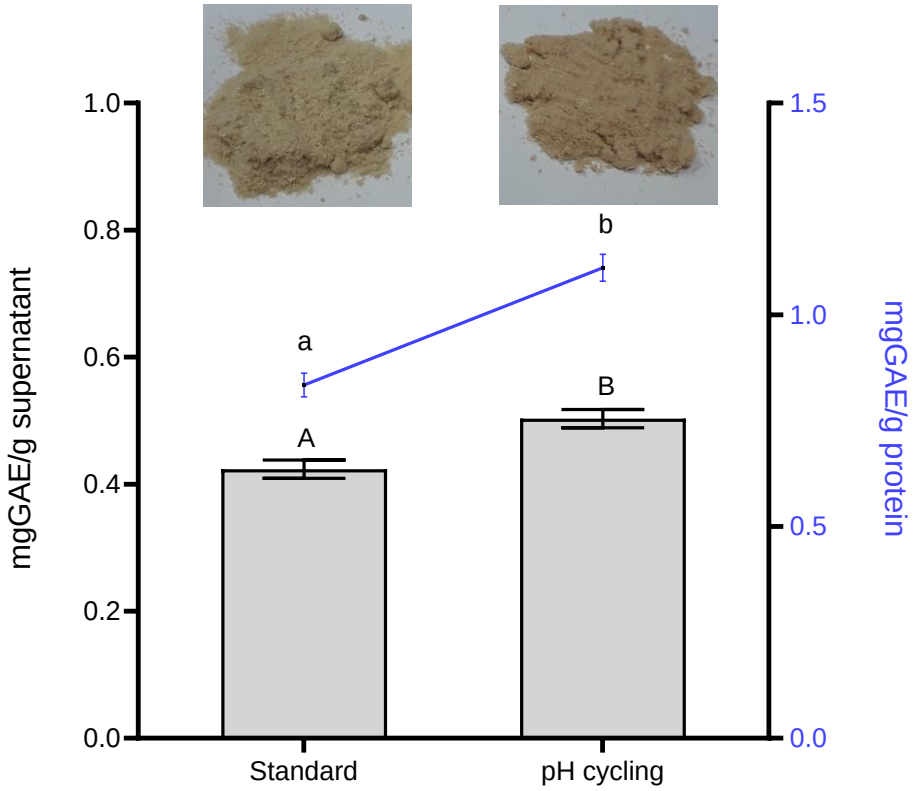

Figure 5.10: Total phenolic content of hemp protein after extraction with and without alkaline pH cycling at pH 9, 65 ^{o}C. GAE, gallic acid equivalent. Histograms with the left ordinate. Curve with the right ordinate. Different letters indicate significant differences (p < 0.05). Error bars represent the standard deviation of two replications. The images capture the extracted protein powder after centrifugation and freeze-drying.

5.2.2.4. Available lysine in the extracts

The available lysine content of the protein retained after extraction with and without pH cycling was investigated, as shown in Figure 5.11. The alkaline pH cycling caused a significant decrease ($p < 0.05$) in the available lysine content of hemp protein. At pH 9, 33.1 mg of lysine was available per gram of protein. However, this amount was reduced to 27.3 mg after alkaline pH cycling extraction, hence decreasing by about 17%.

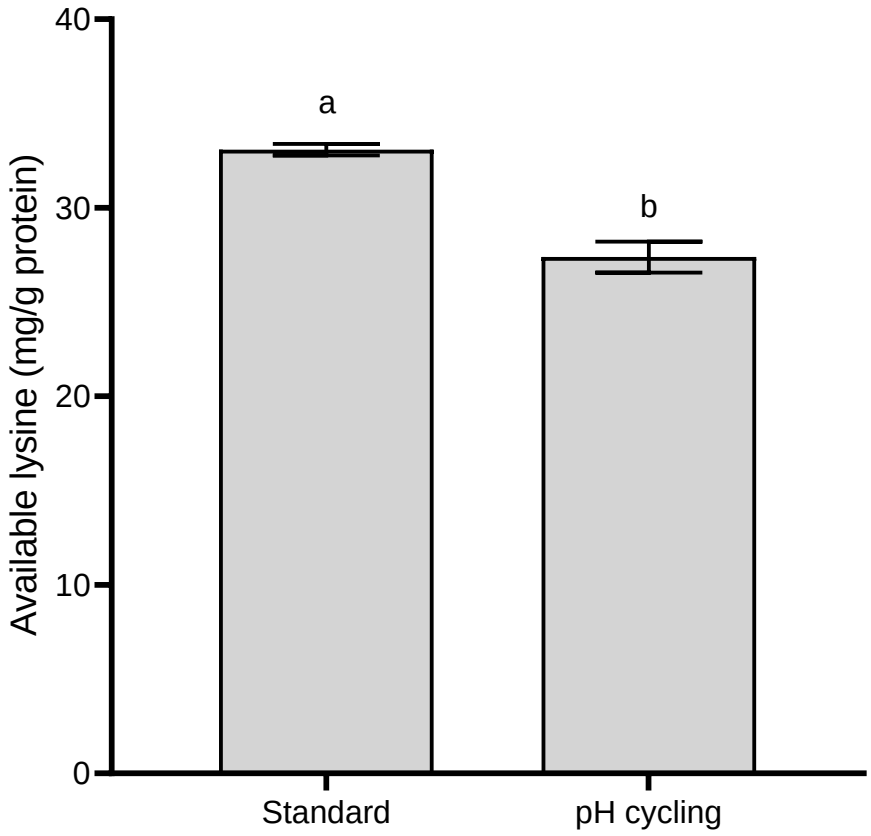

Figure 5.11: Comparison of available lysine content present in the extracted hemp protein with and without pH cycling extraction at pH 9, 65 ^{o}C. Sample without pH cycling marked as standard. Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of two replications.

5.2.2.5. Microstructure of the spent hemp seed meals

The effect of pH cycling on the matrix was studied by scanning electron microscopy (Figure 5.12). It was hard to distinguish on the SEM images, probably because of an effect arising from the sample

preparation method. Hence, the effect of pH cycling on the microstructure of hemp seed meal requires further investigation.

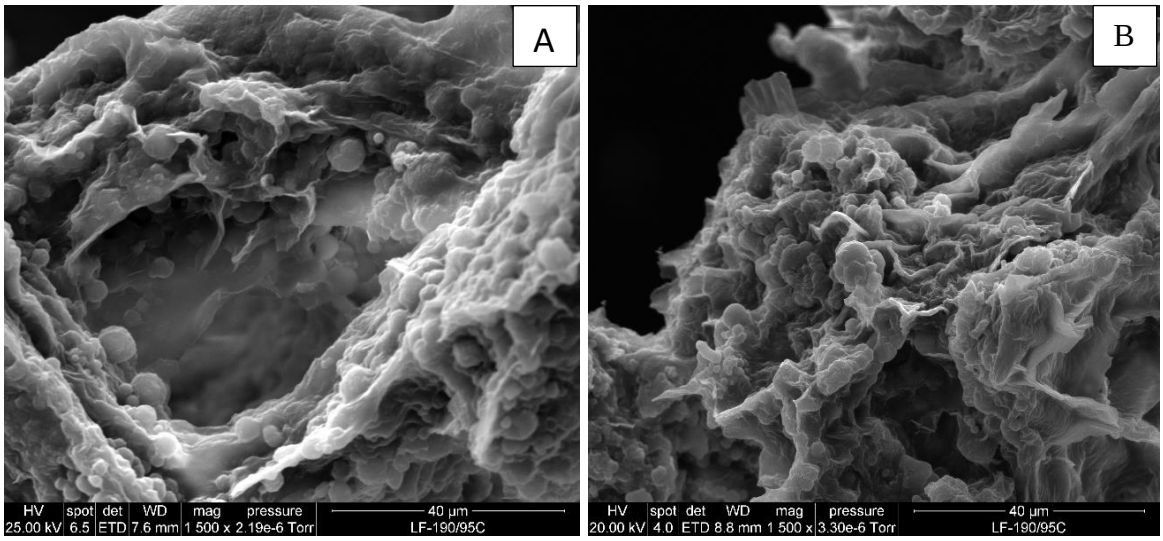

Figure 5.12: SEM images of the pellet after centrifugation of hemp protein extraction with (A) and without (B) cycling treatment.

5.2.3. Effect of combining salts, pH and temperature on the extraction of hemp proteins

5.2.3.1. Extraction yield of hemp proteins

At neutral pH 7 and without the addition of any salt (Figure 5.13 A), the extraction yield was very low (about 13%). The addition of 0.25 M NaCl significantly increased ($p < 0.05$) the protein extraction yield by 62%, compared to the control sample (without salt). Doubling the salt concentration (0.5 M) increased the protein extraction yield by 51%. Nevertheless, this yield was still low (32%) compared to the total protein available for extraction in the material. When the NaCl concentration was 0.8 M a significant increase ($p < 0.05$) in the yield of protein extraction was observed.

The extraction of proteins at different NaCl concentrations at pH 9 followed a similar trend as that at pH 7, except that at all concentrations investigated the observed yields were higher. The yield without any salt was only 20% and adding the 0.25 M NaCl increased the yield only slightly to about 27%. However, there was almost a linear increase in the yield of extracted proteins with an increase in the NaCl concentration at salt concentrations > 0.25 M, with a maximum extraction yield of 78% at 0.8 M NaCl.

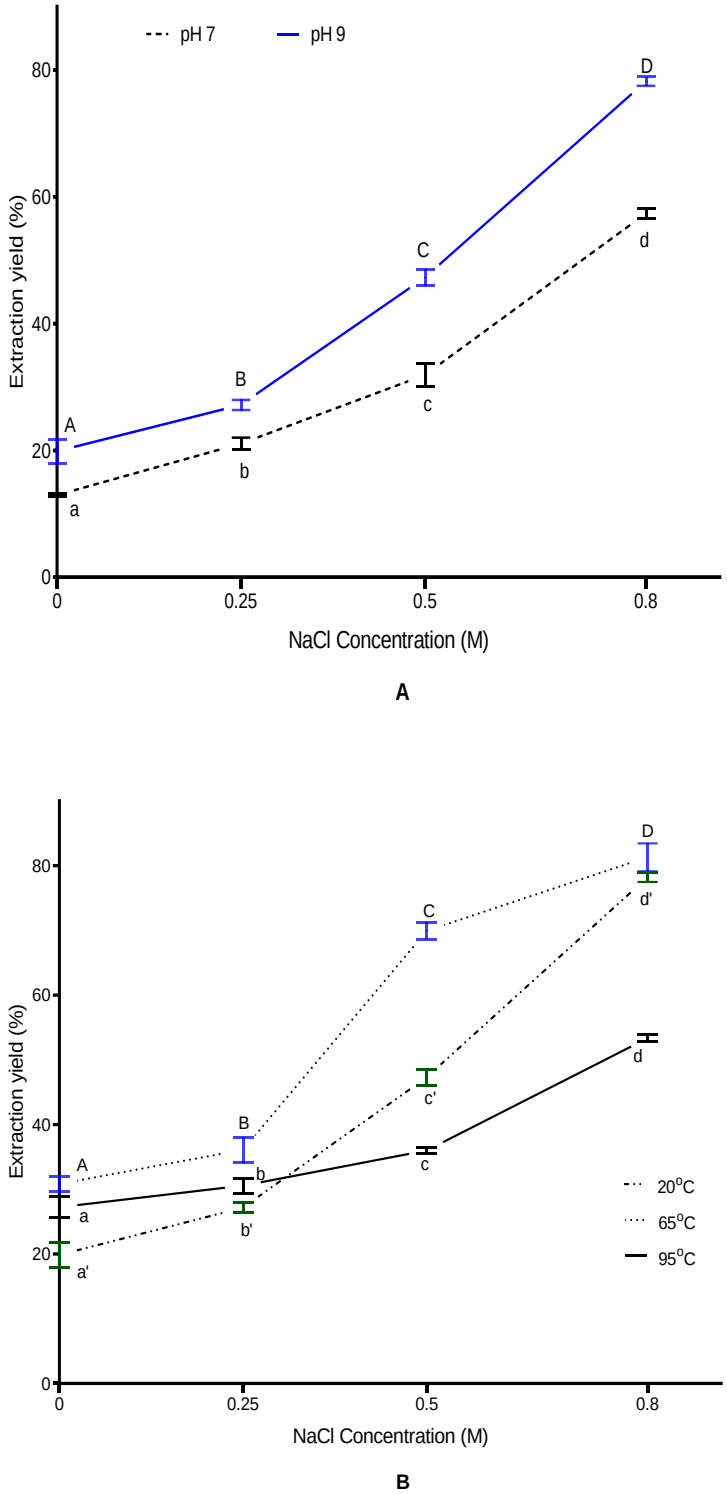

Figure 5.13: Effect of NaCl concentration on the yield of protein extraction at pH 7 and pH 9 at 20 °C (A), and pH 9 at different temperatures (B). Different letters within the same series indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of three replications.

The extraction at pH 9 improved the protein extractability compared with pH 7 (Figure 5.13 A), irrespective of the investigated NaCl concentrations. Therefore, pH 9 was chosen to investigate the effect on the extraction yield as a function of salt concentration at different temperatures, as presented in Figure 5.13 B.

The extraction yield increased along with the increase of salt concentration irrespective of the temperature. At 65 ^{o}C and 0.25 M NaCl (Figure 5.13 B), a slight improvement in protein extractability was observed (17% more) compared to salt-free extraction. A further raising of the salt concentration to 0.5 M resulted in a sharp increase (approximately 94%) in protein extraction yield. The absolute soluble protein content was 36% and 70 % at 0.25 M and 0.5 M NaCl, respectively. When the NaCl concentration was increased from 0.5 M to 0.8 M, about 16% more protein was extracted from the material. This extraction condition (65 ^{o}C and 0.8 M NaCl) led to the highest yield of protein extraction.

Interestingly, however, increasing the temperature to 95 ^{o}C did not improve the extraction yield compared to the unheated sample. Although low salt concentration (≤ 0.5 NaCl) and heating at 95 ^{o}C appeared to improve extraction yield, the yield was significantly diminished at higher salt concentrations. Beyond 0.5 M NaCl concentration, the extraction at 20 °C appeared to be better than that under heating at a very high temperature 95 ^{o}C.

5.2.3.2. Composition of proteins in the extracts

The combination of pH 9 and 65 ^{o}C seemed to be an optimal condition for extraction of hemp protein, as shown in Figure 5.13 B. Therefore, the extracted protein under different NaCl concentration at pH 9 and 65 ^{o}C was characterised under reducing SDS-PAGE condition. As presented in Figure 5.14, the adding of NaCl to 0.25 M did not significantly modify neither the composition of solubilised hemp protein nor the intensity of the bands. The compositions of protein mainly included albumin fractions at molecule weight less than 18 kDa (band D), two subunits of globulin 11s, 11s-AS and 11s-BS corresponding to the band B at 34 kDa and band C at 18-20 kDa, respectively. The component of globulin 7s also presented in the extract with less intensity. Further addition of NaCl (≥ 0.5 M), reduced the intensity of albumin fractions but enhanced the intensity of globulin 11s fraction represented by the two subunits, band B and band C. Meanwhile, the globulin 7s band was a little less intense compared to lower NaCl concentrations (≤ 0.25 M).

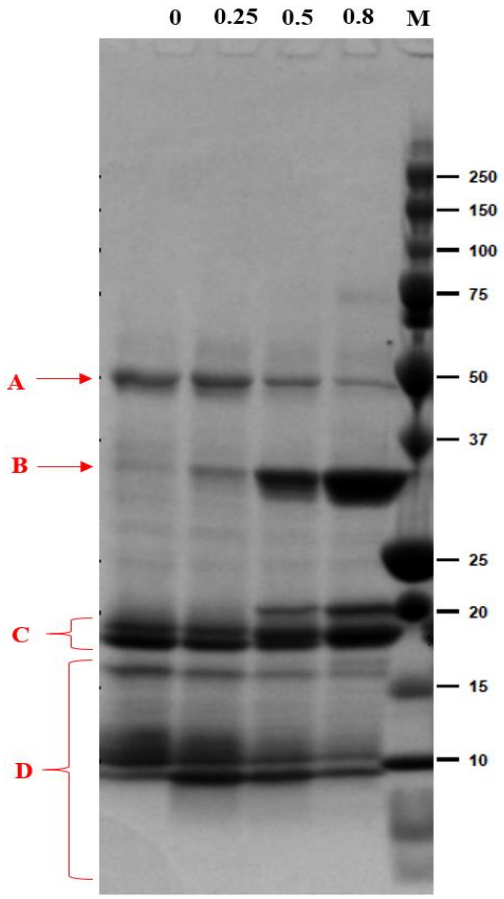

Figure 5.14: SDS-PAGE of hemp protein extracted at different NaCl concentrations at pH 9, 65 ^{o}C. Numbers above the lands indicate the NaCl concentrations (M); M, molecular mass marker in kDa. Bands A-D indicate likely globulin albumin fractions of hemp proteins, see text for more details. The protein concentration for all samples in the SDS-PAGE loading buffer was adjusted to 1 mg/mL before loading.

5.2.3.3. Effect of different types of salt on the extraction yield of proteins

Figure 5.15 below shows the yield of proteins at different salt concentrations and different salts. The extraction yield increased along with the rise of salt concentration, as noted in Figure 5.13. At a given salt concentration, the divalent salts appeared to exhibit more efficiency than the monovalent salts in releasing the protein from the food matrix. The extraction yield resulting from monovalent salts was significantly lower ($p < 0.05$); than approximately half of those produced by divalent salts at the same salt concentration.

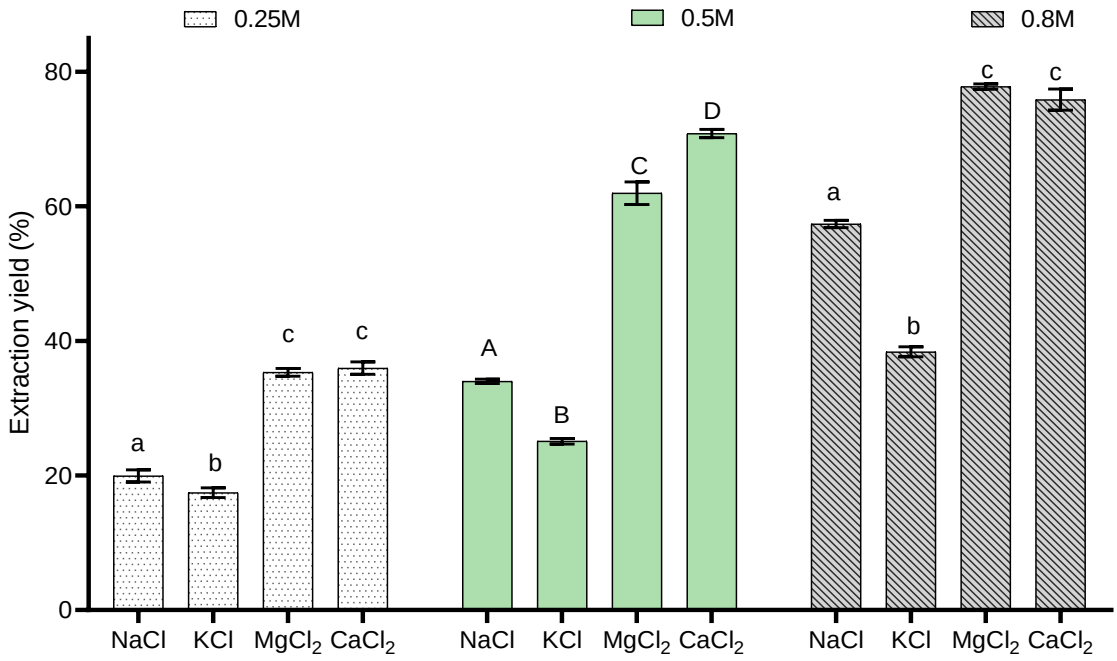

Figure 5.15: Effect of different types of salt (at pH 7 and 20 °C) on the extractability of hemp proteins. Different letters indicate significant differences ($p < 0.05$) at the same salt concentration. Error bars represent the standard deviation of three replications.

Between the two monovalent salts, NaCl and KCl, sodium chloride proved more effective than the potassium chloride in extract hemp protein from hemp seed meal, especially at higher salt concentration. The protein extraction yields using NaCl were significantly higher ($p < 0.05$) than those observed with KCl (14%, 36%, and 50% at salt concentration 0.25, 0.5, and 0.8 M, respectively).

Unlike the monovalent salt group, the two divalent salts did not show much difference in the yield of protein extraction at concentrations from 0.25 to 0.8 M. At the lowest and highest investigated concentrations, the extraction yield of those two salts largely similar, with the difference being within 3%. However, at 0.5 M concentration, the extraction yield of $CalCl_2$ was about 14% higher than that resulted from $MgCl_2$.

5.3. Discussion

5.3.1. Effect of temperature on the extraction of hemp proteins at different pH values

From the results shown in Figure 5.1, the absolute yields at pH 9 were higher than those of pH 7 at all investigated temperatures. The finding suggests that the increase in the pH of extraction resulted in an improvement in the extractability of hemp proteins. The results of the current study were consistent with those of Potin et al. (2019) who also noted an increase in the protein extraction yield (about 53%) when the pH increased from 7 to 9. The positive effect of high pH on the extraction of protein can be explained by the increase in the repulsive force between proteins molecules, caused by a higher net charge. This reduces the protein-protein interactions and makes the protein structure more flexible and more accessible to the aqueous medium (Ruiz et al., 2016).

Hemp proteins consist of approximately 15% albumins, and 85% globulins and their extractability strongly depend on the pH of extraction (Park et al., 2012; Tang et al., 2006). The solubility of hemp albumins and globulins at pH 7 is approximately 85% and 30%, respectively (Malomo et al., 2015a). At pH 9, while the solubility of albumins largely remains unchanged, the solubility of globulins increases to about 55% (Malomo et al., 2015a). Indeed, the SDS-PAGE analysis of extracts from pH 9 showed the presence of additional bands (increased intensity of bands A and B, and regions C and D of Figure 5.2). Therefore, it is likely that the extractability of proteins from hemp seed meal at pH 9 increased due to increased solubility of the globulins.

The increase in the extractability of proteins at high pH was also attributed to the impact of alkaline pH on the matrix. Non-protein components are solubilised at alkaline pH (Niemi et al., 2013); resulting in loosening and an increase in the permeability of the matrix. Therefore, the alkaline pH facilitated the release of protein out of the matrix, subsequently, increased protein extraction yield.

Increasing the extraction temperature increased from 20 ^{o}C to 65 ^{o}C resulting in an improved extractability at both pH values (pH 9 and pH 7) almost to an equal extent. An increase of 53 – 55% was observed. This result was attributed to the increase in the solubility of hemp proteins with heating

(65 ^{o}C). This is in agreement with the results of Wang et al. (2018b), who reported hemp protein solubility increased upon heating conditions. The authors observed an improvement of about 3% in the solubility of hemp seed isolate, upon heating temperature from 20 ^{o}C to 60 ^{o}C at pH 7. However, the absolute increment in yield was still much lower than the result in this chapter. This difference may be because of differences in starting material and in the solubility measurement protocols used in the two studies.

The results are also consistent with Raikos et al. (2015), who revealed that the solubility of hemp seed protein was at its highest at 60 ^{o}C heating. The increase of protein solubility was explained with the assumption that heating might cause loosening of the cell wall in plants (Mason et al., 2017) and disruption in the cell structure (Choi et al., 2006).

In contrast, further increase of the temperature to 95 ^{o}C resulted in a proteins extraction net yield decrease at both investigated pH values. This result was also consistent with the finding of Raikos et al. (2015) who reported the protein solubility dropped when the temperature increased to above 60 ^{o}C. The results might be attributed to heat-induced denaturation and aggregation of proteins at high temperature (95 ^{o}C). The protein extractability may still be improved by high temperature, above 80 ^{o}C, loosening the matrix and weakening the hydrogen bond (Chuang et al., 2019). However, these temperatures being beyond the thermal denaturation temperature of hemp proteins might have caused the unfolding of hemp proteins and further exposed the hydrophobic groups, which were buried inside the structure before (Sirtori et al., 2012). The unfolded proteins may interact with each other via hydrophobic interactions and disulphide bonding, resulting in aggregation and hence reduced solubility in water (Raikos et al., 2015). Additionally, due to the presence of high sulphur-containing amino acids, the heating condition might promote the thiol-disulphide exchange reactions between two adjacent protein monomers, rendering the thermal aggregation of hemp protein (Chuang et al., 2019; Makinen et al., 2016).

Moreover, it was likely that at very high temperatures of the extraction (95 ^{o}C), the thermal aggregation, and subsequently, the extractability was governed by the pH. The mechanism of thermal aggregation was proposed by Markossian et al. (2009) and Chuang et al. (2019). According to this mechanism, protein aggregation starts from the formation of the initial aggregates, contain hundreds of denatured protein molecules. Further growth of the protein aggregation is accomplished as a result of the sticking of the initial aggregates, or that of a higher order, so-called the diffusion-limited cluster-cluster aggregation. Therefore, there is no repulsive barrier between particles in this regime.

Hence, the aggregation is rapid and only limited by the diffusion of aggregate particles, and every collision between proteins results in sticking and aggregation. As observed in section 5.2.1.1, the higher pH resulted in lowering the denaturation speed of protein aggregation. It is possible that the higher charge at pH 9, may have inhibited the heat-induced denaturation and aggregation of proteins during heating, as noted at pH 7. This phenomenon might be explained by the stronger repulsion force, which resulted from the pH 9 that created a stronger barrier for the protein-protein collisions and hence aggregation compared to that at the neutral pH 7. Therefore, pH 9 might result in higher net extraction of proteins that do not undergo aggregation at 95 ^{o}C in comparison to pH 7, as also noted in this study.

Overall, both pH and temperature factors affect protein extractability. The pH 9 is more suitable for extractions than pH 7, and medium heating at 65 ^{o}C is more effective than 20 ^{o}C and 95 ^{o}C. It was observed that pH influences more than the temperature on protein solubility because of the dependence of ionisation of charged amino acid to the pH (Makinen et al., 2016).

The results from SDS-PAGE suggests that the albumins and globulins fractions solubilised at both pH values (pH 7 and pH 9). This result confirmed Potin et al. (2019)'s finding, who reported that all protein fractions were solubilised from pH 7 and above.

Extraction of proteins under more alkaline conditions of pH 9 also facilitated the co-extraction of phenolic compounds as compared to pH 7. At a given temperature, increasing the extraction pH increased the extraction of phenolic compounds. As a result, the recovered powder after extraction at pH 9 was darker than that at pH 7. The different colours were observed in the pictures of the hemp protein powder recovered after centrifugation and freeze-drying, as shown in Figure 5.3 A similar finding was presented by Potin et al. (2019), who showed the higher phenolic content obtained in the extraction supernatant along with an increase of the extraction pH.

Besides the pH, the temperature also affected the co-extraction of phenolic compounds with a higher phenolic content observed with an increase in extraction temperature. These results can be confirmed by Spigno et al. (2007) and Wang et al. (2008a), who investigated the effect of temperature on the extraction yield of phenolic compounds. The picture of hemp protein powder, as shown in Figure 5.3 showed the darker powder retained under higher temperature extraction condition. However, from the result of mg GEA converted to per gram protein, the effect of pH on the phenolic content is significant but not temperature.

Available lysine has a good correlation with the biological value of the protein (Kakade et al., 1969). Heating at 65 ^{o}C and pH 9 seemed to cause a slight positive effect on improving the available lysine content of hemp protein (Figure 5.4). The results were in agreement with Kwok et al. (1998), who described that under controlled optimum heating conditions, the higher available lysine was received. It has been hypothesised that heating causes the unfolding of compact protein structure compared to the native, unheated protein, resulting in an availability of more amino groups' exposure, which facilitates the accessibility of a test agent to the group. The increase in available lysine content was probably due to the complete unfolding and dissociation of the protein, exposing more free amino groups of lysine at the molecular surface (Kwok et al., 1998). It was also assumed that the disturbance of the quaternary structure of proteins under the effect of temperatures led to the "revealing" of lysine that became more accessible (Zilic et al., 2006).

5.3.2. Effect of pH cycling on the extraction of hemp proteins

The pH cycling or sometimes referred to as pH shift has been used by some researchers to improve the protein solubility and, or functionality for different plant materials, such as hemp protein (Wang et al., 2018b), peanut protein (Wang et al., 2020) and, soy protein (Jiang et al., 2010). It has been hypothesized that pH cycling leads to the disassociation of protein multimers and unfolding of the native structure of proteins (Jiang et al., 2018; Jiang et al., 2013). The unfolding of protein molecules is made possible by exposing the material to an extreme alkaline or acidic pH for a period of time. That was followed by the neutralising to pose the partial refolding of protein molecules (Jiang et al., 2013; Wang et al., 2018b). As a result, some functional groups previously hidden inside the compact protein molecular structure were exposed to the solvent.

So far, no study has been done on pH shift which neutralised to pH 9, the highest has only been almost to pH 7. Results from this study revealed that neutralising to pH 9 produced a higher yield compared to doing it at pH 7.

Results from this study further revealed that when performing a pH cycling with the terminal pH 7; although the yield is improved, it is also less than the control sample, as shown in Figure 5.6. This finding also confirms Wang et al. (2018b)'s research that stated pH shift at room temperature slightly reduces hemp protein solubility. However, this result contradicted Jiang et al. (2010)'s finding of another research group working on soy protein isolate. The difference might come from the difference in the raw materials used for extraction. Interestingly, the extraction yield resulted by the cycle pH of 9-12-7 (13.6%) was not much different from the yield created by the extraction condition at pH 7 and

20 ^{o}C (12.9%) as presented in Figure 5.1. It is possible that the terminal pH of the pH cycling plays an important role in governing the yield of protein extraction obtained.

The alkaline pH cycling with heating improved the extraction yield or solubility of hemp proteins significantly. At extreme alkaline pH (pH 12), the protein is more sensitive to thermal effect; therefore, the heating will enhance the effect of alkaline pH cycling on the protein (Wang et al., 2020). The results of the current work were consistent with the findings of previous authors (Wang et al., 2018b). However, the absolute yield in the current work was lower than that reported by the previous authors. The difference might come from the initial hemp materials used by the two authors; also, it might be attributed to the difference in temperatures used. From the results of the current work and the finding of Wang et al. (2018b), it can be concluded that the temperature range optimal for pH cycling applied for hemp protein extraction ranged from 60 ^{o}C to 80 ^{o}C.

The improvement yield resulted from alkaline pH cycling in this study was consistent with the finding of previous authors (Ravichandran et al., 2019). The effect might come from the unfolding of native protein structure, as mentioned above. Besides that, exposing the material at extreme alkaline pH caused the loosening of the food matrix (Niemi et al., 2013), which facilitates the release of protein out of the food matrix, as can be seen in Figure 5.12.

In contrast, the acidic pH cycling led to the reduction of the yield of protein extraction, also noted by Liu et al. (2015). When the material was exposed to extremely low pH, the protein was unfolded, aggregated, as well as exposing to more hydrophobic groups (Yuan et al., 2012). The protein became more susceptible to denaturation, especially under the heating treatment (Liu et al., 2015). The main reason for this phenomenon was the formation of disulphide cross-linking and hydrophobic aggregation (Jiang et al., 2009).

From the SDS-PAGE results, it can be seen that the majority of protein extracted by the acidic pH cycling was the water-soluble fraction, albumin. The result could be explained by the fact that globulin seemed to be aggregated at a pH lower than neutral pH (Kim et al., 2011a; Leonard et al., 2019). Therefore, most of the globulin fraction was aggregated at the stage when the pH was at 2. Then, adjusting the pH back to pH 9 could not re-dissolve the globulin aggregates. The protein profile of alkaline pH cycling of hemp protein observed in the current work was consistent with those found previously (Wang et al., 2018b).

The alkaline pH cycling led to an increase in total phenolic content in the extracts when compared to the treatment without pH cycling. The difference might be attributed to the extreme alkaline pH (pH 12) exposure of protein, which promoted the diffusion of phenolic compound to the aqueous extraction medium together with the hemp protein. Phenolic compounds are favourably solubilised in alkaline pH, as presented by previous authors (Potin et al., 2019; Wang et al., 2019; Xu et al., 2000). Presumably, these differences in phenolic compounds content of samples extracted by alkaline pH cycling and without pH cycling also affected their colour. Hence, the resulted hemp protein powder extracted by pH cycling was darker than that obtained without pH cycling (Figure 5.10).

The combination of extreme alkaline pH with heating is known to promote the formation of nephrotoxic compounds, lysinoalanine (LAL), which contribute to loss of lysine, subsequently reducing the quality of protein (Wang et al., 2019). The results of available lysine content revealed that alkaline pH cycling under heating condition might have negatively affected the protein quality. The alkaline pH was considered a good condition that promoted the cross-linking between protein and oxidised fat or other components, such as phenolic compound via the amino groups of protein (Potin et al., 2019). Although the LAL content was not measured in extracts, the available lysine content showed that there was a slightly negative impact of alkaline pH cycling on the free lysine content. It is possible that the ε-amino groups of lysine were involved in those cross-links and that may have reduced the availability of lysine in the extracted protein, more work needs to be done in future to test this hypothesis.

5.3.3. Effect of combining salt, pH and temperature on the extraction of hemp proteins

The objective of this study was to investigate the dependence of hemp protein extraction yield to salt concentrations under various conditions (pH and temperature). At a given pH, the more NaCl was added, the higher the protein extraction yield created (Figure 5.13 A). In agreement with our results, the positive relationship between salt concentration and protein extraction yield has been previously reported for hemp proteins at pH 9 (Potin et al., 2019), and broad bean proteins at pH 7 (Arogundade et al., 2006). However, the latter authors showed contrasting findings, noting that the extraction yield decreased at alkaline pH 9 when the NaCl salt concentration increased. The differences might be attributed to the different methods and materials (broad bean and hemp seed meal) or method of extraction.

The effect of added salts in improving the protein extraction yield was attributed to the salting-in effect (Potin et al., 2019). Specifically, the increase of NaCl concentration increased the protein

solubility, and subsequently increasing the protein extraction yield. The solubility of proteins with increasing salt levels increases until a critical salt concentration is reached, beyond that point additional salt causes instability of the protein because of the salting-out effect (Beauchamp et al., 2012). In this study, for hemp protein solubilisation, it appeared that the 0.8 M NaCl was still below the critical salt concentration that was beginning the salting-out effect. This result is in agreement with those reported by Schweizer et al. (2014); they stated that 1.0 M is the maximum salt concentration for hemp protein extraction.

The results from this study showed the effect of salt concentration on protein extraction yield was dependent on the pH of the medium, observing that an alkaline pH (pH 9) increased the yield slightly. This is in agreement with previous studies working on different materials, broad bean (Arogundade et al., 2006), soy protein (Jiang et al., 2010), sunflower protein (Pickardt et al., 2009), and hemp protein (Potin et al., 2019). When the pH is close to the electric pH (p*I*), the presence of NaCl ions might allow forming an electric double layer, which increases the protein solubility (the so-called salting-in effect). When the pH is far from the p*I*, the salt ions might diminish the protein net charge that is created by the pH. Therefore, this reduction of electrostatic repulsion causes protein aggregation (Arogundade et al., 2006; Jiang et al., 2010; Roberts et al., 2015). In addition to the effect of salt on the protein, the added salt in the medium also can have an impact on the matrix. The high salt concentration can also contribute to suppress the phenolic-protein interactions and improve protein extractability (Pickardt et al., 2009).

A positive correlation between protein extractability and salt concentration was different at different temperatures (Figure 5.13 B). The extraction of protein at the low salt concentration (≤ 0.25 M NaCl) combined with heating at medium (65 ^{o}C) and high temperature (95 ^{o}C) was more efficient than in unheated samples. A further increase in salt concentration (> 0.25 M NaCl) combined with mild heating (65 ^{o}C) still resulted in a positive effect on the protein extraction yield. However, increasing the temperature to 95 ^{o}C decreased the protein extraction yield. It is possible that at 95^{o}C, the proteins extraction efficiency might still be high under these ionic strength conditions, but the high temperature caused thermal denaturation of proteins that leads to aggregation and protein insolubility.

As discussed in Section 5.1.2, heating improved the extractability of hemp proteins by loosening the cell wall (Mason et al., 2017), and disrupting the cell structure (Choi et al., 2006), even at high temperature such as 95 ^{o}C. However, in the presence of NaCl at a high concentration (> 0.25 M), the opposite outcome was obtained. This phenomenon could be probably explained by the fact that the

raise of salt concentration increases the vulnerability of protein to heat-induced denaturation (Jiang et al., 2010). Hence, the protein extraction yield at high temperature and salt concentrations was lower than that found in the unheated samples at high salt concentration.

When there is less salting-out effect there is more protein extraction, whereas more salting-out effect leads to less protein extraction. According to the order of cation in the Hofmeister series, the two divalent cations Ca^{2+} and Mg^{2+} create less salting-out effect than the two monovalent cations Na^{+} and K^{+} (Roberts et al., 2015). This order was consistent with our findings where the extraction yield resulted from the divalent salts was remarkably higher (i.e. salting-in effect in the same concentration) than that of monovalent salt. According to the above-mentioned order, potassium chloride showed a higher salting-out effect than sodium chloride, when comparing these two monovalent salts. This order also entirely matched the finding of this study in which sodium chloride produced higher extractability than potassium chloride at the same concentration. For the two divalent salts and based on the Hofmeister series, it was assumed that calcium chloride showed less salting-out effect than magnesium chloride, thus the protein extractability with calcium chloride was higher than with magnesium. However, in this study, it was found that at the highest salt concentration (0.8 M) calcium chloride showed a lower protein extraction yield than magnesium chloride.

Additionally, it seems the yield of hemp protein extraction was more affected by the cations than by anions. At the same salt concentration, the amount of Cl^{-} is equal for both monovalent and divalent salts, whereas the amount of cations in divalent salts is double of those in monovalent salts. Remarkably, in this study, the divalent salts showed nearly double extraction yields compared to monovalent salts. Therefore, the assumption that the majority effect is caused by the salt concentration came from its cation content. This assumption was in agreement with the statement presented by Jenkins (1998). Besides the explanation based on the order of cation in the Hofmeister series, the presence of cations in the medium can help break the salt bridges in protein and enhance the extraction (Guzman et al., 2020).

From the results of SDS-PAGE, at high salt concentrations (0.5 M and 0.8 M NaCl), the more salt-soluble fraction of protein (i.e. globulin) was solubilised, which made the bands (Band B & C) more intense. The water-soluble fraction was however still extracted when using salt. In other words, both fractions of hemp proteins, the water-soluble and the salt-soluble were extracted by adding salt. In conclusion, the use of different salt concentration to extract protein did not change the protein composition but may have made the change in the quantity of extracted proteins.

Overall, from the results of chapter 5, the optimal conditions of temperature and pH for hemp protein extraction was 65 ^{o}C and pH 9. The addition of salt in the extraction medium was an effective method to extract protein from hemp seed meals. Significantly, the use of divalent salts was more effective at extracting hemp proteins, compared to monovalent salts. In addition, alkaline pH cycling with the pH cycle 9-12-9 proved to be a promising method in the extraction of hemp proteins.

Chapter 6

6. Ultrasound-assisted extraction of hemp proteins

6.1. Introduction

Ultrasound, a novel processing technique has been increasingly applied for protein extraction from plant materials, e.g., sunflower meal (Dabbour et al., 2018), rapeseed meal (Yagoub et al., 2017), rice Dreg Flour (Li et al., 2017). However, ultrasound-assisted extraction of hemp proteins has not been researched before. This chapter explores the effect of this promising technique on the extraction, either singly or in combination with either salts addition or pH cycling, to explore further approaches to improve the yield of hemp protein extraction.

6.2. Results

6.2.1. Effect of ultrasound on the extraction of hemp proteins

6.2.1.1. Effect of ultrasound treatment on the yield of protein extraction

The ultrasound treatment was carried out by the method described in Chapter 3 (section 3.2.2). A 10% dispersion of hemp seed meal powder was adjusted to the target pH (pH 7 or pH 9) at 20 ^{o}C. It was then subjected to the ultrasound treatment at 20 ^{o}C or 65 ^{o}C for 15 minutes, with varying amplitudes (20% or 80%). Figure 6.1 below shows the effect of ultrasound amplitude on the extractability of proteins at 20 °C and different pH.

In general, ultrasound-treated samples showed higher extractability than control samples (without ultrasound), regardless of the amplitude used. At pH 7 and 20 ^{o}C, the ultrasound treatment with 20% amplitude showed approximately 58% higher yield than that without ultrasound treatment. At the same pH, a higher amplitude resulted in a higher yield, which recorded an increase of about 84% more than the sample without ultrasound treatment. Between the amplitude of 20% and 80%, the later amplitude exhibited a significantly higher ($p < 0.05$) protein extraction yield. The yield was 16% higher in samples treated with 80% amplitude than in those treated with 20% amplitude.

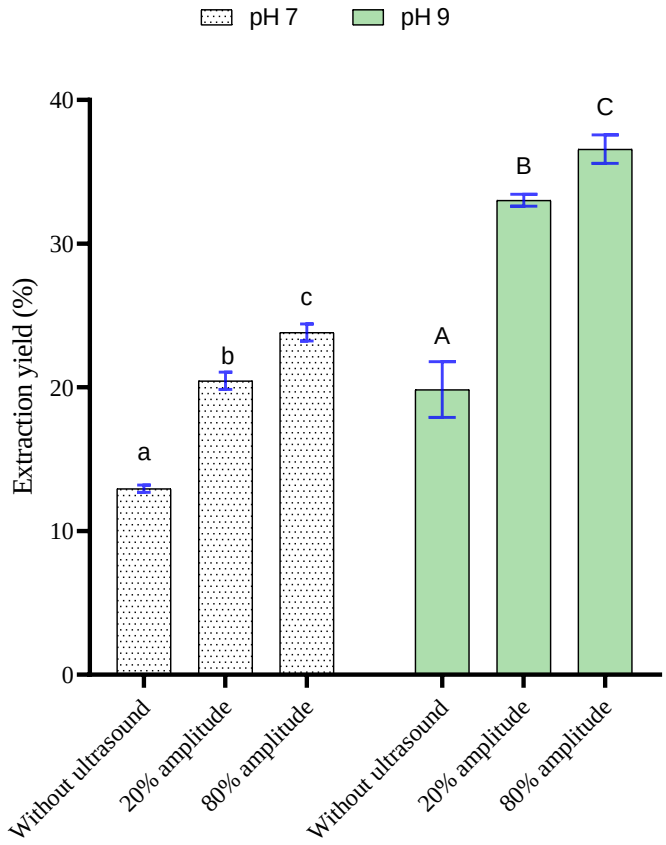

Figure 6.1: The yield of hemp protein extraction under different ultrasound treatments at different pH at 20 °C. Grey bars present pH 7. The different letters indicate a significant difference ($p< 0.05$) at each pH value. Error bars represent the standard deviation of three replications.

Similarly, at pH 9 and 20 °C, the extraction yield of ultrasound-treated samples with 20% amplitude increased by 66%, compared with untreated samples. An increase in yield of about 84% was observed using an amplitude of 80%, compared to untreated samples. Interestingly, this increase in extraction yield was similar to that recorded at pH 7. The highest amplitude also used in this study (80%) led to a significant increase in protein extraction yield (approximately 11%) compared to the 20% amplitude (p<0.05).

Between pH 7 and pH 9, the yield obtained at pH 9 was remarkably higher at both amplitudes investigated (the approximate difference being 54% and 61% using amplitude 80% and 20%

respectively). Therefore, pH 9 was used to investigate the effect of ultrasound at different temperatures, as presented in Figure 6.2.

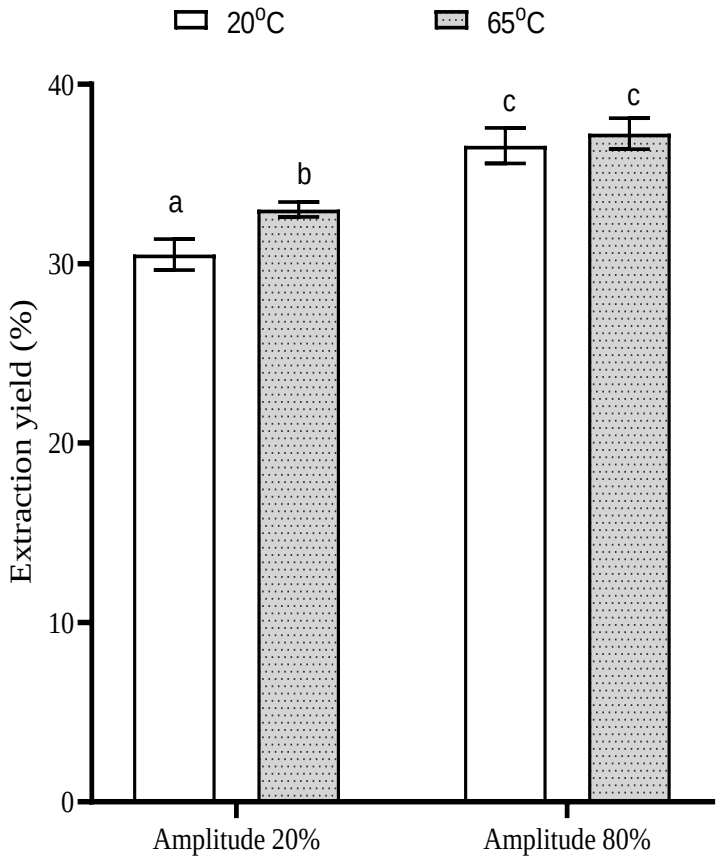

Figure 6.2: The yield of hemp protein extraction under different ultrasound treatments at different temperatures (20 °C and 65 °C) at pH 9. The different letters indicate a significant difference ($p < 0.05$). Error bars represent the standard deviation of three replications.

In general, the treated temperature affected the yield of hemp protein extraction. A higher temperature resulted in a higher extraction yield. At an amplitude of 20%, ultrasound at 65 °C produced about 33% in yield, an approximately 8% higher than that of 20 °C at the same amplitude. At the amplitude of 80%, the ultrasound treatment at 65 °C resulted in a higher yield than that at a lower temperature, the difference is however not significant ($p < 0.05$). Overall, 65 °C appeared to be better than 20 °C in the ultrasound-assisted extraction.

6.2.1.2. Effect of ultrasound duration on the yield of extraction.

Figure 6.3 shows that the ultrasound duration significantly affected ($p<0.05$) the yield of hemp protein extraction. A steady rise in the extraction yield was observed in the first 30 seconds, but after that,

the extraction occurred at a much slower but steady rate. It took 30 seconds to increase the yield by 20% more than that initially obtained (from about 13% to 15.6%). In the next 90 seconds, only a ~3% increase in yield on average was observed every 30 seconds. A further increase in the ultrasound duration time up to 900 seconds, only led to a 1-2% increment every 30 seconds. Then after 900 seconds of ultrasound treatment, the extraction yield was not significantly different ($p < 0.05$).

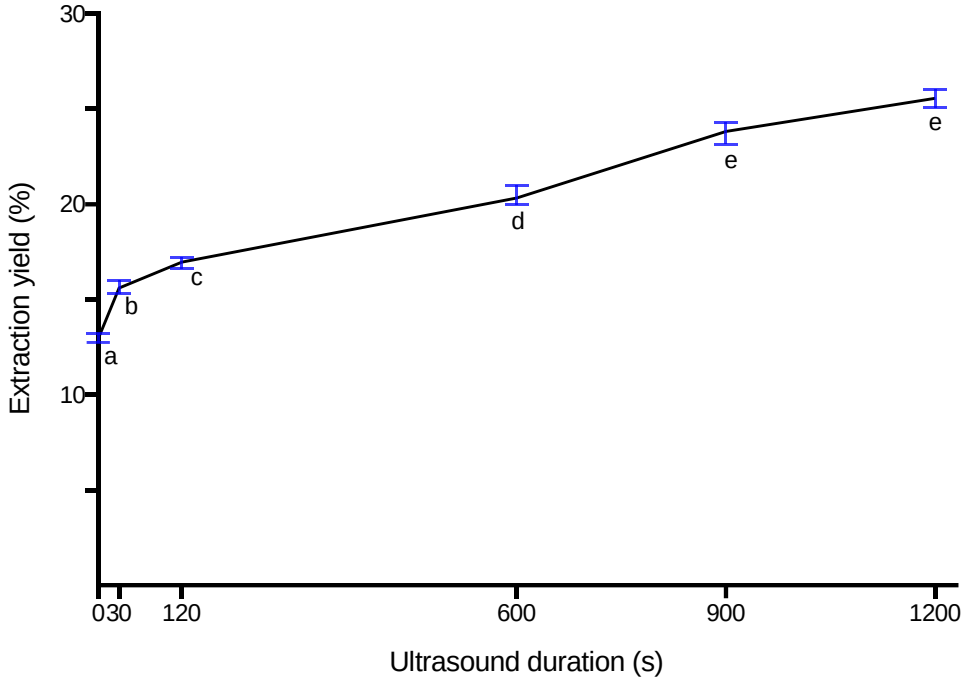

Figure 6.3: Effect of ultrasound duration time on the protein extraction yield, the extraction condition at pH 7, 20 °C, and 80% amplitude. The different letters indicate a significant difference ($p < 0.05$). Error bars represent the standard deviation of three replications.

6.2.1.3. Composition of proteins in the extracts

Figure 6.4 shows the comparison of hemp proteins fractions extracted under different ultrasound treatments (20% and 80% amplitude) and pH conditions (pH 7 and pH 9). The control samples without ultrasound treatment are also presented for comparison. All the samples, with and without ultrasound treatment, showed the same four distinct protein fractions corresponding to bands A-D. Specifically, band A with a molecular mass of approximately 48 kDa corresponds to the globulin 7s fraction; whereas bands B and C at about 34 kDa and 18-20 kDa, represent the two subunits of globulin 11s, acidic and basic subunits, respectively. The water-soluble fraction, albumin, is shown in band D, with molecular weights less than 18 kDa. Additionally, all the bands appeared faint without

ultrasound sample but increased intensity at ultrasound treatment conditions. The ultrasound treatment at amplitude 80% and pH 9 was the most intense band among all other treatments.

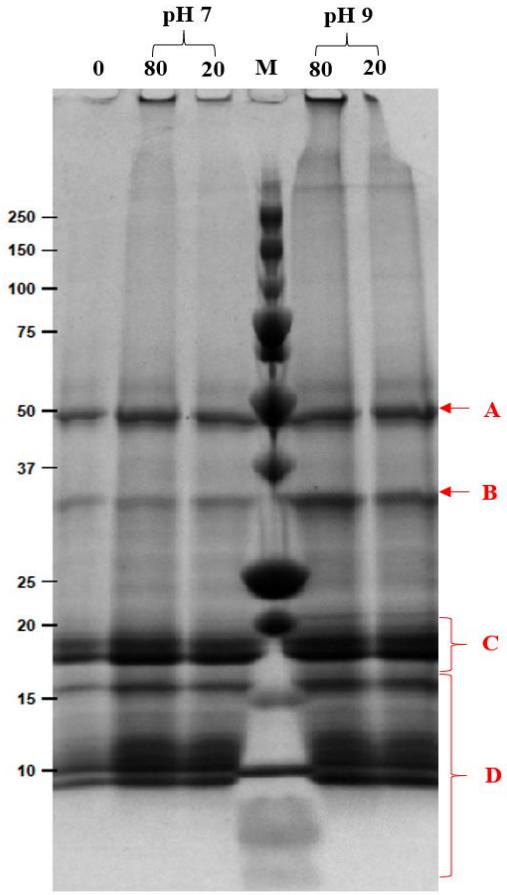

Figure 6.4: SDS-PAGE comparison of hemp proteins extracted by ultrasound at different amplitude (20% and 80%) at different pH (pH 7 and pH 9) at 65 °C. Numbers above the lands indicate the used amplitude in percentage; 0, sample without ultrasound. M, molecular mass weight marker in kDa; Bands A-D most likely indicate globulin and albumin fractions of hemp proteins, see text for more details. The protein concentration for all samples in the SDS-PAGE loading buffer was adjusted to 1 mg/mL before loading.

6.2.1.4. Phenolic content in extracts

The phenolic content of the supernatant obtained after centrifugation (10,000 x *g*, 20 °C, and 20 min) of the sample with ultrasound treatment at different amplitudes at pH 9, 65 °C were analysed and shown in Figure 6.5. Samples without any ultrasound treatment were also presented for comparison. As can be seen, the extraction by ultrasound produced a significantly higher (p <0.05) content of phenolic compounds in the obtained supernatant. Without ultrasound treatment, the supernatants of

samples obtained after extraction at 65 °C and pH 9, the TPC in the sample was 0.42 mg of GAE. After ultrasound treatment, an increase of approximately 44% of TPC was observed, but the amplitude (20% and 80%) did not significantly affect the TPC ($p < 0.05$).

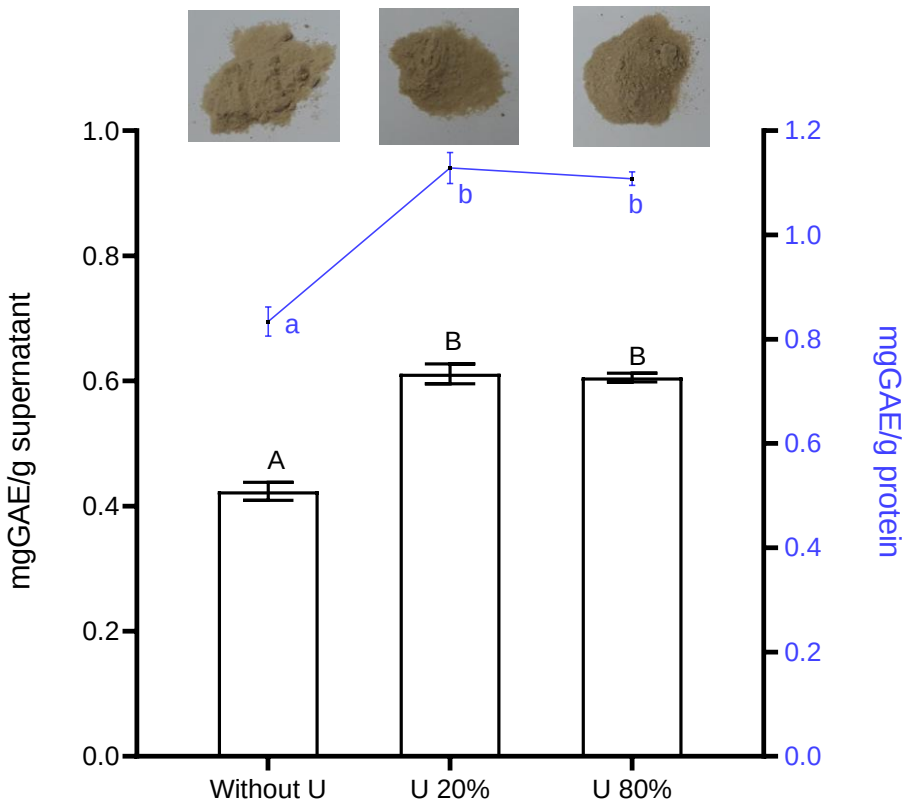

Figure 6.5: Total phenolic content of the extracted hemp proteins under ultrasound treatment at different amplitudes (20 and 80%), pH 9 and 65 °C. GAE, gallic acid equivalent. Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of two replications. The images capture the extracted protein powder after centrifugation and freeze-drying.

A similar trend was observed when the total phenolic content was expressed as GAE per gram of extracted protein (mg GAE/g protein) (Figure 6.5). Without ultrasound, an amount of approximately 0.9 mg GAE was co-extracted per gram of extracted hemp proteins. This amount slightly increased to around 1.1 mg GAE with the ultrasound treatments. Also, there were no significant differences in the total phenolic content of extracted protein when using ultrasound treatments with 20% or 80%

amplitude. The appearance of the extracted protein powder obtained after centrifugation and freeze-drying was darker in ultrasound-treated samples than in untreated ones

6.2.1.5. Available lysine content in extracts

The available lysine content in the extracted hemp protein was analysed and presented in Figure 6.6. An amount of 33.1 mg of lysine available per gram of extracted protein was found under the extraction condition of 65 ^{o}C and pH 9. Interestingly, ultrasound treatment increased the free lysine content by approximately 10% which approximately reached 36.5 mg. However, there was no significant difference between the two ultrasound amplitudes ($p < 0.05$).

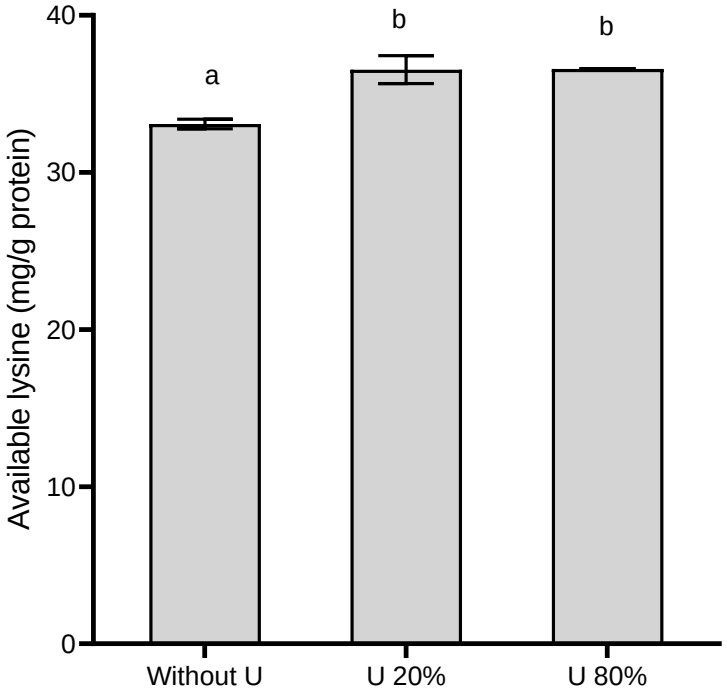

Figure 6.6: Available lysine content presented in the extracted hemp protein under ultrasound treatment at different amplitudes (20% and 80%) at pH 9, 65 ^{o}C. Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of two replications.

6.2.1.6. Scanning electron microscopy of hemp seed meals after extraction

The impact of ultrasound on the food matrix was also investigated by SEM, and micrographs are presented in Figure 6.7. The untreated sample (without ultrasound) appeared to be tighter and closed (Figure 6.7 A) compared to the one obtained after ultrasound treatment (Figure 6.7 B).

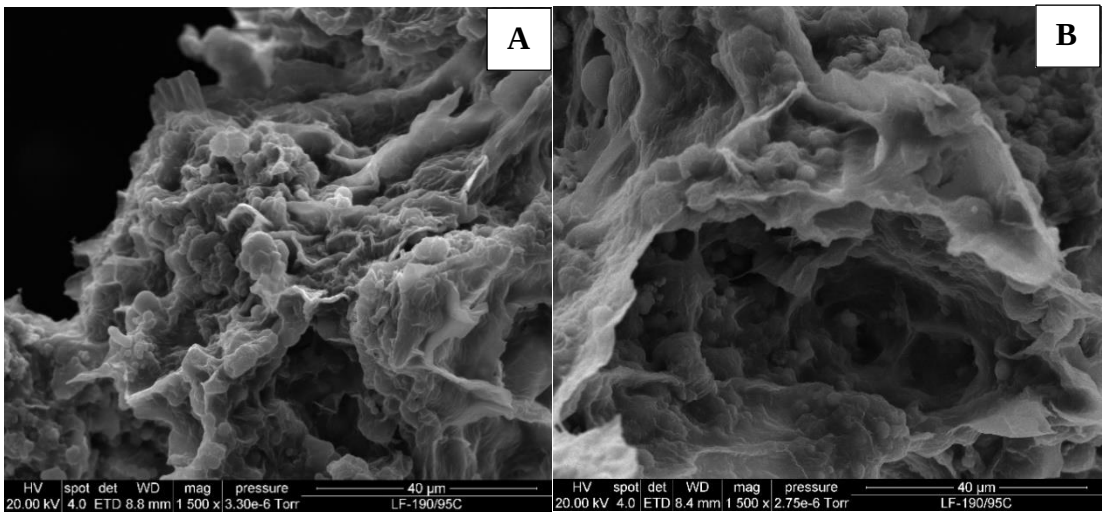

Figure 6.7: SEM images of the spent HSMs obtained by extraction at 65 °C and pH 9 (A) untreated sample (B) ultrasound treated at 80% amplitude.

The SEM images shown in Figure 6.8 compare the disruption level of ultrasound treatment at different amplitudes (20% and 80%). Small differences in the microstructure of the spent mass after ultrasound treatment could be observed at both amplitudes. However, from the SEM images, it is difficult to quantify the major changes in the microstructure and a more sensitive and non-invasive technique must be used to quantify the changes in the microstructure.

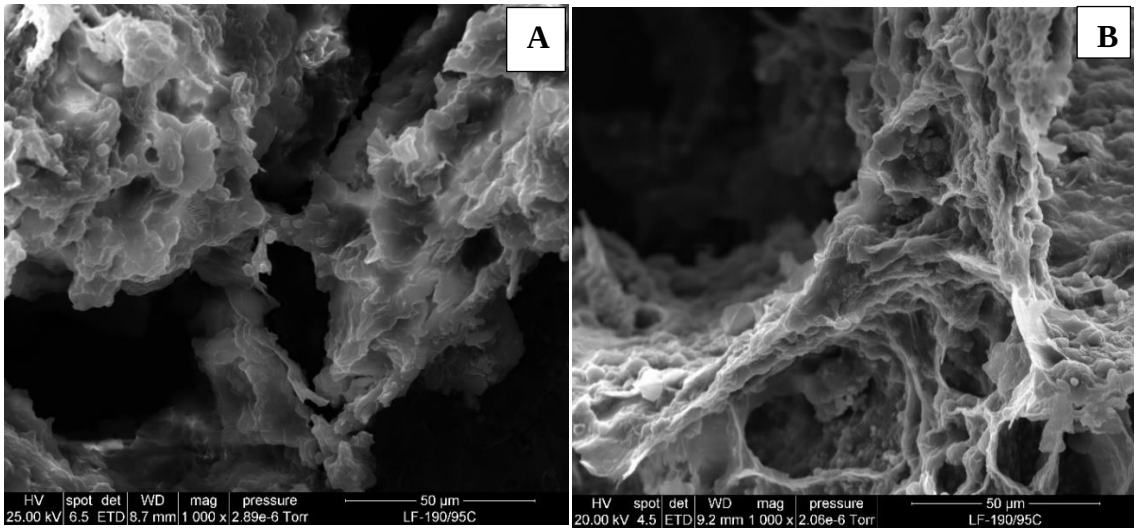

Figure 6.8: SEM images of spent HSMs after ultrasound-assisted extraction at 65 °C and pH 9 using ultrasound at (A) 20% amplitude and (B) at 80% amplitude.

6.2.2. Effect of combining ultrasound and adding salts on the extraction of hemp proteins

6.2.2.1. Effect of ultrasound treatment combined with salt.

Two types of salts, NaCl (representative for monovalent salts) and $CaCl_2$ (representative for divalent salts), combined with an ultrasound treatment were investigated. This was achieved by dispersing the hemp seed meals in distilled water (10% w/w) at different salt concentrations (0, 0.25, 0.5 and 0.8 M), pH 9. The suspension was placed in a shaking water bath at 65 ^oC for 1 h, before subjecting it to ultrasound treatment (80% amplitude, 15 min). The results of protein extraction yield are shown in Figure 6.9.

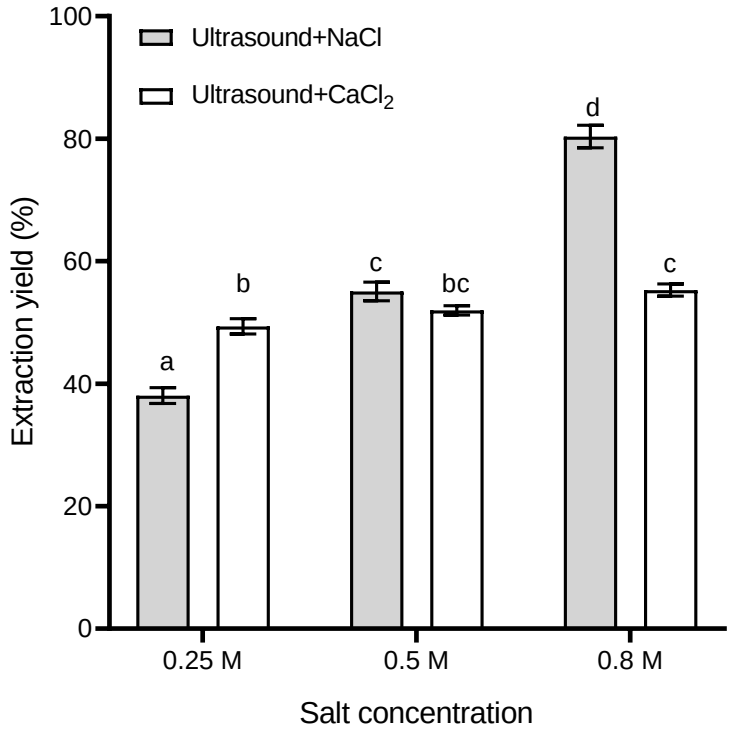

Figure 6.9: The yield of hemp protein extraction of dispersions containing different types of salt (NaCl and $CaCl_2$) and after subjected to ultrasound treatment (80% amplitude, 15 min) at 65 ^oC, pH 9. Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of three replications. For extractability of proteins with NaCl and $CaCl_2$ without ultrasound treatment see Figure 5.15, Chapter 5.

The combination of ultrasound and NaCl as monovalent salt significantly increased the yield of protein extraction, and this effect was enhanced with the increase in salt concentrations. There was a significant difference (p<0.05) between different NaCl concentrations investigated. The yield

resulting from the addition of a divalent salt ($CaCl_2$) almost remain unchanged from 0.25 M to 0.8 M.

At low salt concentration (0.25 M), the yield of extraction with the divalent salt was approximately 30% ($p < 0.05$) than that with the monovalent salt. When the salt concentration was doubled to 0.5 M, there was no significant difference in the yield of extraction in the presence of both salts. A further increase in the salt concentration to 0.8 M resulted in a significant increase in the yield of extraction in the presence of the monovalent salt (approximately 45%), compared with the divalent salt.

6.2.2.2. Effect of ultrasound treatment combined with added salt and different temperatures

From the above results, the monovalent salt (NaCl) seemed to perform better than the divalent salt when combined with the ultrasound treatment. Therefore, NaCl was used to investigate the further combined effect of salt addition and ultrasound on protein extraction yield at different temperatures, as shown in Figure 6.10. The yield of extraction of the sample without ultrasound treatment, i.e., only containing NaCl, was also shown for comparison.

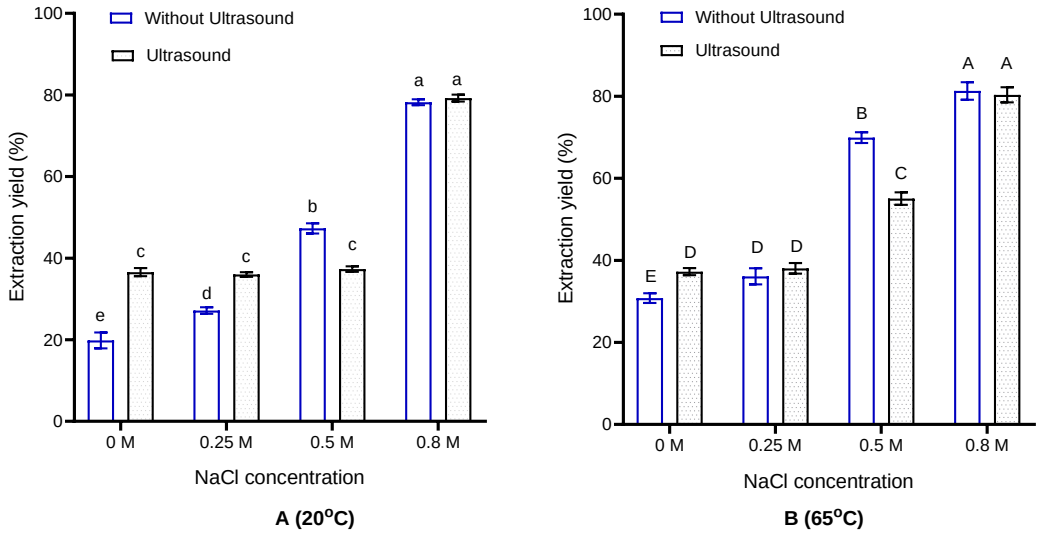

Figure 6.10: Yield of hemp protein extraction from dispersions that contained different concentrations of NaCl and were treated by ultrasound treatment (80% amplitude, 15 min, pH 9) at different temperatures: (A) 20 °C, and (B) 65 °C. Different letters indicate significant different ($p < 0.05$) at each graph. Error bars represent the standard deviation of three replications.

At 20 °C (Figure 6.10 A) ultrasound treatment induced no significant change (p <0.05) in the yield up to 0.5 M NaCl and an absolute yield of approximately 36% was recorded. A further rise in the

NaCl concentration to 0.8 M significantly improved the absolute yield to 79%, meaning that it increased by almost double, compared to the yield at 0.5 M. In comparison with the dispersions that only had NaCl added (without ultrasound), the inclusion of ultrasound for sample treatment created a significant improvement in the yield only at the low NaCl concentration (0.25 M). However, a decrease in the yield was observed at 0.5 M. At the highest NaCl concentration (0.8 M), the combined treatment did not show any additional improvement, compared with the treatment without ultrasound.

At 65 ^{o}C (Figure 6.10 B), without ultrasound, the yield extraction increased significantly (p<0.05) when the salt was added at 0.25 M NaCl; however, when combined with ultrasound, no difference in protein extraction yield was observed. Further addition of NaCl to a concentration of 0.5 M, resulted in a significant increase in the yield (about 45%) for co-treated samples. However, the absolute yield was still remarkably lower than that observed in samples without ultrasound at the same NaCl concentration. At the highest NaCl concentration (0.8 M), the combined treatments showed a significant (p<0.05) increase in the yield by approximately 46%, compared with co-treated samples containing 0.5 M NaCl. However, no significant differences were observed between samples with and without ultrasound, which is in agreement with the results obtained at 20 ^{o}C.

Comparing the extraction yields at 20 ^{o}C and 65 ^{o}C, (Figure 6.10 A and Figure 6.10 B), without ultrasound, the yield at 65 ^{o}C was higher than that at 20 ^{o}C at a salt concentration of 0 – 0.5 M; however, not much difference at 0.8 M was observed. Whereas, with ultrasound, the yield of protein extraction did not show a noticeable difference at all NaCl concentrations except for 0.5 M NaCl and 65 ^{o}C, which produced remarkably high yield (about 49%) compared with that at 20 ^{o}C

6.2.3. Effect of pH cycling and ultrasound treatment on the extraction of hemp proteins

6.2.3.1. Extraction yield of hemp proteins under ultrasound combined with pH cycling

In this experiment, the acidic pH cycling (pH 9-2-9) and alkaline pH cycling (pH 9-12-9) at 65 ^{o}C combined with ultrasound treatment (80% amplitude, 15 min, pH 9, and 65 ^{o}C), were used to test the extractability of proteins as per the protocols described in section 3.2.3.4.3, Chapter 3. The results are presented in Figure 6.11. The effect of ultrasound and pH cycling treatment alone is also presented for comparison.

As can be seen in Figure 6.11 A, the ultrasound treatment alone showed a yield of about 37%, whereas the yield resulting from acidic pH cycling alone, was remarkably lower, at about 18%. The protein yield obtained by co-treatment of ultrasound and acidic pH cycling was about 17% higher than that

obtained by acidic pH cycling alone. However, the absolute yield was still much lower than that resulting from the ultrasound alone.

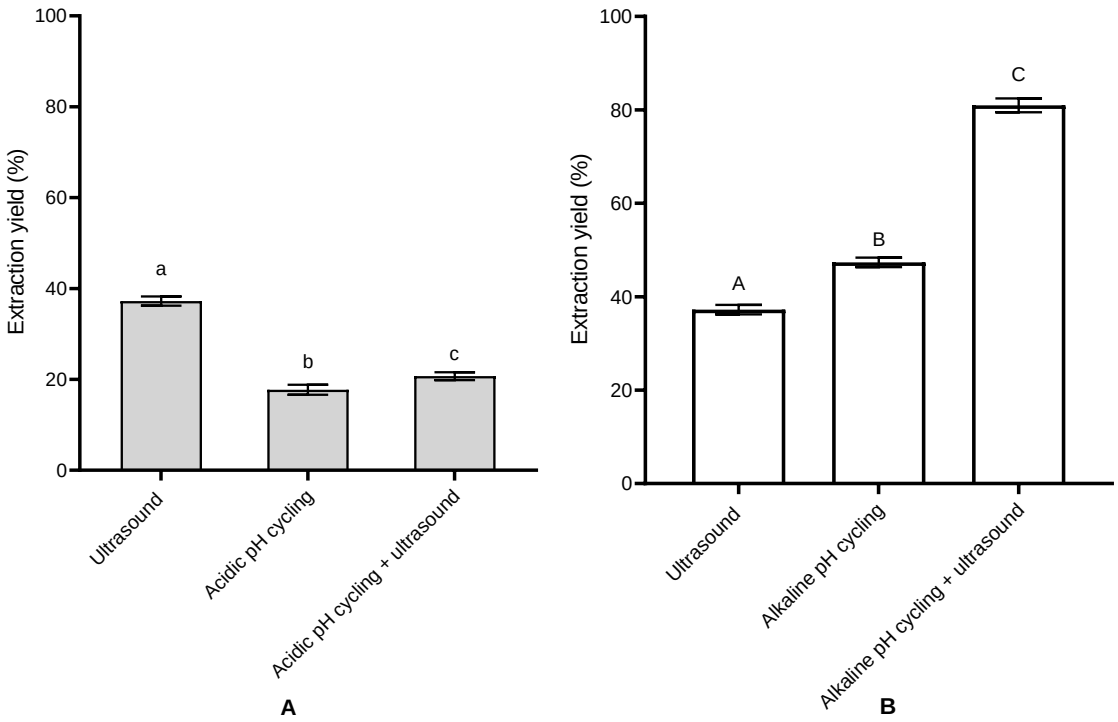

Figure 6.11: Effect of the combination treatment of ultrasound (80% amplitude, 15 min, pH 9) with acidic pH cycling (A) and alkaline pH cycling (B). Different letters indicate significant difference ($p < 0.05$) at each graph. Error bars represent the standard deviation of three replications.

In Figure 6.11 B, the alkaline pH cycling treatment significantly increased the yield of protein extraction, compared with the ultrasound treatment alone; absolute yields obtained were approximately 47% and 37%, respectively. The combination of alkaline pH cycling and ultrasound treatment dramatically improved the yield to about 81%. Between the two treatments (Figure 6.11 A and Figure 6.11 B), the co-treatment with alkaline pH cycling and ultrasound showed a remarkably higher yield (almost 4-fold) than the co-treatment with acidic pH cycling and ultrasound.

6.2.3.2. Composition of hemp protein in the extracts

The composition of proteins extracted using ultrasound under different conditions was investigated by SDS-PAGE (Figure 6.12). The composition of extracts from ultrasound treatment at pH 9 alone showed a protein profile similar to that obtained at pH 9 extraction without ultrasound, including four bands. Among which, band D appeared the most intense corresponding to the albumin fraction of

hemp proteins at molecular mass less than 18 kDa. The bands B and C represented the acidic and basic subunits at 33 and 18 - 20 kDa, respectively, of globulin 11s. The globulin 7s fraction with a molecular weight at 48 kDa also presented faintly.

The hemp proteins extracted by alkaline pH cycling singly and co-treated with ultrasound showed three distinct bands. The bands of two globulin 11s subunits, bands B and C, appeared more intense at the co-treatment sample than those in the pH cycling treatment only. The band of albumin fraction presented the same intensity at both pH cycling and the co-treatment. In these two treatments, the globulin 7s which appeared in the standard and ultrasound treatment singly was diminished.

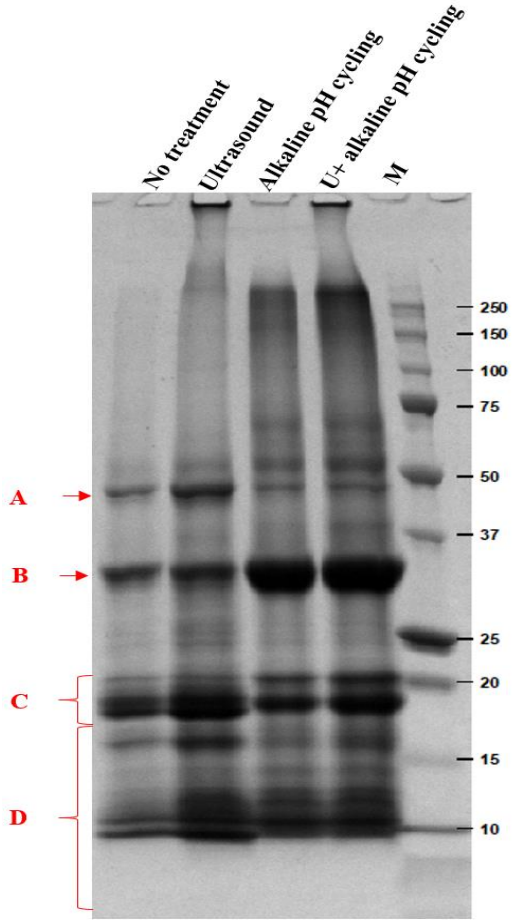

Figure 6.12: SDS-PAGE of soluble hemp proteins under different treatments. M, molecular mass marker in kDa. Labels above the lands indicate the used treatments. No treatment marked as a standard sample at 65 ^{0}C and pH 9. U + alkaline pH cycling, ultrasound co-treatment with alkaline pH cycling. Bands A-D indicate likely globulin albumin fractions of hemp proteins, see text for more details. The protein concentration for all samples in the SDS-PAGE loading buffer was adjusted to 1 mg/mL before loading.

6.2.3.3. Total phenolic content in the extracts

The treatments significantly affected ($p < 0.05$) the TPC of the extracts. The highest total phenolic content was observed when using ultrasound treatment alone (about 0.61 mg GAE/g of supernatant). Applying alkaline pH cycling resulted in a significantly lower protein yield, about 17% less than the ultrasound treatment alone. Interestingly, the co-treatment resulted in the lowest phenolic content, with only about 0.37 mg GAE/g of extract. Images of the supernatant presented in Figure 6.13 indicate that samples darken when phenolic content increases.

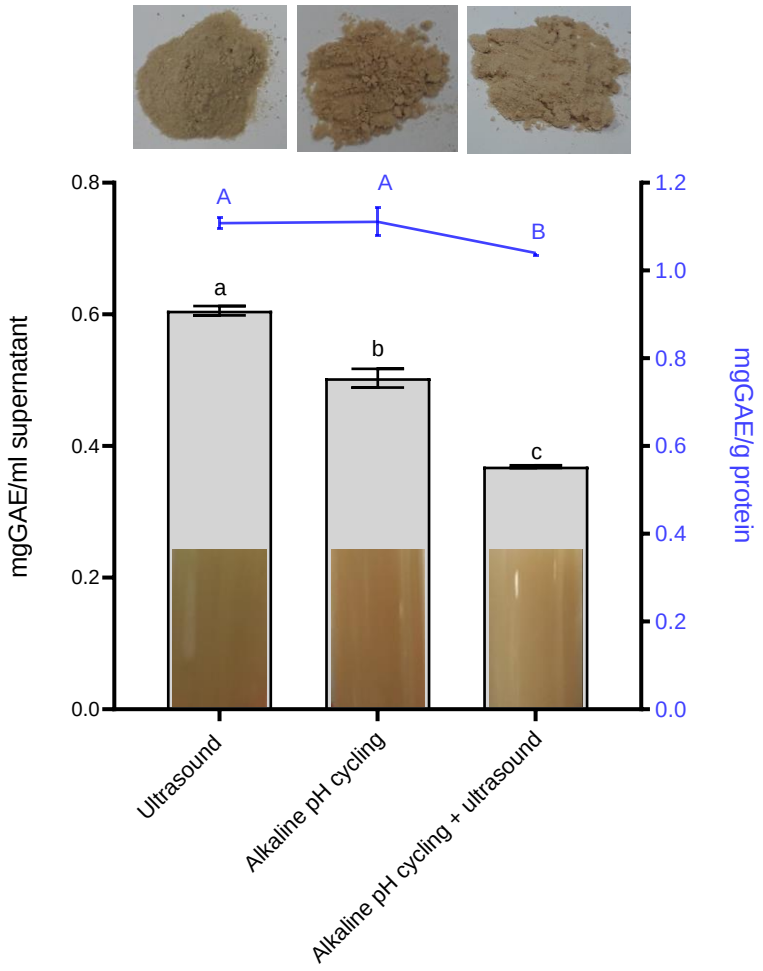

Figure 6.13: Effect of co-treatment of pH cycling and ultrasound on the total phenolic content. GAE, gallic acid equivalent. Histograms with the left ordinate. Curve with the right ordinate. Different letters indicate significant differences ($p < 0.05$). Error bars represent the standard deviation of two replications. The colour bars show the colour of the supernatant. The images capture the extracted protein powder after centrifugation and freeze-drying.

While expressing the TPC as mg GAE per gram of extracted protein, there were no significant differences ($p < 0.05$) between ultrasound and alkaline pH cycling treatments alone. An amount of approximately 1.1 mg GAE was co-extracted under those two treatments with one gram of hemp proteins. The combination of those two treatments resulted in a significant reduction (about 9%) in the phenolic content. Approximately 1 mg of GAE per gram of extracted proteins was recorded. The pictures presented the colour of extracted hemp protein powder obtained after centrifugation and freeze-drying. Specifically, the powder resulting from the co-treatment appeared with a lighter shade compared to alkaline pH cycling and ultrasound treatment singly. The observed colours seemed to be matched with the quantity of phenolic content recorded.

6.2.3.4. Available lysine content in the extracts

The available lysine content in samples co-treatment with ultrasound and alkaline pH cycling is shown in Figure 6.14. The ultrasound treatment alone at pH 9 has an available lysine content of about 37 mg/g of extracted hemp protein. Alkaline pH cycling treatment alone had a lower available lysine with 27 mg/g protein. The combination of ultrasound and alkaline pH cycling treatment had a lower available lysine of about 30 mg/g of extracted protein, as compared to ultrasound alone. But this was slightly more than pH cycling treatment alone.

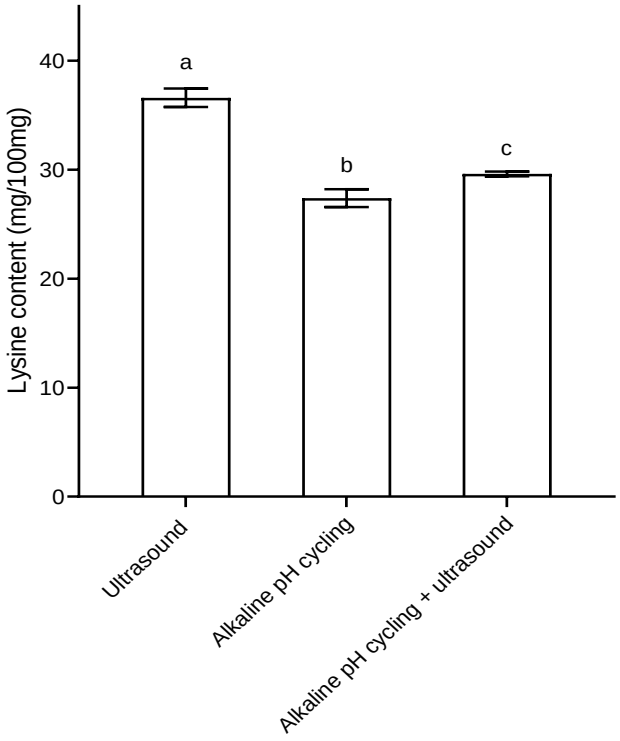

Figure 6.14: Available lysine content in extracted protein obtained under different treatments: ultrasound, alkaline pH cycling, and co-treatment with ultrasound and alkaline pH cycling. Different letters indicate significant difference ($p < 0.05$). Error bars represent the standard deviation of two replications.

6.2.3.5. Scanning Electron Microscopy of spent hemp seed meals

The SEM images of the spent HSMs recovered after alkaline pH cycling treatment alone and after its combination with ultrasound treatment are shown in Figure 6.15. The microstructure of the samples after alkaline pH cycling combined with ultrasound (Figure 6.15 B) presented differently from the microstructure of the alkaline pH cycling alone (Figure 6.15 A). The one with pH cycling alone had fine details of the surface structure were retained while this was absent in the one with ultrasound.

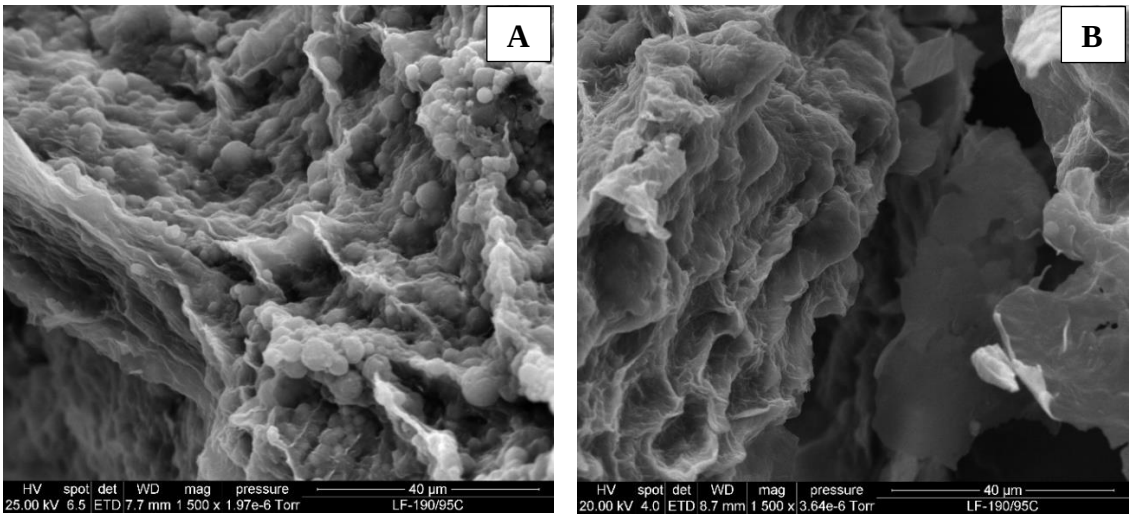

Figure 6.15: SEM images of spent HSMs of alkaline pH cycling alone (A) and alkaline pH cycling treatment combined with ultrasound (B).

6.3. Discussion

6.3.1. Effect of ultrasound on the extraction of hemp proteins

The results suggest that the ultrasound treatment had a positive impact on improving the hemp protein extraction yield. Some previously published studies have proved the effectiveness of ultrasound to improve the extraction of proteins from different plant materials, such as defatted wheat germ (Xue et al., 2009), sunflower meals (Dabbour et al., 2018), defatted rice bran (Ly et al., 2018), rapeseed meal (Yagoub et al., 2017), and rice dreg flour (Li et al., 2017).

The effectiveness of ultrasound in protein extraction could be explained based on its impact on the protein macromolecules and the plant-based matrix. According to Al-Hilphy et al. (2020), via agitating the medium of extraction, ultrasound power advances the contact protein and the medium of extraction leading to the increase of extraction yield. The acoustic cavitation generated by ultrasonic waves can disrupt plant cells and increase the surface porosity of the matrix facilitating the release of proteins (Greenly et al., 2015; Li et al., 2017). The effect of ultrasound treatment on the plant matrix has been previous reported by Xiea et al. (2015). The authors showed that the microstructure of plant material after ultrasound treatment had more damage and less thickness than that without ultrasound treatment. The SEM images in Figure 6.7 and Figure 6.8 attempted to characterize these changes in microstructure, however, these differences were not distinctly evident. This may be due to the artifacts introduced by the sample preparation protocol for the environmental

SEM used for imaging. A more sensitive and non-destructive technique to investigate the effect of ultrasound on the matrix must be used in future to relate the structure vs yield relationships.

Increasing the amplitude of ultrasound means that there is more energy applied to the system (Hashtjin et al., 2015), and more cells disrupted due to cavitation forces (Natarajan et al., 2014). Therefore, a greater amplitude would facilitate the release of protein out of the food matrix. This was consistent with the results of the extraction yield (Figure 6.1 at 20 °C and Figure 6.2 at 65 ^{o}C) where a greater amplitude, i.e., higher intensity used, led to a greater yield of protein extraction and the differences in microstructure as observed in Figure 6.8. The role of temperature also improved the yield of extraction under ultrasound condition as discussed previously in Chapter 5, in which, ultrasound at 65 ^{o}C resulted in a higher yield than that obtained by the 20 ^{o}C (Figure 6.2).

The application of ultrasound has been known to cause a breakdown of protein aggregates (Li et al., 2017). Ultrasound might also cleave peptide bonds and reduce non-covalent interactions (Hu et al., 2013; Huang et al., 2017). As a result, this makes the protein chains smaller and easier to diffuse within the plant-based matrix. However, the SDS-PAGE images of the gels showed no evidence of hydrolysis of proteins.

In addition, the increased porosity of the matrix-induced by ultrasounds allows water molecules to efficiently penetrate inside the material, therefore increasing the mass transfer (Li et al., 2017). Prolonged exposure to ultrasound treatment also decreases the particle size of the material (Hashtjin et al., 2015), which causes largely disruption of cell-matrix (Natarajan et al., 2014) and facilitates the extraction of proteins from the material. Likely, the higher yield of proteins upon ultrasound treatments seen in this chapter resulted from one or more of the above mechanisms.

Longer duration of ultrasound treatments resulted in greater protein extraction yields, but the rate of increase in the yield with additional treatment time was not very large (Figure 6.3). A slight increase in yield has been reported with longer ultrasound treatments, attributed to the decrease in the particle size of the material (Hashtjin et al., 2015), caused by the disruption of cell-matrix (Natarajan et al., 2014). This in turn facilitated the extraction of proteins from the material. In this study, at the beginning of the ultrasound when the equilibrium of protein content in and out plant material has not been established, the extraction yield increase with time. However, once the equilibrium was attained, longer ultrasound treatment times did not increase the yield significantly; and energy cost considerations become prohibitive (Dong et al., 2010; Shirsath et al., 2017).

However, a long ultrasound treatment might negatively affect the extracted protein quality (Dong et al., 2010). Therefore, deciding the ultrasound duration is very important to get the balance between the extraction yields and to maintain the quality of protein under ultrasound treatment. Results from this study show that 600-900 seconds appeared to be an optimal ultrasound duration for hemp protein extraction with ultrasound alone.

From the composition of hemp proteins in the extracts (Figure 6.4), the ultrasound treatment did not change the protein compositions of the hemp proteins. Nevertheless, it is interesting to note that all samples with ultrasound treatment had polypeptides that had molecular weights >250 kDa, which were present in the stacking gels of the respective lanes. All samples were reduced using β-mercaptoethanol in the SDS-PAGE loading buffer, hence it is likely that these high molecular weight aggregates were not disulphide-linked. Since the major peptide bands in the extracts of ultrasound-treated samples were largely similar to those without ultrasound treatment, it is unlikely that these were undisrupted aggregates of major polypeptides detected in the untreated samples. It is likely that these high molecular weight polypeptides were additional proteins that may have been extracted due to ultrasound. Further characterization of the composition of polypeptides in extracts from ultrasound-treated samples is necessary to ascertain this hypothesis.

The results of total phenolic compound content revealed that ultrasound-treated samples exhibited a significant effect in the extraction of phenolic compounds compared with untreated samples. The effectiveness of ultrasound in the extraction of phenolic compounds from plant materials has also been reported by other authors (Bhat et al., 2011a, 2011b). It is possible that the ultrasound-assisted disruption of the cell walls facilitated the release of phenolic compounds into the aqueous environment along with the proteins. The colour intensity of the powder as observed in Figure 6.5 also seemed to be in line with the values of total phenolic content; the higher the phenolic content of the samples the darker the colour of the protein powder.

The results of available lysine indicate that ultrasound-treated samples had a slightly higher available lysine content as compared to samples without ultrasound treatment. One possible reason is that ultrasound helped to unfold the protein. However, the result of the current work was not consistent with the findings reported by other authors (Malik et al., 2017). The authors proposed that an ultrasound treatment might decrease the available lysine content by making them more prone to protein-protein interactions with other protein molecules. As a result, lysine may not be available for

the test protocol. The differences might come from variations in ultrasound treatment conditions and the materials used in the two cases.

6.3.2. Effect of combining ultrasound and adding salts on the extraction of hemp proteins

The results of the effect of co-treatment (addition of salts and ultrasound) on the yield of protein extraction (Figure 6.10) indicated that the combination of these two methods exhibited a synergistic effect, improving the yield of hemp protein extraction with certain salt concentrations. The mechanism behind this synergistic effect has been reported previously by Kim et al. (2017). The use of ultrasound which induced the collapse of the protein structure and the presence of salts helps weaken structure by reducing intra-molecular interactions. The presence of salt also enhances ultrasonic cavitation, which might be one of the reasons for the positive effect of the combined treatment (Zayas, 1986). Those effects contribute positively to the yield of protein extraction. Besides that, as presented previously in Chapter 5 (Section 5.3), the investigated salt concentrations ($\leq$ 0.8 M) was in the range of salting-in effect. Therefore, during the co-treatment of salt and ultrasound process, the salting-in effect was still happening, and it contributed to improve the solubilization of proteins, therefore increasing the yield of extraction.

However, from the results shown in Figure 6.10, it has been noted that NaCl treatment combined with ultrasound showed the synergistic effect on the yield of protein extraction only at the low salt concentration, $\leq$ 0.25 M. Further increase in salt concentration did not result in any apparent differences or even rendered a decrease in yield, compared with the treatment without ultrasound. Therefore, together with the mentioned positive effect, there were negative effects on the yield simultaneously during the co-treatment process. At low salt concentration, the positive effect was prominently increasing extraction yield. However, at higher salt concentration, the negative effect seemed to be greater than the positive. Therefore, no obvious difference or a decrease in yield was observed.

A combination of salting with ultrasound, showed a higher yield by divalent salt in comparison to the monovalent salt at the low salt concentration (0.25 M) as shown in Figure 6.9. In contrast, at higher concentrations ($\geq$ 0.5 M), the reverse was the case. This is also in contrast to the findings of Chapter 5 (Figure 5.15), where the divalent salt resulted in a significantly higher yield than that of monovalent salt at all investigated concentrations. It is therefore possible that the ultrasound treatment impacted the yield negatively and that this impact of the divalent cations was more significant than that of their

monovalent counterparts. It is likely that ultrasound treatment promoted the divalent cation induced aggregation of protein molecules.

The additional ultrasound treatment led to the unfolding of the protein chains (Ren et al., 2017), which exposed most of their functional groups to water molecules. The possibility of cross-links between those groups and the cations provided by salts cannot be excluded. At low salt concentration, the number of cations might not be enough for promoting the cross-linking, thus improving the yield of extraction. However, at high concentration, the number of cations may be sufficient to create cross-linking, resulting in a decrease in the yield of protein extraction. At a certain salt concentration, the cation concentration of divalent salt was double that of the monovalent salt, therefore, the negative effect was greater with the divalent than with monovalent salt at the same concentration. In addition, the protein chains could have been almost fully extracted in the presence of high concentrations of salts; thereby showing no effect by the additional ultrasound (Tang et al., 2020).

The antagonistic effect of ultrasound and the use of high salt concentration observed in this study was consistent with the findings previously reported by (Zayas, 1986) for chymosin and Tang et al. (2020) for actomyosin. In contrast, another study reported a synergistic effect on protein extractability when applying ultrasound at high (1.0 M) NaCl concentration (Kim et al., 2017) for porcine myocardium. These contradictions might be attributed to the different materials and protocols of extractions carried out in these previous studies. Ultrasound treatment at 65^{o}C improved the yield of hemp protein extraction irrespective of the addition of salt. Heating at 65^{o}C resulted in a higher yield than that of 20 ^{o}C however at only the concentration of 0.5 M. As a result, the improvement was not as clear as observed in ultrasound treatment alone. This difference might be attributed to the lack of temperature control during the ultrasound. The ultrasound is expected to generate heat (Wen et al., 2019) which increased the temperature of the sample during operating (data was not shown in this study).

6.3.3. Effect of co-treatment of pH cycling and ultrasound on the extraction of hemp proteins

The combination of pH cycling and ultrasound has been employed before for different plant materials, such as pea protein (Jiang et al., 2017) and soy protein (Yildiz et al., 2017). So far, this combination of treatments has not been explored for hemp proteins. From the results of this study, alkaline pH cycling had a higher protein extraction efficiency as compared to acidic pH cycling when combined with ultrasound treatment. Further, the effect of both alkaline pH cycling and ultrasound was accumulative. The results obtained in this study were consistent with some previous work published by Jiang et al. (2017), which reported a better extraction using ultrasounds treatment and alkaline pH

cycling for soy proteins than that observed with ultrasound and acidic pH cycling. It was proposed that the acidic pH cycling occurring at the pH close to the p*I* of hemp proteins (around pH 5) did not cause much change in the protein structure. As a result, the ultrasound treatment added later did not show much difference in the outcome. This confirmed Jiang et al. (2017)'s study working on pea protein.

In contrast, for alkaline pH cycling, an extreme alkaline pH (at pH 12) posed more extensive structural changes in the protein extensive structural changes due to strong intra-molecular repulsive forces. Subsequently, the ultrasound treatment following this provided the shearing force and wave shocking, resulting in the further alteration of the protein structure. As a result, the additional ultrasound treatment facilitated the release of protein in the water, increasing the yield of protein extraction (Jiang et al., 2017). Yildiz et al. (2017) revealed that an ultrasound treatment after alkaline pH cycling also contributed to the breakdown of the non-covalent and covalent bonds and creates smaller protein particles. This can improve the protein solubility and subsequently, the yield of protein extraction. The addition ultrasound after alkaline pH cycling contributed to disrupting the material surface (Figure 6.15), thus facilitating the release of protein from the matrix.

From the SDS-PAGE results, the albumin fraction was not affected by any treatment as proved by the same intensity observed in all bands. However, the intense bands of globulin fraction observed for pH cycling and the co-treatment (ultrasound plus alkaline pH cycling) revealed the effect of those treatments on the protein fractions. In addition, in Figure 6.12, intense bands were observed at the top of the stacking gel of the samples of ultrasound treatment alone and ultrasound combined with pH cycling. This phenomenon might be attributed to the formation of high molecular weight component which could not get in the wells.

According to Xu et al. (2000), the presence of the phenolic compound in the extract darkened the extracts. A similar observation was recorded in the current study, the lower content of phenolic resulted in the lighter shade of extracts as presented in Figure 6.13. This relationship has also been previously presented by Potin et al. (2019). From the results of this study, the ultrasound treatment alone was more effective at extracting the phenolic compounds than the alkaline pH cycling treatment alone. However, their combination significantly reduced the total phenolic content. It is likely that ultrasound extracted the phenolic compounds due to one or more of the mechanisms discussed for the extraction of proteins above (Bhat et al., 2011b).

The dependence of the colour on the pH observed in this study has previously reported by Potin et al. (2019). In which, the shade of the extract increased along with the increase of the pH. This trend was also observed in this study, as presented in Appendix 1, where the colour changed to a darker shade when the pH was adjusted from pH 9 to 12 and returned to a lighter shade after adjusting back to pH 9. The authors proposed that the colour caused by the phenolic compound was reversible by changing the pH. Interestingly, the phenolic content of the extracts resulting from pH cycling combining with ultrasound was lower than that obtained by pH cycling and ultrasound alone.

From the results of available lysine, the employment of ultrasound after alkaline pH cycling improved the quality of extracted hemp protein through the increase of available lysine content. As discussed in section 6.3.1, the mechanical effect of ultrasound might have contributed to exposing more amino group of lysine. Therefore, this effect can be used to explain the results observed in the current section, where the co-treatment resulted in higher available lysine content. The alkaline pH cycling reduced the available lysine content in the extraction of hemp protein, as discussed in section 5.3.2, due to the forming of protein crosslink and the adverse reaction. Therefore, resulting in the low availability of lysine content as observed in this section.

6.4. Conclusion

Overall, ultrasound showed as an effective method to extract hemp proteins out of the hemp-based materials. The conditions of amplitude 80%, at 65 ^{o}C, pH 9 and ultrasonic duration in 15 minutes appeared to be optimal ultrasound conditions. The combination of ultrasound under the optimal condition with salts exhibited synergetic effects at a low salt concentration (0.25 M). Especially, the effect of ultrasound with alkaline pH cycling (pH 9-12-9) showed an effective method to extract hemp protein.

Chapter 7

7. General discussion and avenues for future work

7.1. Introduction

The application of hemp protein in the food industry has not been fully researched and exploited. One of the factors which limit the use of this good quality protein in food formulation is its low extractability due to poor solubility in water. The improvement of the yield of hemp protein extraction is necessary to popularise this source of protein in the food industry. However, the studies that have been done on hemp protein extractability is not sufficient and still leave some gaps for further research. This work aims to find the optimal conditions of the extraction of hemp protein, and explore novel method technique, ultrasound-assisted extraction itself and combination with other treatment for the extraction of hemp proteins from hemp seeds meals.

This study explored different strategies to improve hemp protein extractability under different conditions. This was achieved using two approaches and optimal conditions for the maximum extraction of hemp proteins in an aqueous medium were determined. In the first approach, the different treatments were used singly and their effect on the yield of protein extraction was investigated. The different treatments included heating, change in pH, ultrasound, adding salts and pH cycling. In the second approach, the combination of effective treatments together was explored. These included a combination of ultrasound and addition of salts, and ultrasound and pH cycling which were used to investigate their effect on the extraction yield. Figure 7.1 presents an overview of the investigated experiments in this study.

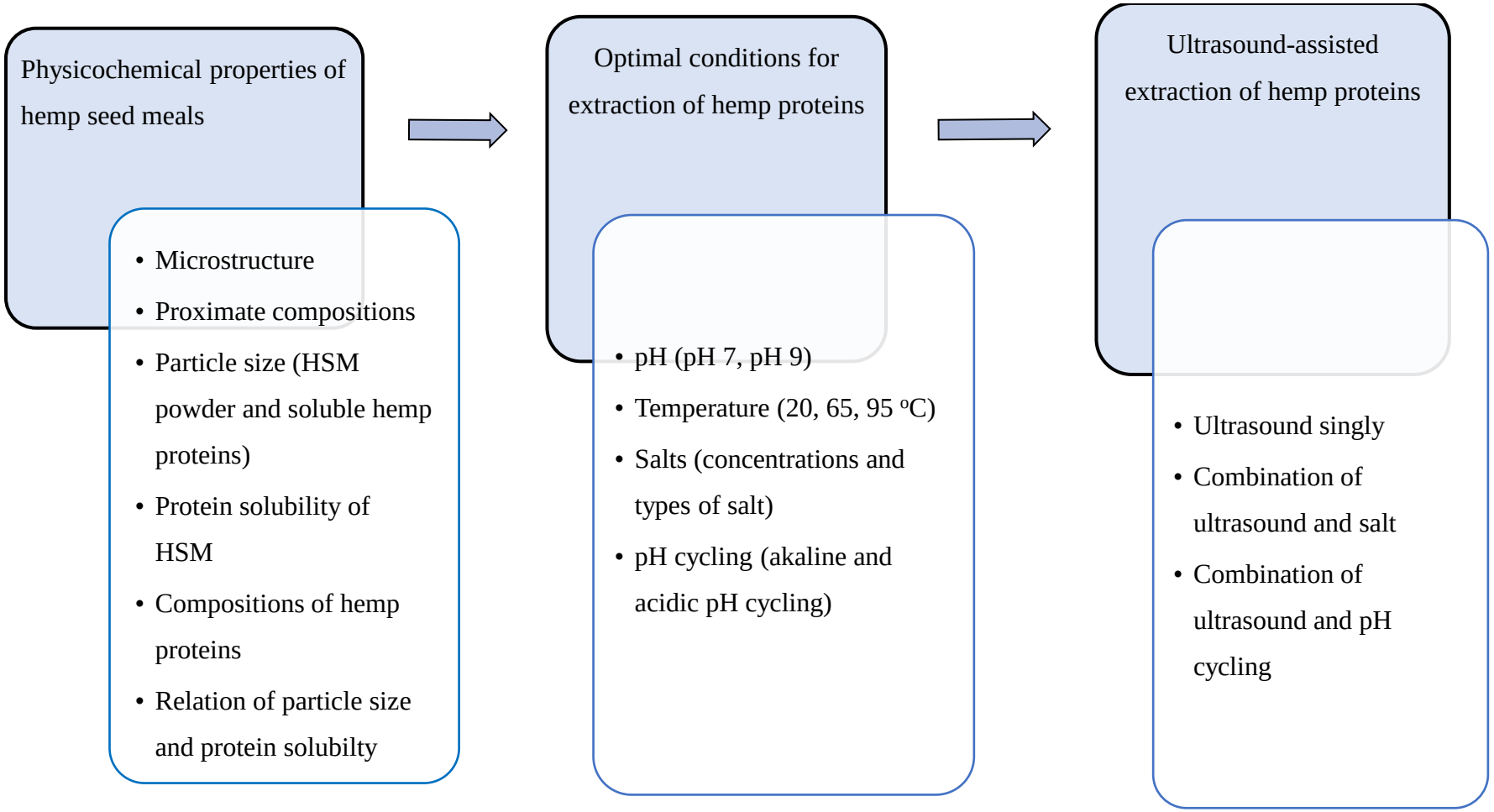

Figure 7.1: Overview of the investigated experiments in this study

7.2. Overall discussion

7.2.1. Hemp seed meals as a suitable source of hemp protein

Hemp seed meals are high content of protein (48 – 65%). They also contain a significant amount of fibre (6 – 23%) and fat (5 – 20%). The use of hem seed meal as a functional protein ingredient is limited because of the presences of non-protein components, i.e., fat and fibre. The extraction of hemp protein out of hemp seed meals is necessary to broaden its application.

Hemp proteins are stored inside rounded-shaped protein bodies (Schultz et al., 2020), which envelop crystalloids where the enormous crystalline structure of edestin are contained (Chuang et al., 2019). Due to the compact structure of globulins, hemp proteins exhibit low solubility and subsequently low extractability in the aqueous medium at neutral pH, of 65% protein contained in hemp seed meal only about 13% is extractable at pH 7 in water. This is challenging for hemp seed protein extraction and therefore, improving the protein extraction yield is required.

Chapter 4 compared the physicochemical characteristics and solubility of commercial hemp seed meals. For the first time, this study explored the relationship between particle size and the extractability of hemp proteins. The reduction of particle size improved the solubility of hemp protein as was observed in Chapter 4. An increase of 22% in hemp protein solubility was observed when the range of particle size decreased from 90 µm - 600 µm to 75 µm - 180 µm.

7.2.2. Optimal pH and temperature for extraction of hemp protein

Chapter 5 investigated the optimal conditions for the extraction of hemp proteins. This chapter investigated the effect of different extraction conditions of pH (pH 7 and 9), temperatures (20˚, 65˚ and 95 ^{o}C) on the yield of hemp proteins systematically. Heating at 65 ^{o}C at pH 9 was found to be the optimal condition for hemp protein extraction.

Controlled heating (65 ^{o}C) and high pH extraction (pH 9) may disintegrate the plant-seed matrix and contribute to the releasing of more proteins out in the aqueous phase. Besides, strong alkaline pH may inhibit the interaction between the extracted protein molecules (Guzman et al., 2020). Further heating at 95 ^{o}C, did not increase extraction yield, this may be explained by the denaturation of hemp proteins at high temperatures which may have caused aggregation or precipitation, thus reducing the yield of extraction. This finding is in agreement with a previous study have done by Raikos et al. (2015), who reported that during the heating at pH 6.8, the temperature of heat treatment should not exceed 80 ^{o}C to preserve the stability and solubility of hemp proteins. Nevertheless, the conditions of the

experiments used in the previous study were different to this study (Heating temperature, pH, aqueous conditions) and heat stability of globular proteins is shown to vary depending on experimental protocols (Nielsen et al., 1996), and aqueous medium properties, e.g., pH which strongly affects the rate of aggregation of proteins (Chi et al., 2003; Jacob et al., 2006). There is no data available looking at the denaturation and aggregation profile of hemp protein as a function of pH, therefore; a hypothesis that hemp protein at pH 9 is more stable against the thermal denaturation and aggregation than at pH 7 requires further investigation.

To further improve the yield of extraction, the impact of pH cycling under the heating condition was employed. As compared to pH 9 alone, alkaline pH cycling (pH 9-12-9) further enhanced the protein extractability. The pH cycling has been previously studied by Wang et al. (2018b) to improve the functionalities of hemp protein. Exposing protein in extreme alkaline pH (pH 12) condition induces unfolding of the proteins and the reduction of pH after that aids in protein refolding (Jiang et al., 2009; Wang et al., 2018b). By using a conventional pH cycling 7-12-7, (Wang et al., 2018b) presented a significant increase in the solubility of the hemp protein isolate. As seen in Chapter 5 the yield resulted from the pH cycle 9-12-7 was significantly lower than the cycle of 9-12-9. From the results of this study, the pH cycling ending at pH 9 proved more effective than pH 7 in the extraction of hemp protein out of the hemp seed meal. It is believed that the end pH of pH cycling regulates the protein-protein interactions caused by extreme alkaline pH. It is possible that the higher electrostatic charge on proteins at pH 9 prevented aggregation and loss of solubility. Therefore, the end pH after pH cycling seems to be a critical parameter to design the pH cycling process in future for plant protein extraction.

7.2.3. Di-valent salts were more effective in improving the extraction of hemp proteins

Chapter 5 systematically explored the effect of salt concentrations (0.25, 0.5 and 0.8 M) and for the first time, the different types of salt (di-valent and mono-valent salts) on the yield of hemp protein extraction. The results showed that salts improved the extractability and were most effective at 0.8 M concentration. This study is the first study that investigated the effect of different types of salts on the yield of hemp protein extraction. Interestingly, at a given concentration, the divalent salts ($CaCl_2$ and $MgCl_2$) produced a greater increase in the yield as compared to monovalent salts (NaCl, KCl) which have conventionally been used for protein extractions.

The presence of cation by adding salt contributes to breaking salt bridges in protein (Guzman et al., 2020), and thereby interrupting the protein-protein interactions resulting in the improvement of protein extraction. The finding in Chapter 5 of this study is in agreement with a previous study (Potin

et al., 2019), where an increase in salt concentration resulted in an improvement in the yield of hemp protein. The extraction yield resulted from the divalent salt is promising or might make the divalent salt being used more commonly, however, new challenges arise with this approach. The occurrence of the cations in the extracted protein might cause a negative effect on the further application of extracted protein, e.g., resulting in an unstable emulsion against coalescence. Therefore, the stability of hemp protein and the functional properties of hemp protein extracted by divalent salt are interesting areas for future research.

7.2.4. Ultrasound-assisted extraction enhanced extraction of hemp proteins

In Chapter 6, for the first time, the application of ultrasound was investigated for hemp protein extraction. The optimal condition was found to be a combination of 80% amplitude, pH 9 and 65 ^{o}C for 15 minutes duration.

The application of ultrasound alone under the above conditions resulted in an approximately 3-fold increase in yield of extraction compared to the neutral pH at 20 ^{o}C as presented in Chapter 6. The effectiveness of this method in extraction plant proteins has been confirmed by previous authors (Dabbour et al., 2018; Dong et al., 2010; Li et al., 2017; Malik et al., 2017). However, the yield resulted from ultrasound was still lower than other investigated extraction methods in this study (adding salts at 0.8 M concentration, alkaline pH cycling). In this work, the uncontrolled temperature during ultrasound operation might be one of the reasons which reducing the sonochemical effects caused by cavitation bubbles collapse (Chemat et al., 2017). In further studies on ultrasound-assisted hemp protein extraction, therefore, a water bath can be used to control the temperature during ultrasound running to prevent the temperature from rising.

The combination of ultrasound with salts showed a synergistic effect on the yield only at low salt concentration; meanwhile, the combination with alkaline pH cycling improved the yield remarkably.

Figure 6.4 and Figure 6.12 show the bands in stacking gel of the samples under ultrasound singly or in combination with other treatments. This phenomenon reveals a hypothesis that the effect of ultrasound resulted in soluble aggregation with high molecule weight (> 250 kDa) that cannot get in the gels. Further investigation relating to the aggregation by ultrasonic effect is necessary.

Generally, the novel method ultrasound is a promising method in hemp protein extraction. It clearly shows an advantage in term of yield extraction, however, further work needs to be done before upscale and integrate this method in the industry.

7.2.5. Extraction methods that promoted co-extraction of phenolic compounds

In agreement with previous work (Hadnađev et al., 2018), the finding of this study revealed the co-extraction of hemp protein and phenolic compounds. It appeared that the conditions which produced a high yield of protein also resulted in high content of phenolic compound in the extracted medium. Higher pH and higher temperature increased the extraction yield of both *Rosa rubiginosa* seed protein and the phenolic compound was reported by a previous study done by (Mourea et al., 2001). The application of ultrasound resulted in an increase in coextracted phenolic compounds.

It is likely that in the aqueous extraction medium and at neutral pH, and the proteins interacted with polyphenols. Although this interaction is strong at low pH; however, can be broken by heating (Ozdal et al., 2013). According to the authors, the interaction might be either reversible or non-reversible depending on the kinds of protein and phenolic compounds. The interaction of phenolic compound and protein may induce the crosslinking of proteins, change in the secondary and tertiary structure of protein resulting in the change of hydrophobic/hydrophilic balance and impact the protein functionality, i.e., foaming capacity, emulsifying capacity, solubility (Ozdal et al., 2013). Although, such extensive crosslinking of hemp proteins was not observed from the SDS-PAGE images. It is possible that the crosslinked proteins were insoluble. Nevertheless, a mixture of PVPP (1.5 % w/w) and sodium metabisulfite (0.1 %) may prevent the protein-phenolic compounds crosslinking during extraction (Chuang et al., 2019). Therefore, further work needs to be done on the availability of protein after extraction conditions with and without adding the above mixture during extraction.

7.2.6. Effect of extraction methods on protein quality

Heating under alkaline pH conditions is known to cause negative impacts on the protein such as, an undesirable compound cross-linked amino acids by the reaction of an ε-amino group of lysine with dehydroalanine residue resulting from the β-elimination of cysteine, serine, which results in lysinoalanine (LAL) (Friedman, 1982; Hou et al., 2017; Luo et al., 2014; Struthers, 1981). Similarly, Hou et al. (2017) reported the increase of LAL when extraction of rice proteins at high pH was combined with temperature. This resulted in LAL having some health concerns. During food processing, the racemization of essential amino acids and loss of amino acids (lysine, cysteine, and threonine) reduce the digestibility and bioavailability of amino acids (Luo et al., 2014; Moure et al., 2006). Another concern is the ability of LAL in the inactivation of metalloenzymes (Luo et al., 2014). Although the formation of LAL was not investigated in this study, the effect of different treatments on the availability of lysine was measured.

The loss of available lysine has been widely used as an indicator of the nutritional loss of processed food (Pereyragonzales, 2003). The loss of this value is mainly due to the consequence of the Maillard reaction under process (Pereyragonzales, 2003; Rutherfurd et al., 2012). In the current study, the investigated methods affected this value. The optimal conditions of pH and temperature (65 ºC, pH 9) improved available lysine significantly compared to the untreated condition, room temperature and neutral pH. The alkaline pH cycling alone or combined with ultrasound reduced the availability of lysine. Interestingly, extraction by ultrasound alone increased the availability of lysine. On the contrary, Malik et al. (2017) reported a decrease of available lysine content in sunflower protein isolates under high-intensity ultrasound. The different results may have come from the difference in material and experiment conditions in these two studies.

The key question that needs to be investigated in detail is whether there is any impact of extraction method condition on the functionality of the hemp protein. Although it was included in the initial scope of this study, the study could not be implemented due to time constraints.

However, it may be possible to predict the impact of different treatments used in this study on the functionalities of hemp protein-based on previous studies on hemp proteins. Hadnađev et al. (2018) presented a higher fat absorption of hemp seed protein extracted by alkaline pH compared to salt extraction. The alkaline pH shift during heating at (50-60 °C) improved the solubility and surface hydrophobicity and emulsifying properties of hemp protein (Wang et al., 2018b). Previous authors reported these properties for different oilseed proteins. Krause et al. (2002) reported that the water retention of flaxseed protein resulted from salt extraction was lower than that resulted from alkaline extraction. Alkaline pH cycling was also found to be improving the gel properties of peanut protein (Wang et al., 2020).

The ultrasound treatment caused the partial unfolding and a decrease in intermolecular interaction hence increased the solubility of soy protein (Hu et al., 2013). Malik et al. (2017) also reported the solubility, emulsifying capacity, emulsion stability, foaming capacity, foam stability and oil binding capacity of sunflower protein were improved under high-intensity ultrasound treatment. However, the binding capacity was reduced. The combination of ultrasound and pH shift improved emulsifying properties of soy protein as reported by Yildiz et al. (2017). Comparable to hemp seed proteins, those oilseed proteins also contain globular proteins, however, further investigation for hemp proteins is required.

7.2.7. Concluding remarks

Overall, this study has explored some new extraction conditions aiming to improve the extraction of hemp proteins.

- The microstructure of the spent hemp meal protein was studied to understand the effect of treatment on the plant-based matrix that impact the extract of protein out of the plant material.
- The effect of the alkaline pH under the heating condition on the yield of hemp proteins was studied.
- Divalent salts were studied in the extraction of hemp proteins.
- A novel method for protein extractions: use of ultrasound was used for the extraction of hemp proteins. The ultrasound treatment was also applied in combination with other treatments such as adding salt and pH cycling.
- The pH cycling process was carried out with the end pH value of pH 9 instead of pH 7, which has been studied conventionally.

From the summarised results of the hemp protein extraction yield by different methods, as presented in Figure 7.2, there are three treatments that give a similarly high yield of extraction, from 80 – 81%, including,

1. The use of NaCl 0.8 M at 65^{o}C and pH 9.
2. The combination of alkaline pH cycling (pH 9-12-9) and ultrasound (80% amplitude, 15 min) at 65^{o}C.
3. The combination of NaCl 0.8 M and ultrasound (80% amplitude, 15 min) at 65 ^{o}C.

No.	Temperature		pH		Alkaline pH cycling	Salt 0.8 M	Ultrasound 80% amplitude	Yield (%)
	20°C	65°C	7	9				
1	✓		✓					13.0
2	✓			✓				19.8
3		✓	✓					20.0
4		✓		✓				30.8
5	✓			✓	✓			26.5
6	✓		✓			✓ *NaCl*		57.4
7	✓		✓			✓ *KCl*		38.4
8	✓		✓			✓ $MgCl_2$		77.8
9	✓		✓			✓ $CaCl_2$		75.9
10	✓		✓				✓	23.8
11		✓		✓	✓			47.4
12		✓		✓		✓ *NaCl*		**81.3**
13		✓		✓			✓	37.2
14		✓		✓	✓		✓	**81.0**
15		✓		✓		✓ *NaCl*	✓	**80.4**

Figure 7.2: Summary and comparison of the effectiveness of different methods used in the extraction of hemp protein in this study

Based on the results from this thesis a pilot-scale process for the extraction of hemp proteins can be predicted as below (Figure 7.3).

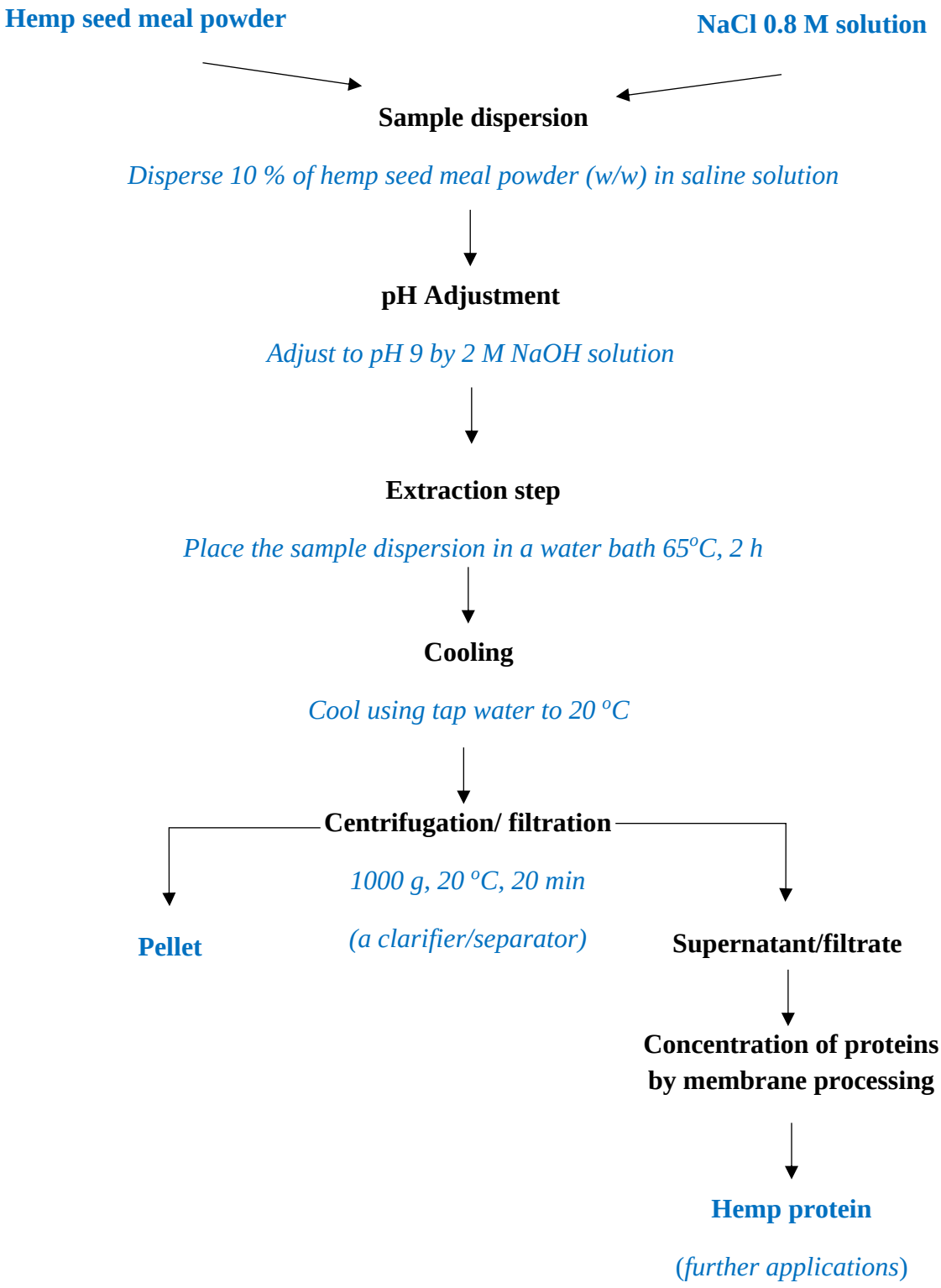

Figure 7.3: Process suggested for hemp protein extraction

7.3. Application

The findings from this study provide valuable fundamental knowledge for the scientific community and food industry. Some of them are summarised below,

- The exploration of different extraction methods allows constructing a roadmap of the most suitable processes and conditions that can be used to improve the extraction of hemp proteins from hemp seed meals.
- The extraction methods of hemp proteins studied in this work can be applied to other plant protein sources.
- The methods found to be favourable for extracting phenolic compounds from hemp seed meals could be used as basic knowledge to develop extraction protocols of phenolic compounds from hemp or other plant-based materials.
- The extraction of hemp proteins at a commercial scale for different food applications could be built up based on the insights provided in this study.
- Ultrasound-assisted extraction combined with a low concentration of salts in the extraction media represents a novel method to improve the extraction of hemp protein that could be applied in reduced-sodium products. Instead of using NaCl to improve the protein extractability, the combination with ultrasound might produce the same extraction yield but at a lower NaCl concentration.

7.4. Avenues for Future Work

Developing suitable methods for extracting plant proteins from plant-based materials is critically important for the food industry and scientific community. This study provided a systematic investigation showing the impact of different extraction methods on the yield of hemp protein extraction, and which conditions/processes can lead to greater amounts of soluble proteins from hemp seed meals. There are some limitations in the current study that have opened up opportunities for future research and have been summarised below.

- The extracted mass contained not only protein but other solubilised components as well, therefore the purifying steps to separate protein from the others need to be done. It is also not known whether the occurrence of contaminated components interacts with extracted hemp protein in the extraction.
- The functionalities of extracted hemp protein affected by extraction methods need to be studied further to choose a suitable method for a specific application.

- Some extraction conditions can solubilise the lignin component of the cell wall. Lignin solubility depends on conditions, such as pH and temperature or ionic strength (Evstigneev, 2011; Evstigneyev et al., 2018). For future studies, it may be necessary to investigate whether lignin is present in the extraction media and/or if it affects the protein functionality.
- The interpretation of SEM images in this study was limited. Further investigation relating to the effects of the extraction method on the microstructure of hemp seed meal is necessary.
- The stability of protein after extraction needs to be taken into account as well.

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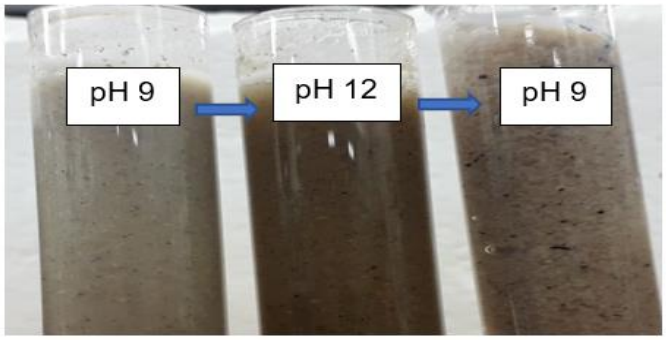
Appendix



Appendix 1. *The changing colour of the extraction suspension of hemp protein accordingly with the change of pH during the alkaline pH cycling process*

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