STRUCTURAL AND FUNCTIONAL PROPERTIES OF HEMP SEED STORAGE PROTEINS

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ABSTRACT

This study aimed to determine the structural and functional properties of hemp seed storage protein fractions (2S, 7S, and 11S) in comparison to hemp seed protein isolate (HPI). The protein content of 11S, 7S, 2S, and HPI were 91%, 90%, 83%, and 90% respectively. The 11S fraction was the predominant protein in hemp seed and accounted for 72% of the total seed protein. In vitro protein digestibility of the hemp seed proteins ranged from 72.54 to 88.28%. Hemp seed proteins were rich in glutamic acid and sulfur-containing amino acids while limiting in tryptophan and lysine. The SDS-PAGE profiles revealed that the 7S fraction consists of basic subunits (18 kDa to 20 kDa) and high MW polypeptides (47 kDa and 85 kDa). The 11S and HPI fractions had similar polypeptides bands that consist of the basic subunit (18 to 20 kDa), acidic subunit (30 to 40 kDa) and other high MW polypeptides. The 2S profile has seven polypeptides (15 kDa, 19, 80 and 125 kDa) with the 15 kDa in the highest proportion. The intrinsic fluorescence and near-UV data showed that the aromatic amino acids of the protein samples were more exposed to the polar environment. The far-UV data showed that the secondary structures of all the proteins were mostly dominated by β-sheet conformation at all the pH values. The 2S fraction was relatively soluble at all the pH values. The 11S fraction and HPI were more soluble at the acidic pH while 7S fraction was soluble at alkaline pH. The 2S and HPI exhibited good oil absorption capacity. The 7S fraction showed better gelling properties when compared to HPI and other fractions. The 2S fraction exhibited higher foaming capacity at all the pH values when compared to HPI and other fractions. The 2S, 7S, and 11S fractions formed better emulsions when compared to HPI emulsions. Overall, the hemp seed protein fractions differ from one another in terms of amino acid composition, polypeptide composition, and protein conformation while their functional properties are superior to those of HPI.

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DEDICATION

This work is dedicated to the God Almighty, the master of the universe.

TABLE OF CONTENTS

ABSTRACT.....II
ACKNOWLEDGMENTS III
DEDICATION IV
TABLE OF CONTENTS.....V
LIST OF TABLES VII
LIST OF FIGURES IX
LIST OF ABBREVIATIONS.....XI
CHAPTER ONE
1. INTRODUCTION 1
1.1 Hypothesis…….....7
1.2 Objective……. …….7
CHAPTER TWO…….9
2. LITERATURE REVIEW.....9
2.1 Industrial Hemp…….....9
2.2 Industrial Hemp seed: Uses and Composition……10
2.3 Nutritional and chemical properties of Hempseed protein……..11
2.4 Structural conformation of protein……...14
2.4.1 Intrinsic Fluorescence……15
2.4.2 Extrinsic Fluorescence: Surface Hydrophobicity……...18
2.4.3 Circular dichroism……..19
2.4.4 Electrophoresis……21
2.5 Food proteins functionality……..23
2.5.1 Protein solubility……....24
2.5.2 Water absorption capacity (WAC)……25
2.5.3 Oil absorption capacity (OAC)……..26
2.5.4 Gelation properties (least gelation concentration)……26
2.5.5 Foaming capacity……...27
2.5.6 Emulsifying activity and stability……..29

CHAPTER THREE

3. MATERIALS AND METHODS 32
3.1 Materials.....32
3.2 Methods 32
3.2.1 Preparation of defatted hemp seed flour (DHF).....32
3.2.2 Preparation of Hemp Seed Protein Isolates (HPI).....32
3.2.3 Preparation of 11S, 7S, and 2S hemp protein fractions.....33
3.2.4 Determination of Protein Content of HPI, 11S, 7S, and 2S.....33
3.2.5 Proximate composition analysis.....34
3.2.6 In vitro protein digestibility method.....34
3.2.7 Amino acid composition analysis.....35
3.2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).....35
3.2.9 Total and exposed sulfhydryl contents.....36
3.2.10 Total carbohydrate content.....37
3.2.11 Determination of Surface hydrophobicity (So).....37
3.2.12 Intrinsic fluorescence emission.....38
3.2.13 Measurements of circular dichroism (CD) spectra.....38
3.2.14 Determination of Functional Properties.....39
3.2.14.1 Protein solubility (PS) of HPI, 2S, 7S, and 11S.....39
3.2.14.2 Water and oil holding capacities (WHC and OHC).....39
3.2.14.3 Least gelation concentration.....40
3.2.14.4 Foam capacity (FC).....40
3.2.14.5 Emulsion formation and oil droplet size measurement.....40
3.2.15 Statistical Analysis.....42

CHAPTER FOUR
4. RESULTS AND DISCUSSION 43
4.1 Proximate compositions of defatted flour (DHF), spent flour (SF), HPI, 7S, 11S and 2S…….43
4.2 Percentage protein content as determined by Kjehdahl, Lowry and amino acid methods……..45
4.3 Percentage gross yield, protein yield and protein digestibility of HPI, 11S, 7S, and 2S……..45
4.4 Total and exposed Sulfhydryl (SH)……...49
4.5 Amino acids composition……..49
4.6 Electrophoresis (SDS-PAGE)……52
4.7 Surface hydrophobicity (So)……..56
4.8 Intrinsic Fluorescence emission…….58
4.9 Secondary structure conformation…….61
4.10 The effect of pH on tertiary structure conformation……64
4.11 Protein solubility profiles of hemp seed proteins……67
4.12 Water and oil absorption capacity……69
4.13 Least Gelation Concentration……..72
4.14 Foaming properties……..72
4.15 Emulsion formation and stability……76
CHAPTER FIVE
CONCLUSION.....84
SIGNIFICANCE OF RESEARCH 87
FUTURE RESEARCH 88
REFERENCES 89

LIST OF TABLES

Table 2.1: Functional properties and underlying mechanisms for the applications of proteins in food products……31

Table 4.1: Proximate composition and protein digestibility of 11S, 7S, 2S and HPI of hemp seed proteins……..44

Table 4.2: Percentage protein content as determined by Kjehdahl, Lowry and amino acids methods……..46

Table 4.3: Protein content, percentage recovery and protein digestibility of HPI, 11S, 7S and 2S……..48

Table 4.4: Percent amino acid composition of HPI and its fractions…….50

Table 4.5: Molecular weight (MW) and relative content of the major subunits of HPI, HPI-7S and HPI-11S under non-reduced condition……54

Table 4.6: Molecular weight (MW) and relative content of the major subunits of HPI, HPI-7S and HPI-11S under reduced condition……...55

Table 4.7: Circular dichroism-derived protein secondary structure composition of 2S, 7S, 11S and HPI……..63

Table 4.8: Oil and water absorption capacity (g) and least gelation concentration (%) of hemp seed proteins……...70

LIST OF FIGURES

Figure 4.1: SDS-PAGE of hemp seed proteins under reducing (A) and non-reducing (B) conditions…….....53

Figure 4.2: pH-dependent surface hydrophobicity of HPI and its fractions…….57

Figure 4.3: Intrinsic fluorescence intensity (arbitrary units) of hemp seed proteins at different pH values…….59

Figure 4.4: Figure 4–Far-UV circular dichroism spectra of hemp seed proteins at different pH values……62

Figure 4.5: Near-UV circular dichroism spectra of hemp seed proteins at different pH values…….65

Figure 4.6: Protein solubility profile of hemp seed proteins at different pH values…….68

Figure 4.7: Foaming capacity of hemp seed proteins at different pH values: (A) 20 mg/ml, (B) 40 mg/ml, and (C) 60 mg/ml protein concentrations…….73

Figure 4.8: Foaming stability of hemp seed proteins at different pH values: (A) 20 mg/ml, (B) 40 mg/ml, and (C) 60 mg/ml protein concentrations……..75

Figure 4.9: Oil droplet sizes of emulsions formed by hemp seed proteins at different pH values: (A) 10 mg/ml, (B) 25 mg/ml, and (C) 50 mg/ml protein concentration……77

Figure 4.10: Emulsion stability formed by hemp seed proteins at different pH values: (A) 10 mg/ml, (B) 25 mg/ml, and (C) 50 mg/ml protein concentrations……..79

Figure 4.11: Oil droplet size distribution of emulsions stabilized by 2S, 7S, 11S and HPI at 10 mg/l at pH 3.0, 5.0, 7.0 and 9.0……81

Figure 4.12: Oil droplet size distribution of emulsions stabilized by 2S, 7S, 11S and HPI at 25 mg/l at pH 3.0, 5.0, 7.0 and 9.0……82

Figure 4.13: Oil droplet size distribution of emulsions stabilized by 2S, 7S, 11S and HPI at 50 mg/l at pH 3.0, 5.0, 7.0 and 9.0……83

LIST OF ABBREVIATIONS

AAA	Aromatic amino acids
ANS	1-anilinonaphthaline-sulfate
BCAA	Branched- chain amino acids
CD	Circular Dichroism
DHF	Defatted hemp seed flour
FC	Foaming capacity
FI	Fluorescence Intensity
FS	Foaming stability
HAA	Hydrophobic amino acids
HPI	Hemp protein isolate
MW	Molecular weight
NCAA	Negatively charged amino acids
OAC	Oil absorption capacity
PAGE	Polyacrylamide gel electrophoresis
PCAA	positively charged amino acids
PS	Protein solubility
SCAA	sulfur-containing amino acids
SDS	Sodium dodecyl sulfate
SH	Sulfhydryl
SF	Spent flour
So	Surface hydrophobicity
THC	Delata-9-tetrahydrocanabinol

WAC	Water absorption capacity

CHAPTER ONE

1. INTRODUCTION

Proteins are one of the macronutrients besides carbohydrates and fats that are vital for life and required for the proper functioning of cells, tissues, organs and body systems as a whole. Food proteins are nontoxic molecules that are derived from both animal and plant tissues, can be digested by the human body and provide nutrition in the form of amino acids to the body (Rodrigues et al., 2012). The global demand for food-derived proteins continues to grow and is expected to increase from a value of $25.62 billion in 2016 to $48.77 billion by 2025 (Grand View Research, 2017). Consumers' negative perception of fat and sugar and their awareness about the nutritional and health benefits of proteins are some of the factors for increased demand for proteins. Many consumers are becoming aware of the health benefits such as increase satiety, building lean muscle mass and improving glycemic control that are associated with consumption of protein-rich diets (Paddon-Jones et al., 2008). Thus, food-derived proteins have become prominent ingredients in the food industry.

Globally, plant-based foods have been a major source of protein, which contributes 65% of total protein in the human diet while the remaining 35% is derived from animal-based foods (Wu et al., 2014). On the other hand, Western traditional diets focus on animal-based foods as a major source of protein, contributing 65% of the total protein while 35% are derived from plant-based protein (Wu et al, 2014). Recently, there is a growing interest in plant-based protein ingredients. Focus has shifted from traditional animal protein sources to plant-based protein sources. Several reasons have led to the shift, which include plant-based protein sustainability and potential health benefits (Baroni et al., 2007; Mattila et al., 2017) growing body of evidence shows that the production of plant-based foods requires less water, land, energy and release less

hydrofluorocarbon such as methane, carbon dioxide into the environment when compared to the animal-based foods production (Day, 2013). This could reduce the financial cost of plant protein production and have less impact on the environment (Baroni et al., 2007; Day, 2013; Vliet et al., 2015). Although animal-based proteins contain higher quality protein in terms of essential amino acid profile and digestibility when compared to plant-based proteins (Vliet, 2015), their high intake, such as red and processed meat has been associated with higher incidence of cardiovascular disease, type 2 diabetes (T2D) and some types of cancers (Chan et al., 2011; Pedersen et al., 2013; Mattila et al., 2017). Also, plant-based foods contain no cholesterol, less saturated fat and packed with high dietary fibers and phytochemicals that are beneficial to human health (Day, 2013; Vliet et al., 2015). The increased consumer awareness about the benefits associated with consumption of plant-based foods has led to a growing consumer preference for plant-based protein products such as meat substitutes and nondairy milk, as well as other plant-based entrees and convenience foods (Vliet et al., 2015). Hence, plant-based proteins have been identified as cheaper and sustainable alternatives to animal proteins in the human diet.

In spite of the aforementioned health benefits of plant-based foods or proteins, many of the plant-based food proteins are still underutilized (Day, 2013). One of the reasons is their low protein content and deficiency in one or two essential amino acids most especially sulfur-containing amino acids, which make them of lower nutritional value when compared to animal-based protein sources (Wu et al., 2014). Also, the presence of anti-nutrient compounds in plant-based foods reduces the nutritional value of nutrients in the diet. These anti-nutrient compounds which are derived from secondary metabolism can cause detrimental effects on human health by inducing discomfort and stress or impairing the intake, uptake, and utilization of other foods in humans (Pihlanto et al., 2017). An example of this is the protease inhibitor (trypsin inhibitor).

Protease inhibitors are widely distributed in plant foods (Philanto et al., 2017; Aluko, 2017). They form very stable complexes with proteolytic enzymes in the intestinal tract and reduce protein digestion (Pihlanto et al., 2017). Other factors that limit the use of plant proteins as functional ingredients in the food system have been attributed to their large molecular weight and size, poor solubility in water, and the cost of isolation and recovery of protein fractions (Day, 2013).

Over the years, there has been considerable research effort to improve the nutritional and functional properties of plant proteins, some of which have yielded tremendous results. An adequate combination of plant proteins from different sources can provide sufficient amounts of essential amino acids required for optimal human health (Wu et al., 2014). Antinutrient compounds can be inactivated or reduced to a safe level by using different processing methods such as soaking, cooking, germination, fermentation, selective extraction, irradiation, and enzymatic treatments. These methods could also enhance digestibility of plant proteins (Sun-Waterhouse et al., 2014). Many physical, chemical and enzymatic treatments have been widely applied to modify the protein structure and improve their functional properties (Zhao, 2011; Suppavorasatit et al., 2011). Cost-effective recovery and separation processes such as air classification, isoelectric precipitation, salt-in, salt-out precipitation, and ultracentrifugation have been employed to extract, isolate and fractionate proteins. Separation of plant proteins into their fractions has produced protein fractions with specific nutritional and functional properties (Karaca et al., 2011, Park et al., 2012, Boye et al., 2010; Taherian et al., 2011; Ajibola et al., 2016; Malomo and Aluko, 2015).

One of the plant proteins that has been extensively studied and modified through research is soy protein. Innovative scientific research has added value to soy proteins by diversifying their use

into a wide variety of food products. Many novel products such as meat and dairy substitutes using soy protein are available in the markets (Asgar et al, 2010; Day, 2013). These products have increased the consumer's awareness of the nutritional value of plant-based proteins and increased the market for plant-based protein products. While soy protein products are the most popular plant-based protein products, a range of new products from other legumes and grain proteins are emerging in the market (Asgar et al, 2010). The potential of other plant proteins such as canola (*Brassica napus*), cowpea (*Vigna unguiculata*), flaxseeds (*Linum ussitatissimum*), yellow pea *(Pisum sativum* L.), peanut (*Arachis hypogaea* L.), lentil (*Lens culinaris* L.), kidney bean (*Phaseolus vulgaris* L.) and hemp seeds (*Cannabis sativa* L.) (Tan et al., 2014; He et al., 2014; Mundi and Aluko, 2012, 2013; Avramenko et al., 2013; Malomo and Aluko 2014, 2015) as food hydrocolloids has been extensively studied by examining their structural and functional properties in model food systems.

Industrial hemp (*Cannabis sativa* L.) is a multipurpose crop with numerous applications in different industrial sectors. It has been recognized as a sustainable crop that does not require fertilizer, herbicides, and pesticides for its production (Aiello et al., 2016). Industrial hemp is a variety of *Cannabis sativa* with a low content of delta-9-tetrahydrocannabinol (THC) (Aiello et al., 2016). A THC level of 0.3% is adopted to differentiate the industrial hemp from its marijuana sibling, which contains about a 3-30% THC level (Lu et al., 2010; Callaway, 2004). The major industrial hemp cultivars grown in Canada are high-grain yielding and dual-purpose (seed and fiber) cultivars. Canada is a recognized leader in the global production and processing of industrial hemp food products. Although the fiber-rich hemp plant is widely used for paper and clothing productions, an increase in demand for hemp seed and its products (oil, meal, and

protein) for food and the nutraceutical market is the driver for Canadian hemp production (Lu et al., 2010).

The hemp seed contains more than 30% oil in which about 80% is polyunsaturated fatty acids (Da Porto et al., 2012; Teh and Birch, 2013) and is also a rich source of proteins (25% dry weight) (Callaway, 2004). Over the years, hemp seed oil has been the major industrial interest due to its numerous applications either in food or body care products. However, hemp seed proteins are becoming very popular due to the high nutritional quality. The main storage proteins in hemp seed are edestin, which accounts for 60 - 80% of the total protein while the remaining protein is albumin (25%) (Callaway, 2004; Raikkos et al., 2015). Hemp seed protein is highly digestible because of the absence of protease inhibitors (Tang et al., 2006). Although limited in lysine and tryptophan, the protein is superior to soy protein with a significant amount of most of the essential amino acids especially sulfur-containing amino acids (House et al., 2010). Hemp seed protein is a good source of bioactive peptides with many health benefits such as antioxidant, antihypertensive, oxidative apoptosis protective agent and hydrogen peroxide-induced apoptosis protective agent (Girgih et al., 2014a, b; Chakrabarti et al., 2014; Lu et al., 2010). While much is known about the nutritional and health benefits of hemp seed proteins, there are knowledge gaps regarding its structure-function relationship under given conditions.

Currently, the available hemp seed protein in the market is a protein concentrate that is obtained from the defatted meal, which is a by-product of hemp seed processing during which the seeds are cold-pressed to remove the oil. The protein-rich meal is milled into powder and passed through several sieves to obtain hemp protein concentrate with less than 70% protein content. Although the hemp protein concentrate has been successfully incorporated into a variety of products such as protein shakes, hemp milk, energy bars and defatted meals, its use as a

functional ingredient in food application is still limited due to poor functional properties. The poor functional properties of hemp seed concentrate are attributed to its high fiber and phytate content and poor solubility (Tang et al., 2006). Another factor that has been implicated in poor functionality of hemp seed protein is the oil extraction method. Protein denaturation may occur during oil extraction due to the type of processing technique employed to extract oil from the hemp seed (Malomo and Aluko, 2014). While supercritical fluid extraction at 40^{o}C may have less denaturation effect on the protein native structure, the mechanical force applied during cold press extraction may result in severe protein-protein interaction and thus reduce their solubility (Wang et al., 2008; Malomo and Aluko, 2015). The method of oil extraction that will maintain the native structure of the protein is needed to fully understand the physicochemical and functional properties of hemp protein. Tang et al. (2006) examined the functional properties of hemp seed protein isolate (86.9% protein content) obtained from the defatted meal using alkaline solubilization followed by isoelectric precipitation. The results showed that the hemp seed protein isolate exhibited lower functional properties when compared to those of soy protein isolate. Hemp seed protein was subjected to limited enzymatic hydrolysis with trypsin. Although this technique was effective in improving the protein solubility, there were decreases in other functional properties (Yin et al., 2007). Protein isolate (mHPI) obtained by membrane protein concentration (after digested with carbohydrase and phytase to remove non-protein material) was reported to have significantly higher protein solubility and foaming capacity but lower emulsifying capacity when compared to that of protein isolate (iHPI) obtained by isoelectric precipitation (Malomo and Aluko, 2015). It has been demonstrated that the separation of plant proteins into different fractions could produce protein fractions with good functional properties. A comparative study of structural and functional properties of hemp seed albumin and globulin

protein fractions showed that albumin fractions with flexible structure could serve as a better ingredient in food foam formulation when compared to globulin with highly ordered structure (Malomo and Aluko, 2014). The characterization of 11S (legumins) and 7S (Vicilins) fractions of hemp seed protein showed that these proteins differ in physicochemical and nutritional properties (Wang et al., 2008). Similarly, 11S of soy protein has been shown to differ noticeably from 7S fractions in physicochemical, nutritional and functional properties and play different roles in food and nonfood soy protein products (Liu et al., 2007). While some structural information is available for 11S and 7S hemp seed protein fractions, to the best of our knowledge, information is scant on the structural-functional relationships of 11S, 7S, and 2S of hemp seed proteins. Additional studies on the structural-functional properties of these hemp seed protein fractions could enhance their utilization as functional ingredients in food formulations.

1.1 Hypotheses

I. Defatting of hemp seed flour using solvent extraction (acetone) will have minimal effect on the protein native structure.

II. The 11S, 7S and 2S hemp protein fractions will differ in terms of polypeptide composition, amino acid composition and protein conformation.

III. Functional properties of the protein fractions will differ from each other and be superior to those of the protein isolate.

1.2 Objective

- Overall aim of this research was to determine the structural and functional properties of hemp seed storage protein fractions (11S, 7S and 2S).
- Therefore, the specific objectives of the proposed study were to:
    - Determine the purity and yield of hemp seed protein fractions- 11S, 7S, and 2S.

- Determine the physicochemical properties of each protein fraction using electrophoresis, intrinsic fluorescence, and circular dichroism.
- Determine food formulation-related functional properties of each protein fraction.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Industrial hemp

Hemp (*Cannabis sativa* L.) is an herbaceous plant that belongs to the family of Cannabinaceae and native to Asia. Its cultivation dates back centuries during which it was used as a source of fiber, food, medicine and psychoactive drug (Docimo et al., 2014). Hemp cultivation was banned in Canada and other countries in the 1930s due to the presence of the psychoactive compound known as delta-9-tetrahydrocannabinol (THC) in the plant flower and leave (Tang et al., 2006). Hemp genotypes with low-THC content, typically less than 0.3% were first produced in Europe and introduced into the Canadian production system in 1998 (House et al., 2010). Hemp is classified into two categories based on their THC contents which are industrial hemp (non-drug with less than 0.3% THC level) and marijuana (a drug with 3-30% THC level) (Aiello et al., 2016). At present, both industrial hemp and marijuana are legalized in Canada (Sawler et al., 2015). In Canada, hemp is cultivated for its seeds and fiber while marijuana is typically cultivated for its psychoactive component and has been shown to have medicinal benefits (Sawler et al., 2015).

Industrial hemp is a multipurpose crop with numerous applications in different industrial sectors and has been recognized as a sustainable crop, not requiring fertilizer, herbicides, and pesticides for its production (Aiello et al., 2016). The major industrial hemp cultivars grown in Canada are high-grain yielding and dual-purpose (seed and fiber) cultivars. While hemp is cultivated in both eastern and western Canada, it is mainly concentrated in the prairie provinces and Manitoba is a leading producer (Aiello et al., 2016). Currently, there are two main Manitoba hemp seed processing companies (The Hemp Oil Canada, St. Agathe; and Manitoba Harvest,

Winnipeg), which has made Canada a recognized leader in the global production and processing of industrial hemp food products. Although the fiber-rich hemp plant is widely used for paper and clothing productions (Lu et al., 2010), the driver for Canadian hemp production is the increasing demand for hemp seed and its products (oil, meal, and protein) for food and in nutraceutical markets.

2.2 Industrial hemp seed: uses and composition

Hemp seeds are achene fruits that consist of a single seed within a hard shell, which are usually brownish with darker brown stripes (Clark, 2007). Following harvest, the seeds are threshed to remove the outer surface. The cleaned seeds, which are light brownish-grey in color are dried to a moisture content of less than 10%, which prevents them from germination during storage (Aluko, 2017). Over the years, hemp seed is recognized and valued as food for both animals and humans. In different parts of the world, hemp seeds are processed and consumed in various forms. For instance, in India, the seeds can be eaten whole, pressed to extract cooking or lighting oil, roasted and consumed as a snack or processed into flour to make different dishes (Clark, 2007; Aluko, 2017). In North America, specifically Canada, hemp seeds are processed into hemp food and natural health products. Various commercial hemp seed-derived products such as snacks, defatted meals, protein shakes, hemp milk, energy bars, and oils are available in the market (House et al., 2010).

Hemp seed contains more than 30% oil in which about 80% is polyunsaturated fatty acids. The oil is an excellent source of linoleic acid (omega-6) and alpha-linolenic acid (omega-3) and present in ratio 3:1 (n6/n3) (Da Porto et al., 2012; Teh and Birch, 2013). Omega-3 and 6 are essential polyunsaturated fats that can only be sourced through food and are required for normal growth and development of human beings (Simopoulos, 2011). Consumption of food

with a 2:1 to 4:1 of omega 6:3 is recommended by the nutritionist and this makes hemp seed oil of high nutritional value (Callaway, 2004). Hemp seeds contain 20-30% carbohydrate, 27.6% total fiber (5.4% digestible and 22.2% non-digestible fiber) and 5.6% ash (Callaway, 2004). Although anti-nutritional factors such as phytic acid, condensed tannins and trypsin inhibitors are found in hemp seed, they are present in low concentrations (Russo and Reggianin, 2015). Hemp seed is also a rich source of proteins (approx. 25% dry weight), which contain an appreciable amount of all the essential amino acids (Tang et al., 2006).

2.3 Nutritional and chemical properties of hemp seed proteins

The nutritional value of protein depends mainly on its quality. Protein quality is defined as the ability of a food protein to meet the body's metabolic demand for amino acids and nitrogen (Boye et al., 2012). The quality of a protein is determined by its amino acids and essential amino acid composition, the digestibility of the protein as well as the bioavailability of the individual amino acid (Boye et al., 2012). Hemp seed proteins contain significant amounts of all the essential amino acids, especially sulfur-containing amino acids, which are limiting in most plant proteins (Callaway, 2004). Although limited in tryptophan and lysine, most of the essential amino acids in hemp seed proteins are sufficient for the FAO/WHO suggested requirements of children (Tang et al., 2006). The ratio of essential amino acids to the total amino acids (E/T) of hemp proteins was reported to be significantly higher than that of soy protein isolates (Wang et al., 2008). Hemp seed protein is also a rich source of arginine and glutamine. Therefore, the consumption of hemp seed protein could enhance cardiovascular vascular health as arginine is a precursor of nitric oxide, a vasodilator that helps normal blood flow and pressure (Malomo and Aluko, 2014). The digestibility of a protein is defined as the proportion of the dietary protein that is effectively digested and absorbed in the body (Boye et al., 2012). In an in vitro digestion of

hemp seed proteins using pepsin and trypsin, 88-90% digestibility was observed, which were significantly higher than the 71% for soy protein isolate (Wang et al., 2008). Hence, hemp seed protein isolate can be utilized as a good source of protein nutrition for human consumption (Wang et al., 2008). Protein digestibility is affected by methods used in food preparation and processing and the presence of anti-nutritional factors such as proteinase inhibitors. The high digestibility observed in hemp proteins was attributed to the absence of proteinase inhibitors (Aluko, 2017).

The seed protein contents of ten industrial hemp cultivars approved for production in Canada were evaluated by Vonapartis et al. (2015). A noticeable variation was observed in crude protein content among the cultivars, which range between 238 and 280 g/kg, respectively. Proteins are classified based on their solubility and extractability in different solvents (Osborne, 1924). Based on this classification, albumins are soluble in water and can be coagulated by heat. Globulins are insoluble in water but soluble in dilute salt solutions. Prolamins are insoluble in either water or salt solutions but soluble in concentrated aqueous alcohol solutions (i.e. 60 - 70% v/v) while glutelins are not soluble in neutral aqueous solutions, saline or alcohol but soluble in dilute aqueous acid or alkali solutions. Hemp seed proteins consist mainly of the storage proteins (Wang et al., 2008). Storage proteins are defined as a group that comprises proteins generated mainly during seed development and stored to serve as a nitrogen source during germination. They are characterized by the absence of enzymatic activity, their multimeric nature and also present at 5% or more of total protein fraction (González-Pérez and Vereijken, 2007). The two storage proteins found in hemp seed are globulin (edestin) and albumin. Edestin has been found to constitute most of the hemp seed protein, ranging from 67-75%, while albumin is 25 – 37%. Edestin, with an estimated MW of 300 kDa has been shown to consist of six identical subunits

and each subunit contains acidic and basic subunits linked by a single disulfide bond (Yin et al. 2009). The basic subunit mainly consists of two subunits of about 20.0 and 18.0 kDa, while the acid subunit is relatively homogeneous with a MW of 34.0 kDa (Wang et al., 2008; Yin et al., 2009). Hemp seed proteins are also classified based on their sedimentation coefficient (Wang et al., 2008). The sedimentation coefficient is a measure of the rate at which a suspended protein in colloidal solution sediments during ultracentrifugation (Gonz´alez-P´erez and Vereijken, 2007). It characterizes the behavior of the protein during centrifugation and is usually expressed as the Svedberg's (S) unit. Wang et al. (2008) identified two protein fractions, 11S and 7S from hemp seed proteins that belong to the edestin group. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the 11S fraction consists of both the acidic and basic units of edestin while 7S consists of a basic unit and a 4.8 kDa subunit. While the 11S and 7S exhibited similar protein digestibility, the amino acid profile showed that the 11S contains higher contents of sulphur-containing amino acids, arginine, and other essential amino acids when compared to 7S. This shows that 11S fraction is of higher nutritional value than the 7S fraction.

Albumin, a water-soluble fraction of hemp protein with a sedimentation coefficient of approximately 2S has been reported to account for 25% of total hemp seed protein. Malomo and Aluko (2014) separated hemp seed proteins into water-soluble (albumin) and salt soluble (globulin) fractions. Gel electrophoresis showed that the albumin contains fewer disulfide bonds indicating flexible structure. The intrinsic fluorescence and circular dichroism data showed greater exposure of tyrosine residue, which makes albumin more flexible when compared to globulin with rigid structure; these results were directly correlated to the gel electrophoresis data.

2.4 Structural conformation of proteins

Protein functionality in the food system is often associated with structural changes in secondary and tertiary structures, which involve unfolding of protein in solution or at interface and denaturation during gelation. Determination of specific structural transitions during folding and unfolding of food proteins is essential to the understanding of the molecular basis of functionality. Protein structure is divided into four levels: primary, secondary, tertiary and quaternary structures. The primary structure is a linear sequence of amino acids that are bonded together by a covalent bond, through the peptide bond (Ustunol, 2015). The physical, chemical, structural, biological and functional properties of a protein are influenced by the chain size and the order in which the amino acids bind together (Rodrigues et al., 2012). The secondary structure is related to the spatial disposition of the amino acid sequence in certain segments of the peptide chain while the tertiary structure is a complex and irregular folding of the peptide chain in three dimensions, that is, geometric shape that a protein assumes. The quaternary structure refers to a cluster of more than one protein molecule or polypeptides (Rodrigues et al., 2012).

Over the years, effective detection methods such as computer vision, spectroscopy and hyperspectral have been developed to evaluate conformational changes in protein and understand the relationships between its structural and functional characteristics during processing (Wang et al., 2017). The most widely used among these methods is spectroscopic techniques which include fluorescence and circular dichroism (CD), Nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) (Mao et al., 2014; Zhang et al., 2015; Chandrapala et al., 2012; Ruffin et al., 2014). They are simple, fast and convenient techniques that are used for probing and monitoring structural conformational changes such as folding and unfolding of

proteins during processing or modification. The underlying principles of two of these spectroscopic techniques, fluorescence and CD are summarized below.

2.4.1 Intrinsic fluorescence

Fluorescence spectroscopy is one of the oldest and powerful analytical methods that has been extensively used to elucidate the molecular structure, function, and conformation of proteins. Fluorescence is the emission of light due to the absorption of ultraviolet (UV) or visible (Vis) light of chromophores that can emit light. The aromatic amino acids are the chromophores in proteins. The chromophore absorbs energy in the form of light at a specific wavelength and liberates energy in the form of light emission at a higher wavelength. The principles of fluorescence generation as elucidated by a Jablonski diagram (Karoui and Blecker, 2011) involve three stages. "The first step (1) is the excitation state in which the chromophore absorbs light and undergo transition from ground state to excited state, the second step (2) is the vibrational relaxation or internal conversion, in which the chromophore undergoes a transition from an upper electronically excited state to a lower one without any radiation while the final step (3) is the state where the emission occurs and the electron return to its more stable ground state, which is usually between 10-8s after excitation" (Karoui and Blecker, 2011). Hence, fluorescence emission is observed when an excited electron reverts from the first excited state to the ground state.

The components of a spectrofluorimeter system consist of a light source, which is usually mercury or xenon lamp for emitting UV or Vis, a sample compartment, two monochromator and /or filters(s) in which one is for selection of the excitation wavelengths while the other is for selection of the emission wavelengths; a detector for converting emitted light to an electric signal; and a data acquisition unit (Karoui and Blecker, 2011). The fluorescence of proteins

originates from phenylalanine, tyrosine and tryptophan residues. In a protein that contains all three aromatic amino acids, the fluorescence intensity depends on the intrinsic fluorescence of tryptophan (Trp) residues because the absorbance at the wavelength of excitation and the quantum yield of emission is considerably greater than the respective values for tyrosine (Tyr) and phenylalanine (Phe). Hence, the intrinsic fluorescence spectrum is determined mainly by the polarity of the environment of the Trp residues (Yin et al., 2011). Fluorescence emission is much more sensitive to changes in the environment of the chromophore than is light absorption. As lifetime of the excited state is long, a broad range of interactions or perturbations can influence this state and thereby the emission spectrum. Thus, fluorescence provides a sensitive means of monitoring conformational changes in proteins, protein-protein interactions as well as ligand-protein interactions (Tang and Wang, 2010). For a tightly packed protein in which the Trp residue is partially or fully buried in the hydrophobic core of protein interior, Trp fluorescence emission is maximum at < 330 nm (Jiang et al., 2014). Changes in protein conformation such as unfolding of protein often lead to large changes in fluorescence emission. In the case of a protein that does not contain Trp, changes in Tyr emission are responsible for changes in intensity with fluorescence maximum around 303 nm irrespective of the molecular environment of the chromophore (Jiang et al., 2014). Although Tyr residues are more fluorescent than Trp residues in solutions, the fluorescence quantum yield significantly decreases when Trp is present in proteins. The quenching of Tyr fluorescence within the protein has been associated with the protein tertiary and quaternary structure, which suppresses the Try fluorescence and the transfer of energy from Tyr to Trp residues (Karoui and Blecker, 2011). It has been shown that Tyr fluorescence is not sensitive to the polarity of the environment. Hence, Tyr could only be used as an intrinsic fluorescent probe in studying Trp-lacking proteins (Munishkina and Fink, 2007). The

fluorescence emission of Phe is maximum near 280 nm and is rarely used as a fluorescent probe due to its relatively low quantum yield. Several factors related to the nature and the concentration of chromophore of food samples can influence the fluorescence intensity. Some of the factors are quenchers such as oxygen and high temperature, which decrease fluorescence intensity by deactivating the excited molecules. Factors such as the concentration, the molar absorptivity and the optical depth of the sample or chromophore can also influence the fluorescence intensity. The molecular environment is another important factor that can influence the intensity of intrinsic fluorescence. In hydrophilic environments, the chromophore in an excited state will relax to a lower energy state, which makes the fluorescence emission to be shifted towards longer wavelengths and is referred to as a red shift (Karoui and Blecker, 2011). The opposite effect occurs in a hydrophobic environment, which causes fluorescence emission shifts to shorter wavelengths (blue shift). The structure and location, temperature, pH and color of proteins have been shown to have a strong effect on the fluorescence emission and quantum yield of a chromophore. Increased temperature leads to increased movement of the molecules, and thereby more collisions, thus reducing fluorescence emission (Karoui and Blecker, 2011). Variation in sample temperature may affect the fluorescence intensity, therefore it is paramount that all samples in an experiment be evaluated at the same temperature. Dark samples will reabsorb more of the fluorescence than bright samples while hydroxyl aromatic compounds fluoresce better at high pH (Karoui and Blecker, 2011). Malomo and Aluko (2014) measured the conformational changes of hemp seed protein meal (HPM) and hemp seed protein isolate (HPI) at different pH values using intrinsic fluorescence spectroscopy technique. For both HPI and HPM, the characteristic fluorescence profile of Trp residues is within a hydrophilic environment

at pH 3.0 and pH 5.0 while the Trp residues are located in more conserved protein structure and within a hydrophobic environment at pH 7.0 and 9.0.

2.4.2 Extrinsic fluorescence: surface hydrophobicity

While intrinsic protein fluorescence derives from natural chromophores such as Trp and Tyr to provide information on the conformational changes of protein, extrinsic fluorescence dyes such as 1-anilinonaphthaline-8-sulfonic acid (ANS) offer additional information on protein conformation most especially on surface hydrophobicity (Jiskoot et al., 2005; Ladokhin, 2000). Extrinsic fluorescence dyes attach to proteins by covalent interactions through the amino group lysine, N-terminus, the thiol group of cysteine or interact non-covalently with protein and protein degradation products through hydrophobic and electrostatic interactions. The binding mechanism of ANS to proteins is through hydrophobic and electrostatic interactions (Hawe et al., 2008). The ability of the ANS dye to assess non-polar surface hydrophobicity or posttranslational modification of protein has been used extensively to measure surface hydrophobicity of native and denatured proteins. Surface hydrophobicity can be relevant for the activity, aggregation, and adsorption of proteins (Hawe et al., 2008). ANS fluorescence is sensitive to the dye's environment in terms of polarity, viscosity, and temperature. Both conformational and functional properties of food proteins can be influenced by inherent (e.g. amino acids composition and external (e.g. pH, temperature and ionic strength) factors.

The shape and overall hydrophobicity of proteins depend on the amino acid composition and the chemical properties of the amino acid side-chain groups. Proteins tend to assume globular shape when they contain a large number of hydrophobic residues while they assume an elongated rod-like shape when they contain a large number of hydrophilic amino acid residues distributed uniformly in their sequences (Wang et al., 2017). For most native proteins, many

hydrophobic residues are buried in the interior of the protein, although, some hydrophobic groups may be at the surface. When proteins are denatured, these hydrophobic groups are exposed at the molecular surface. The surface hydrophobicity and hydrophilicity characteristics of the protein surface influence their solubility characteristics, which govern several functionalities such as thickening, foaming, emulsification, and gelation properties (Hettiarachchy et al., 2012). In principle, there exists a relationship between water solubility and surface hydrophobicity; the higher the surface hydrophobicity, the lower the water solubility and this is due to protein tendency to aggregate by hydrophobic interactions as well as the incompatibility of hydrophobic groups with the hydrophilic water. Wagner et al. (2000) found a trend contrary to this principle, in which the greater the surface hydrophobicity, the greater the solubility. This result was based on the conditions in which the soy protein isolates were prepared.

2.4.3 Circular dichroism

Circular dichroism (CD) is a phenomenon that results when chromophores in an asymmetrical environment interact with polarized light. Polarized light will split into two circularly polarized components with one rotating left-handed (counter-clockwise) and the other right-handed (clockwise), which then passes through a modulator that is subjected to an alternating 50 kHz electric field (Kelly et al., 2005). The sample must be optically active to generate a CD signal. In proteins, the major optically active groups are the amide bonds of the peptide backbone and the aromatic side chains. CD signals are observed in the same spectral regions where the absorption bands of a particular compound are found, provided that the respective chromophores or its molecular environment are asymmetric. CD bands of protein occur in two spectral regions. The far-UV or amide region (190-250 nm) is dominated by the contributions of the peptide bonds,

while CD bands in the near-UV region (250-300 nm) originate from aromatic amino acids (Kelly et al., 2005). Also, disulfide bonds give rise to monitored CD bands around 250 nm. The two spectral regions give different kinds of information about protein structure. Circular Dichroism bands in the amide region contain information about the peptide bonds and the secondary structure of a protein and are frequently employed to monitor changes in secondary structure in the course of structural transitions. In general, the CD spectrum of α-helix has an intense positive band at 191-193 nm and a typical double negative at 208-210 and 222 nm while β-sheet has a fairly intense positive band at 195-200 nm and negative band at about 216-218 nm whereas random coils have a strong negative band at 195-200 nm and a much weaker band (either positive or negative) between 215 and 230 nm (Tang and Wang, 2010; Wang et al., 2017).

CD bands in the near-UV region are observed when aromatic side chromophores (Phe, Tyr, and Trp residues) are immobilized in a folded protein and thus transferred to an asymmetric environment. It has been widely used to assess the tertiary and occasionally quaternary structures of proteins during processing. The CD of the aromatic residue is very small in the absence of ordered structure (e.g. in short peptides), hence the farther away from zero (negative or positive) the more organized the tertiary structure (Wang et al., 2017). Each of the amino acids tends to have a characteristic wavelength profile. The Trp residue shows a peak close to 290 nm with fine structure between 290 and 305 nm; the Tyr residue peaks between 275 and 282 nm, with a shoulder at longer wavelengths; the Phe residue shows weaker but shaper bands with fine structure between 255 and 270 nm (Kelly et al., 2005; Martin and Schilstra, 2008). The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on the number of each type of aromatic amino acids present, their mobility, the nature of their environment (H-bonding, polar groups, and polarizability) and their spatial disposition in the protein (Yin et al.,

2011). An increase in the band magnitude and intensities, which are an indication of structural change has been associated with the loss of native-like structure and increasing interactions of the aromatic amino acid residues during processing (He et al., 2014).

2.4.4 Gel electrophoresis

Electrophoresis is the process of moving charged molecules, including large molecules such as DNA and proteins in solution under the influence of an electric field. The electric field and the charges on the molecule are the driving force that moves the molecule within the separation gel (Gallagher, 2006). The mobility of the molecules in an electrical field depends on their charge, shape, and size. Larger proteins and proteins with a lower net charge move more slowly. Electrophoresis has been extensively used to evaluate molecular sizes of proteins and separate proteins based on their molecular sizes (Rosenberg, 2005). Electrophoresis is a method of choice to gain insight into changes in protein composition, hydrolysis, or any modifications that may occur and can be used to identify and characterize food proteins. Electrophoresis is used to separate complex mixtures of proteins, compare samples, evaluate the protein purity, determine physical characteristics of proteins such as molecular weight, isoelectric point, and subunit composition, and purify small amounts of proteins for further analysis (Rosenberg, 2005). In addition to molecular size of the proteins, electrophoretograms of the proteins provide information on the presence or absence of protein cross-linkings such as intermolecular disulfide bonds.

In polyacrylamide gel electrophoresis (PAGE), macromolecules move through pores in the gel matrix under the influence of an electric field. Different species of molecules in the sample move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. The gel consists of polymers of

crosslinked acrylamide. The gel pore size is determined by acrylamide concentration; for example, a 10% polyacrylamide gel will have larger pore sizes than a 15% gel. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein (Gallagher, 2006). A matrix is required because the electric current passing through the electrophoresis solution generates heat, which causes diffusion and convective mixing of the bands in the absence of a stabilizing medium.

The most widely used method for protein electrophoresis is the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970). This method separates proteins according to their molecular weights range from 1 to 1000 kDa. The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptides backbone in a ratio of approximately 1.4 g SDS/g protein. The bound SDS masks the charge of the proteins themselves, forming anionic complexes with constant net negative charge per unit mass. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and partially unfolds the protein molecules, minimizing differences in molecular conformation by eliminating the secondary and tertiary structures (Omstein, 1996). In the presence of disulfide bond reducing agents such as mercaptoethanol, the protein unfolds completely. The reducing agent cleaves the disulfide bonds, which can form between cysteine residues, and the polypeptides become flexible rods of negative charges with equal charge densities, or charge per unit length. Treating proteins with both SDS and a reducing agent can result in separations that are exclusively based on molecular weight. SDS-PAGE system is a modification of a discontinuous system described by Omstein (1996) and Davis (1964), which was originally devised to separate proteins under non-denaturing (native) conditions. Under the

native condition, the migration rate of protein is dependent on both its intrinsic charge and size. The molecular weight of the protein, therefore, cannot be directly determined by its migration in a single gel. The resolution of non-denaturing electrophoresis is generally not as high as SDS-PAGE, but the technique is useful when one wishes to retain the native structure or enzymatic activity of a protein, following electrophoresis.

2.5 Food proteins functionality

In addition to providing amino acids for human nutrition, proteins also serve several non-nutritional functions in foods. The most common non-nutritional function of proteins is known as techno-functionality, which is the protein's ability to provide and/or stabilize the characteristic structure of foods during preparation, processing, storage, and consumption and thereby contribute to the overall quality, sensory attributes and consumer acceptability of the food products (Foegeding and Davis, 2011). Functional properties have been defined as 'those physical and chemical properties that influence the behavior of proteins in food systems during processing, storage, cooking and consumption' (Moure et al., 2006). Food protein functionality is influenced by both intrinsic factors such as protein size, shape, amino acid composition and sequence, net charge, charge distribution, conformation (e.g, surface hydrophobicity, hydrophobicity/hydrophilicity ratio), structure (secondary, tertiary and quaternary) and extrinsic factors that include pH, temperature, salt concentration and interactions with other food constituents (Boye et al., 2010). The ability of a protein to create networks to hold water, form gels, develop films, absorb fat, foam, emulsify oil droplets and dissolve under various pH conditions are some of the indices used to measure protein functionality (Aryee et al., 2017). In addition to these indices, proteins also contribute to sensory characteristics of food products such as flavor, texture, and taste which determine consumer acceptability. Hence, functional

properties of proteins refer to the overall behavior or performance of proteins in foods and reflect the various interactions that involve protein participation. Functionality may vary with the source of protein, its composition, the method of preparation, its thermal history and the prevailing environment, i.e. pH, ionic strength, temperature, and presence of salts (Boye et al., 2010). Some of the important protein functionalities of interest are discussed below. Their summary and food applications are provided in Table 2.1

2.5.1 Protein solubility

Protein solubility is an important property in protein dispersion, which influences other functional properties of proteins such as water and oil holding capacity, foaming, emulsification, and gelation. Protein solubility is the amount of protein that goes into solution or colloidal dispersion under specified conditions and does not sediment by moderate centrifugal forces (Gonz´alez-P´erez and Vereijken, 2007). The solubility of proteins has been associated with the balance of hydrophobicity/hydrophilicity residues, which depends on the amino acid composition, particularly at the protein surface. Hence, the more polar its surface, the more soluble a protein is likely to be (Gonz alez-Perez and Vereijken, 2007). Other factors that influence protein solubility include protein denaturation, surface charge, and electrostatic repulsion, pH and salt concentration of the solvent. The presence of a lower number of surface hydrophobic residues, the elevated charge and the electrostatic repulsion and ionic strength of the solvent at pH above and below the isoelectric point enhance protein solubility (Moure et al., 2006). Over the range of pH values, amino acids exist in three different forms. At the isoelectric region of a protein, zwitterions or dipolar nature predominate, which result in minimum solubility because of minimum repulsion among the constituent amino acids. The balance in positive and negative charges minimizes the electrostatic repulsion and enhances protein-protein

interactions, which reduce protein solubility at the isoelectric pH. When the pH of the solution reduces below the isoelectric point, cations predominate while anions dominate in an alkaline medium. In both extremes of the pH scale, electrostatic repulsion improves and this enhances solubility. Hemp seed protein isolate was reported to be sparingly soluble at acidic pH and least soluble at pH 4.0 to 5.0, which represents the isoelectric point for hemp protein, while solubility increased gradually at pH above isoelectric point (Tang et al., 2006; Malomo and Aluko, 2014). Hemp seed proteins have been shown to exhibit lower functional properties when compared to soy protein. The poor functional properties of hemp seed proteins have been linked to poor solubility, formation of covalent disulfide bonds between individual proteins and subsequent aggregation at neutral or acidic pH due to its high free sulfhydryl content from sulfur-containing amino acids (Tang et al., 2006; Malomo and Aluko, 2014). Generally, the low solubility of plant proteins in acidic medium is associated with the dominance of attractive forces and molecular association, which limit their ability to form an emulsion in acidic media and limits their utilization in food systems (Boye et al., 2011; Barbana and Boye, 2013).

2.5.2 Water absorption capacity (WAC)

The behavior of proteins in food systems is related to the degree of its interaction with water. Water absorption capacity is the amount of water that can be absorbed per gram of protein material (Boye et al., 2010). The water retention capacity of proteins in the food system is their ability to retain water against gravity and includes bound water, hydrodynamic water, capillary water and physically entrapped water (Moure et al., 2006). Non-polar amino acids are insoluble in water and have very low interaction with water while polar amino acids are good sites for protein-water interactions (Chavan et al., 2001). Factors that affect water binding capacity (WBC) of food proteins include amino acid composition, protein conformation, surface

hydrophobicity, temperature, ionic strength and protein concentration (Moure et al., 2006). WBC/WAC measurements are important for food processing applications. Materials that have low WBC/WAC may not be able to hold water effectively while materials having high WBC/WAC may reduce brittleness and dryness of food products during storage (Boye et al., 2010). The application of WHC in food industries is to maintain the moistness and softness of bakery products. WHC is also very important in the meat industry, especially in the production of meat analogs, because it affects texture, juiciness, and taste.

2.5.3 Oil absorption capacity (OAC)

Fat or oil absorption capacity (FAC, OAC), also sometimes referred to as fat or oil binding capacity (FBC, OBC), is calculated as the amount of oil absorbed per weight of protein powder or sample flour. A protein's ability to entrap oil depends on protein source, size, concentration, number of hydrophobic amino acids, degree of hydrolysis, flexibility of the protein network, processing methods and protein-lipid interactions (Tomotake et al., 2002). Plant proteins contain a high amount of hydrophobic amino acids, which enable them to bind hydrocarbon chains and accounts for their high oil absorption capacity. Oil absorption capacity is an important property in fat and flavor retention as well as texture and finds several applications in batter, flavor and emulsions (Aryee et al., 2017)

2.5.4 Gelation properties (least gelation concentration)

In the food system, the gel can be described as an intermediate state between solid and liquid states in which the solid states can be proteins, polysaccharides or a mixture of both while the liquid states are usually water (Moure et al., 2006). Large molecules such as proteins are capable of forming crosslinks in three dimensions and are more efficient gelling agents than small molecules like carbohydrates (Moure et al., 2006). The ability of proteins to form gels is

traditionally measured by the least gelation concentration (LGC), which may be defined as the lowest protein concentration required to form a self-supporting gel. Hence, the lower the LGC, the better the gelling capacity of the protein. Protein gelation is a complex process that often involves several reactions such as denaturation, aggregation and network formation (Gonzalez-Perez and Vereijken, 2007). This process can be induced by heat, chemical and enzymatic treatments. The process of gel formation involves partial denaturation of proteins during which the protein adopts a more unfolded conformation and functional groups such as sulfhydryl or hydrophobic groups become exposed. These exposed groups can thereby interact with each other to form irreversible aggregates through disulfide bridges, hydrogen bonds, hydrophobic, and/or van der Waals interactions. At high protein concentrations, a three-dimensional network may be created, which is known as a gel (Gonz alez-Perez and Vereijken, 2007; Aryee et al., 2017). Properties of gels depend on the type of interactions between the solvent and the molecular network. A highly ordered gel is stabilized by hydrogen and hydrophobic interactions. Transparent gels are formed by proteins that contain a high amount of hydrophilic amino acid residues while coagulant gels are formed by proteins that contain non-polar amino acid residues. Other factors that can influence the properties of a gel include pH, ionic strength, reducing agents, urea, temperature, the presence of non-protein components and the mechanical forces applied to the system (Sathe, 2002). Protein gelation is important in the preparation of many foods such as puddings, jellies, and many desserts and meat applications.

2.5.5 Foaming capacity

Foaming is responsible for the desired rheological properties of many foods, such as the texture in bread, cakes, whipped cream, ice cream, and beef froth. Food foams consist of dispersed gas bubbles that are entrapped in a continuous liquid or semi-solid phase. For such a system to be

stable, surfactant molecules are required at the air/water interface to prevent coalescence of air bubbles (Makri et al., 2005). The surfactant molecules such as proteins unfold to form an interfacial thin film around the dispersed air bubbles and prevent their collapse (Boye et al., 2010). Proteins are the main surface-active agents that are used to stabilize the gaseous dispersed phase in food products. Foaming requires a large interfacial area to facilitate the incorporation of air into the liquid phase and the formation of an interfacial film that is resistant to internal and external forces. The ability of a protein to form an interfacial thin film is enhanced by the unfolded structure rather than a globular compact structure (Aryee et al., 2017).

Foam expansion (FE), foam capacity (FC) and foam stability (FS) are the most frequently used indices for measuring foaming properties. The ability of proteins to reduce surface tension depends on molecular flexibility, solubility and physicochemical properties such as hydrophobicity, net charge, and charge distribution, as well as hydrodynamic properties (Moure et al., 2006). Good foaming proteins must (i) rapidly adsorb during whipping and bubbling, (ii) have a rapid conformational change, rearranging at the air-water interface with reduction of surface tension and (iii) form a viscoelastic cohesive film through intermolecular interactions, which is thermodynamic (Ptaszek et al., 2015; Żmudziński, et al., 2014). While foaming capacity of a protein is its ability to form a foam under a specific condition, foam stability indicates how well such protein maintains the foam volume over a specific period (Barac et al., 2010). Foam stability is expressed as the time required for a 50% reduction in foam volume, which indicates the ability to stabilize against gravitational and mechanical stresses (Damodaran, 1997). The albumin fraction of hemp seed protein was reported to have exhibited higher foaming capacity when compared to the globulin fraction (Malomo and Aluko, 2014). The high foaming capacity of the albumin was attributed to its flexible structure and its solubility over a wide pH range.

Hence albumin fraction of hemp protein is a potential ingredient for food foam formation (Malomo and Aluko, 2014).

2.5.6 Emulsifying activity and stability

An emulsion may be defined as a dispersion/suspension of two immiscible liquids, in which one of the liquids is dispersed as small spherical droplets in the other liquid (McClements, 2005). Food emulsions are either oil-in-water (O/W) mixtures such as milk, creams, salad dressings, mayonnaise, and soup, or water-in-oil as found in margarine and butter. Emulsion formation requires the mechanical shear of the two immiscible liquids using homogenizer, which creates small droplets of one liquid dispersed in the other (Schultz et al., 2004). Such a system is thermodynamically unstable because of increased interfacial surface tension and requires emulsifiers/stabilizers at the oil/water interface to reduce interfacial surface tension and prevent coalescence of the dispersed small droplets. An emulsifier acts as a stabilizer by forming viscoelastic films around dispersed oil droplets to prevent flocculation or coalescence of the emulsion. Food proteins are commonly used in the food industry to stabilize food emulsions due to their amphiphilic nature and film-forming ability. The amphiphilic nature of proteins allows them to be adsorbed to the oil/water interface, form an interfacial layer around dispersed oil droplets, lowers the surface tension and prevents structural changes in the emulsion such as coalescence, creaming, flocculation or sedimentation during processing or storage (Boye et al., 2010). The physicochemical properties of proteins such as molar mass, hydrophobicity/hydrophilicity ratio, conformation stability, charge, and external factors such as pH, ionic strength and temperature play an important role in determining their emulsifying capacities (Papalamprou et al., 2010). Once proteins are absorbed to the interface, they unfold

and re-align to position their hydrophobic amino acids to the oils phase and hydrophilic amino acids to the aqueous phase, which consequently reduce the interfacial tension (Walstra, 2003). The formation of a viscoelastic film at the oil-water interface due to the protein-protein interactions provides resistance to mechanical stress and coalescence. Also, electrostatic and steric interactions contribute to emulsion stabilization (Lam and Nickerson, 2013). Emulsifying activity (EA) and emulsifying stability (ES) are the two indices often used to evaluate the emulsifying properties of a protein.

Table: 2.1 Functional properties and underlying mechanisms for the applications of proteins in food products

Functionality	Description	Main mechanism	Application
Solubility	Ability to dissolve in a solvent	Hydrophilicity	Beverages
Water holding	Protein interaction in water, water binding and swelling	Hydrogen bonding	Meat, sausage, cake, bread
Fat holding	Flavor interaction/flavor binding and fat retention	Hydrophobic binding	Bakery products, yogurt, fish, meat products
Gelation	Gel formation	Network formation	Meat, sausage, pasta, baked goods
Foaming	Protein adsorption at the interface, coating of air cells	Interface adsorption, film formation	Whipped toppings, cakes, mousse, meringues
Emulsification	Protein adsorption at the interface, coating of oil droplets	Interface adsorption, film formation	Salad dressing, soups

Table Adapted from: Proteins in Food Processing, edited by Rickey Y. Yada, Elsevier Science & Technology, 2017. ProQuest Ebook

CHAPTER THREE

3. Materials and Methods

3.1 Materials

Hemp seed hearts (dehulled) were purchased from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada) and stored at -20 ^{o}C. Other analytical grade chemicals and reagents such as sodium hydroxide, hydrochloric acid, sodium meta bisulfite, sodium carbonate, sodium tartrate, sodium phosphates Sodium dodecyl sulfate, copper sulfate, bromophenol blue, 2-mercaptoethanolm, trichloroacetic acid, 2-nitro-5-thiobenzoate, 1-anilino-8-naphthalenesulfonate, Folin Ciocalteu, Bovin serum albumin (BSA), urea, Ellman's reagent, Anthrone reagent, were procured from Fisher Scientific (Oakville, ON, Canada).

3.2 Methods

3.2.1 Preparation of defatted hemp seed flour (DHF)

Hemp seed flour was obtained by grinding the hemp seed hearts in a laboratory blender (Oster pro 1200 plus), which was followed by defatting using acetone extraction at 1:10 (w/v) for 1 h at room temperature. The mixture was allowed to settle after which the acetone was decanted. The defatting process was repeated a second time followed by air-drying of the residual flour in a fume hood at room temperature (23 ^{o}C) for 16 h. The resultant defatted meal was milled using laboratory blender to obtain defatted hemp seed flour (DHF).

3.2.2 Preparation of hemp seed protein isolate (HPI)

Hemp seed protein isolate was produced from the DHF according to the method described by Tang et al. (2006) with slight modifications. The DHF was dispersed in deionized water (1:20, w/v) and the dispersion adjusted to pH 10.0 using 2 M NaOH to solubilize the proteins while

stirring at 37 ^{o}C for 2 h; this was followed by centrifugation (7000 x *g*, 60 min at 4 ^{o}C). The precipitate was discarded and the supernatant filtered with cheese-cloth (grade 90, 40 x 36 thread count), adjusted to pH 4.2 with 2 M HCl to precipitate the proteins and thereafter centrifuged (7000 x g, 60 min at 4 ^{o}C). The resultant precipitate was redispersed in deionized water and adjusted to pH 7.0 with 2 M NaOH and freeze-dried to obtain the HPI.

3.2.3 Preparation of 11S, 7S, and 2S hemp seed protein fractions

HPI-11S, HPI-7S, and HPI-2S were prepared at room temperature as described by Wang et al. (2008). One hundred grams of DHF was dispersed in distilled water (1:20, w/v), and adjusted to pH 10.0 with 1 M NaOH. The dispersion was then stirred at 37 ^{o}C for 1 h and centrifuged at 10,000 x *g* for 30 min (at 4 °C) to obtain the supernatant. Then, $NaHSO_3$ was added to the supernatant (at a concentration of 0.98 g /l), and the supernatant adjusted to pH 6.4 with 1 M HCl (to precipitate the 11S fraction) and kept overnight at 4 °C. The resultant dispersion was centrifuged at 6500 x *g* for 25 min at 4 °C. The obtained precipitate (HPI-11S) was suspended in de-inonized water, adjusted to pH 7.0 with 1 M NaOH, and then dialyzed and freeze-dried. The obtained supernatant was further adjusted to pH 4.6 with 1 M HCl (to precipitate the 7S fraction) and thereafter centrifuged at 6500 g for 20 min at 4 °C. The obtained precipitate (HPI-7S) was suspended in de-inonized water, adjusted to pH 7.0 with 1 M NaOH, and then dialyzed and freeze-dried while the supernatant was collected (2S), dialyzed and freeze-dried.

3.2.4 Determination of protein content of HPI, 11S, 7S, and 2S

The protein contents of HPI and its fractions were determined using the Lowry's method (Lowry et al., 1951) as modified by Markwell et al. (1978). The HPI and its fractions (10 mg/ml) and bovine serum albumin (BSA) (10 mg/ml), which is the standard were separately dissolved in 0.1 M NaOH and mixed thoroughly using a magnetic stirrer. The protein solution was further diluted

to obtain 100 µg/ml. An aliquot (1 ml) of the samples and the standard were prepared in 20-100 µg concentration range. A volume of 3 ml of reagent C was added to 1 ml of each sample and BSA, and allowed to stand and incubate for 1 h at room temperature. Reagent C consists of reagent A and reagent B. Reagent A consists of 2% Na_2CO_3, 0.4% NaOH, 0.16% Sodium tartrate and 1% Sodium dodecyl sulfate while reagent B consists of 4% $CuSO_4.5H_2O$ dissolved in distilled water. After a 1 h incubation, 0.3 ml of reagent C (1 part Folin Ciocalteu reagent mixed with 1 part distilled water) was added to the samples and thoroughly mixed using a vortex. The samples were incubated for 45 min at room temperature. Using a spectrophotometer, the absorbance was measured at 660 nm. The concentrations of the proteins in the HPI and its fractions were then determined using the standard curve obtained from the BSA concentrations.

3.2.5 Proximate composition analysis

The moisture, dry matter, crude protein, and ash contents of the albumin and globulin samples were determined using relevant methods of the Association of Official Analytical Chemists (Horwitz and Latimer, 2005), while their crude fiber and fat contents were analyzed according to the methods of the American Oil Chemists' Society (Mehlenbacher et al., 2009).

3.2.6 In vitro protein digestibility method

The in vitro protein digestibility of the HPI and its fractions was carried out according to the method described by Hsu et al. (1977) with slight modifications using an enzyme system consisting of trypsin and chymotrypsin. A 10 ml aliquot of aqueous protein suspension (6.25 mg protein/ml) in double-distilled water was adjusted to pH 8.0 with 0.1 M NaOH while stirring at 37 °C. The enzyme solution (containing 1.6 mg trypsin and 3.1 mg chymotrypsin/ml) was maintained in an ice bath and 1 ml of the solution was then added to the protein suspension. The

pH drop was recorded over a 10 min period. The % protein digestibility of each protein sample was calculated using the regression equation predicted by Hsu et al. (1977) as follows:

$$\% \text{ Protein digestibility (Y)} = 210.46 - 18.10Xf$$

where Xf is the final pH value of each sample after a 10 min digestion.

3.2.7 Amino acid composition analysis

The amino acid profiles of the hemp seed protein fractions were determined using the HPLC Pico-Tag system according to the method previously described after samples were digested with 6 M HCl for 24 h (Bidlingmeyer et al., 1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke et al., 1985) and the tryptophan content was determined after alkaline hydrolysis (Landry and Delhaye 1992).

3.2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis (SDS-PAGE) of HPI and its fractions was determined according to the method of Aluko & McIntosh (2004). The protein samples (10 mg) were dispersed in Tris-HCl buffer containing (pH 8.0) containing 10% SDS and 0.01% bromophenol blue (non-reducing condition), or 10% SDS, 0.01% bromophenol blue containing 5% (v/v) 2-mercaptoethanol (ME) for the reducing condition. The samples were heated in boiling water for 10 min, cooled to room temperature and then centrifuged at 16000 x g for 10 min. After centrifugation, 1 µl of supernatant of each sample was loaded onto the 8-25% gradient gels and electrophoresis was performed with a Phastsystem Separation and Development unit according to the manufacturer's instructions (GE Health Sciences, Montréal, Canada). A mixture of protein standards (14.4-116 kDa) was used as the molecular weight marker and the gels were stained with Coomassie brilliant blue. The gel was scanned using GE Health labscan 5 and the bands intensity were calculated using Image QuantTZ

3.2.9 Total and exposed sulfhydryl contents

Sulfhydryl and total cysteine contents were determined by a modification of the method of Beveridge et al. (1974). For the sulfhydryl group, samples (15 mg each) were suspended in 3 ml of the reaction buffer (Tris-glycine buffer, pH 8.0) as follows; i) without 8 M urea (exposed SH), ii) with 8 M urea (total SH). After the addition of 50 µl of the Ellman's reagent, the resultant suspensions were incubated for 1 h at room temperature with occasional shaking and then centrifuged for 25 min at 10,000 x g. The absorbance of the supernatant was measured at 412 nm using the mixture of reagent buffer and Ellman's reagent as the blank. To determine the total cysteine level, 0.5 ml mercaptoethanol was added and incubated for 1 h at 25 ^{o}C followed by centrifugation for 25 min at 10,000 x g. An aliquot (1 ml) of the supernatant was transferred into a test tube and 10 ml of 12% trichloroacetic acid (TCA) was added and held for another hour at 25 ^{o}C. The mixture was centrifuged for 15 min at 10,000 x g and the precipitate was suspended twice in 5 ml TCA, centrifuged each time and then dissolved in 3 ml Tris buffer without urea. The color was developed with 50 µl Ellman's reagent and absorbance read at 412 nm.

The sulfhydryl concentration (total and exposed) in µmol/g of protein was calculated by using the extinction coefficient of 2-nitro-5-thiobenzoate (NTB) at 412 nm (13 600 mol l^{-1} cm^{-1}):

µmol SH/g protein = 73.53A x D/C

Where A = the absorbance at 412 nm; C = the sample concentration in mg solids/ml; D = dilution factor; and 73.53 is derived from 106 / (1.36 × 104); 1.36 × 104 is the molar absorptivity and 106 is for conversions from the molar basis to the µM/ml basis and from mg solids to g solids. The absorbance at 412 nm for samples without the Ellman's reagent and the absorbance for the Ellman's reagent in the sample buffer were deducted from the absorbance for samples together with the Ellman's reagent

3.2.10 Total carbohydrate content

Total carbohydrate content was determined as described by Mundi and Aluko (2012). The standard glucose stock solution was prepared by dissolving 100 mg in 100 ml water, and then 10 mL of stock was further diluted to 100 ml with distilled water. The solution was refrigerated until analysis after adding a few drops of toluene. Sample (100 mg) was weighed into the boiling tube and hydrolyzed in a boiling water bath for 3 h with 5 ml of 2.5 71 M HCl and cooled to room temperature. Solid sodium carbonate was added until the effervescence ceased. The volume was made up to 100 ml and centrifuged; the supernatant (0.5 and 1 ml aliquots) was taken for analysis. A standard curve was prepared by dispensing varied volumes of the glucose standard (0, 0.2, 0.4, 0.6, 0.8 and 1 ml) where '0' served as blank. The volume was made up to 1 mL in all the tubes including the sample tubes by adding distilled water. Then 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath, cooled rapidly and the green to dark green color was read at 630 nm. From the plot of concentration of standard versus absorbance, the amount of carbohydrate present in the sample tube was calculated as the amount of carbohydrate present in 100 mg of the sample = mg glucose x 100 Sample vol (ml)

3.2.11 Determination of surface hydrophobicity (So)

Surface hydrophobicity of HPI and its fractions were determined as described by Haskard and Li-Chan (1998), using 1-anilino-8-naphthalenesulfonate (ANS) as the probe. Protein samples (10 mg of albumin, globulin and protein isolate) were dissolved in 5 ml of 10 mM phosphate buffer (pH 7.0). The solutions were stirred for 1 h at room temperature and centrifuged at 7000g for 20 min. The supernatant was diluted with 10 mM phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 30 to 250 μg/ml. A 40 μl aliquot of 8 mM ANS in 10 mM phosphate buffer (pH 7.0) was added to every 4.0 ml of protein solution. Spectrofluorometer

(JASCO FP-6300) was set at an excitation wavelength of 390 nm and an emission wavelength of 470 nm. The coefficient of linear regression analysis of the FI versus protein concentration (mg/ml) was used as an index of protein surface hydrophobicity (So).

3.2.12 Intrinsic fluorescence emission

The Intrinsic fluorescence measurements were carried out as described by Agboola and Aluko (2009) on a JASCO FP-6300 spectrofluorimeter (JASCO Corporation, Tokyo, Japan). Protein stock solution was prepared by dispersing 10 mg in 1 ml of 0.1 M sodium phosphate buffer, followed by centrifugation and determination of protein content of the supernatant. The supernatant was diluted to 0.02% and 0.002% (w/v) with buffer (0.1 M phosphate buffer, pH 3-8). Protein samples were excited at 257 nm (phenylalanine), 275 (tyrosine), or 295 (tryptophan) nm and emission recorded up to 500 nm at 25 ^{o}C. Emission of the buffer blanks was subtracted from those of the respective samples. The fluorescence intensity (FI) was expressed in arbitrary units. Fmax is the maximum obtained during the wavelength scan, while λmax is the wavelength at Fmax.

3.2.13 Measurements of circular dichroism (CD) spectra

Far- and near-UV CD spectra were measured as described by Agboola and Aluko (2009), using a J-815 spectropolarimeter (JASCO Corporation, Tokyo, Japan) at 25 ^{o}C. The far- UV CD spectrum was measured on 2 mg/ml protein fraction in 0.1 M phosphate buffer (pH 3.0 – 9.0) at 190-240 nm, using a quartz cell with a path length of 0.5 mm. The near-UV spectrum was measured at 250-320 nm under the same condition as above but using a 4 mg/ml protein concentration and quartz cell path length of 1 mm. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of respective buffer spectra. Since the samples contain protein mixtures, the obtained spectra reflect the sum of individual

conformation of the various polypeptide components. Secondary structure fractions were calculated from the far-UV data using the SELCON3 (optimized for 190-240 nm) secondary structure determination algorithm (Lobley, Whitmore, & Wallace, 2002; Whitmore & Wallace, 2004, 2008) available on the DichroWeb website (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

3.2.14 Determination of Functional Properties

3.2.14.1 Protein solubility (PS)

Protein solubility of HPI and protein fractions was determined according to the method described by Ajibola et al. (2016) with modifications. Briefly, 10 mg of protein sample was dispersed in 1 ml of 0.1 M phosphate buffer solutions at a pH 3.0-9.0 range and further diluted to obtain 0.1% (w/v), the resulting mixture was vortexed for 2 min and centrifuged at 10000 x *g* for 20 min. Total protein content was determined by dissolving the protein samples in 0.1 M NaOH solution. The total protein content and the concentration of soluble proteins across pH 3-9 in the phosphate buffer were determined by the Lowry method at 660 nm. Percent protein solubility (PS) was expressed as follows:

PS (%) = (Protein content of sample at certain pH/Total protein content of sample) x 100

3.2.14.2 Water and oil holding capacity (WHC and OHC)

The WHC and OHC were determined using the method of Adebiyi and Aluko (2011) with some modifications. A 3 g sample was dispersed in 25 ml distilled water (or oil) contained in a 50 ml pre-weighed centrifuge tube. The dispersion was vortexed for 1 min, allowed to stand for 30 min, and then centrifuged at 7000 x *g* for 25 min at room temperature. The supernatant was decanted and excess water or oil was drained for 15 min; the milliliters of water or oil retained per gram of sample were calculated.

3.2.14.3 Least gelation concentration

Least gelation concentration was determined as previously described (Aluko et al., 2009) by suspending the protein samples in water at different concentrations (2–20%, w/v). The mixtures were vortexed, placed in a water bath at 95 ^{o}C for 1 h, cooled under tap water and left in the refrigerator (4 ^{o}C) for 14 h. The sample concentration at which the gel did not slip when the tube was inverted was taken as the LGC.

3.2.14.4 Foaming capacity (FC)

Foams were formed as previously described (Aluko et al., 2009) using slurries that were prepared by dispersing 0.1–0.3 g samples in 50 ml graduated centrifuge tubes containing 5 ml 0.1 M phosphate buffer, pH 3.0, 5.0, 7.0, and 9.0. Sample slurries were homogenized at 20,000 rpm for 1 min using a 20 mm foaming shaft on the polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The capacity of the continuous phase to include air (foam capacity) using the mean of three measurements was determined thus;

Foam Capacity (FC) = (volume after whipping-volume before whipping/volume before) x 100.

3.2.14.5 Emulsion formation and oil droplet size measurement

The oil-in-water emulsion was prepared according to the method described by Adebiyi and Aluko (2011) with some modifications. Protein slurries of 10, 25, or 50 mg/ml concentrations were separately prepared in 5 ml of 0.1 M phosphate buffer pH 3.0, 5.0, 7.0, or 9.0 followed by addition of 1 ml of pure canola oil. The oil/water mixture was homogenized at 20,000 rpm for 1 min, using the 20 mm non-foaming shaft on a Polytron PT 3100 homogenizer. The oil droplet size ($d_{3,2}$) of the emulsions was determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as a dispersant. Under constant shearing, emulsion sample taken from the emulsified layers of the samples was added to about 100 ml of water contained in

the small volume wet sample dispersion unit (Hydro 2000S) attached to the instrument until the required level of obscuration is attained. The instrument was set to automatically measure the oil droplet size of each emulsion in triplicate and each sample was prepared in triplicate; the results were used as indicators of emulsifying capacity (EC). Emulsions were kept at room temperature for 30 min without agitation and the oil droplet size distribution and mean particle diameter was measured again to assess emulsion stability (ES) as follows.

Emulsion Stability (ES) = Oil droplet size at 0 min ($d_{3,2}$) x 100 / Oil droplet size after 30 min ($d_{3,2}$)

3.2.15 Statistical analysis

All data were reported as mean ± standard deviation from 3 replicates. One-way ANOVA and Duncan's multiple range tests were used to evaluate statistical significance of difference Differences were considered to be statistically significant at $p < 0.05$. All analyses were conducted using SPSS version 9.4.

CHAPTER FOUR

4. Results and Discussion

4.1 Proximate composition

The proximate compositions of DHF, HPI, 11S, 7S, 2S and spent flour (SF, the residue obtained after extracting protein from the defatted hemp flour) are shown in Table 4.1. The moisture content ranged from 10.23 to 3.11% with DHF having the highest moisture content followed by 2S while SF had the lowest moisture content. The higher moisture content (8.45%) observed in 2S when compared to other fractions could be associated with its high carbohydrate content but lower fat since sugars easily absorbs moisture from the environment. The 11S had significantly higher crude protein content of 97.23% when compared to 7S, 2S and HPI with protein contents of 57.70, 66.34 and 87.14% respectively. The protein contents for HPI and 11S in the present study are similar to those previously reported by Wang et al. (2008) for HPI (90.53%) and 11S (93.02%) while the protein content for 7S (57.70%) in the present study is lower than the one reported for 7S (87.67) by the same author. The low protein contents observed in 7S and 2S could be attributed to high carbohydrate contents and other food components such as fat, ash, and polyphenols that might have been co-extracted with the proteins. Similar results have been obtained for 2S (Ajibola et al., 2016; Mundi and Aluko, 2012) and 7S (Kimura et al., 2008; Wang et al., 2008) of different legumes showing that the two proteins are glycoproteins with covalently bound carbohydrate moieties. The protein content obtained for DF (61.99%) is significantly ($p < 0.05$) higher than the corresponding value (34.10%) for SF, which is expected as most of the proteins had been removed during extraction. The fat content obtained for 11S (6.46%) was significantly higher than those for 7S (5.33%), 2S (0.67%) and HPI (2.1%). The high-fat content observed in 11S and 7S when compared to 2S fraction could be attributed to

Table 4.1: Proximate composition of hemp seed products

	Moisture content	Crude protein	Crude fibre	Fat	Ash	Protein bound CHO
11S	4.84±0.03^{d}	97.23±0.04^{a}	1.12±0.47^{cd}	6.46±0.06^{c}	1.50±0.17^{e}	2.07±0.09^{c}
7S	5.18±0.06^{c}	57.70±0.19^{e}	1.04±0.12^{bc}	5.33±0.42^{d}	8.66±0.04^{c}	10.36±0.53^{a}
2S	8.45±0.03^{b}	66.34±0.01^{c}	0.01±0.01^{e}	0.67±0.09^{f}	6.31±0.06^{d}	10.05±0.49^{a}
HPI	4.11±0.01^{e}	87.14±0.08^{b}	0.11±0.13^{de}	2.14±0.01^{e}	8.63±0.01^{c}	5.16±0.95^{b}
SF	3.11±0.10^{f}	34.10±0.16^{f}	2.60±0.02^{a}	19.55±0.20^{a}	25.34±0.01^{a}	
DF	10.23±0.11^{a}	61.99±0.50^{d}	0.58±0.07^{cd}	10.83±0.19^{b}	10.37±0.43^{b}	

Each value is the mean and standard deviation of triplicate determinations. Different superscript characters (a, b, c, d, e, and f) indicate significant differences at $P < 0.05$ level within a column.

the fact that these proteins were first isolated from the protein solution and there is a possibility of the protein precipitate to contain some of the residual oil present in the defatted hemp seed flour. The fat, ash and crude fiber contents of SF were significantly higher than those of the defatted flour. As expected, a decrease in protein content of SF due to the protein extraction would increase the percentage of other flour components.

4.2 Percentage protein content as determined by Kjehdahl, Lowry and amino acid composition methods

Accurate protein concentration measurements are required when studying the structural and functional properties of proteins. The protein content results from the amino acid analysis are compared with those obtained from Kjedahl and Lowry method (Table 4.2). Except for the 2S protein content that was overestimated by the Lowry method (84 %), the three methods give values that are in relatively good agreement with one another. It has been shown that the presence of other food components such as carbohydrate (Fountoulakis et al., 1992; Noble et., 2007) and polyphenols (Ponomerava et al., 2015; Nwachukwu and Aluko, 2019), which may have been co-extracted with the 2S could have interfered with color development and resulted in overestimation of the protein content.

4.3 Percentage gross yield, protein yield and protein digestibility

Protein recovery of HPI, 11S, 7S and 2S relative to the flour weight (Gross yield) were 41.05%, 37.79 %, 0.83 %, and 1.98% while recovery based on the protein content of flour (Protein yield) were 82.72, 72.70, 1.29 and 3.92%, respectively (Table 4.3). The results showed that 11S globulin is the predominant protein in hemp protein. The summation of the protein yield of all the three fractions (11S, 7S, and 2S) were approximately 78%, which was relatively close to 80%

Table 4.2: Percentage protein content of 11S, 7S, 2S and HPI as determined by Kjeldahl, Lowry and amino acid composition methods

	Kjeldahl method	**Lowry method**	**Amino acids analysis**
11S	97.23±0.04[a]	91.62±1.25[a]	94.36
7S	57.70±0.19[d]	58.00±1.80[c]	54.64
2S	66.34±0.01[c]	84.00±1.72[b]	63.15
HPI	87.14±0.08[b]	91.60±4.04[a]	84.46

Each value is the mean and standard deviation of triplicate determinations. Different superscript characters (a, b, c, d, e, and f) indicate significant differences at $P < 0.05$ level within a column.

protein recovery obtained for HPI. This is in agreement with the previous studies that reported that 11S, 7S, and 2S are the major storage proteins in hemp seed. Employing a similar extraction method to the present study, Tang et al. (2008) reported 71.96 % protein recovery while Malomo et al. (2014) obtained 37.90% yield for hemp seed protein isolate. The low protein recovery was attributed to the processing history of the hemp meal that was used in the Malomo et al. (2014) study. The extent of mechanical force applied during the pressing/extraction of oil from the hemp seed could have a significant impact on the protein structure by causing protein/protein interactions and thus decrease their solubility during extraction and isolation (Malomo and Aluko, 2015).

Protein digestibility is one of the parameters that are used in evaluating protein quality. The complete digestion of food protein requires a combination of proteases rather than single protease. In the present study, a combination of proteases was used to simulate the gastrointestinal enzymatic process that occurs in the normal human digestion of food proteins (Hsu et al., 1977). The cleavage of the peptide bond by proteolytic enzymes during digestion produces H+, which causes a decrease in pH of the reaction mixture. Hence, the faster the pH reduction, the higher the rate of digestion and this can be used as an index of protein digestibility (HS et al., 1977; Malomo and Aluko, 2015). In vitro protein digestibility of HPI, 11S, 7S, and 2S are 88.10, 88.28, 84.48 and 72.54% respectively (Table 2). Protein digestibility of 2S was lower when compared to the other hemp seed proteins. The high content of cysteine (Table 3) and sulfhydryl (Table 2) found in 2S could be responsible for its low digestibility since the presence of disulfide bonds imposes structural restrictions to enzyme attack. The 2S (albumins) are storage proteins with a conserved skeleton of cysteine residues, which form four intermolecular

Table 4.3: Protein content, percentage recovery and protein digestibility of HPI, 11S, 7S and 2S of hemp seed

Samples	**Protein recovery (% defatted flour wt)**	**Protein recovery (% defatted flour protein wt)**	**Protein digestibility (%)**	**Total Sulfhydryl content (μmol/g)**	**Exposed sulfhydryl (μmol/g)**
11S	37.79±1.25^{b}	72.70±2.30^{b}	88.28	1.55±0.22^{b}	0.57±0.04^{c}
7S	0.83±0.07^{c}	1.29±0.11^{c}	84.48	1.51±0.12^{b}	1.32±0.07^{b}
2S	1.98±0.19^{c}	3.92±0.15^{c}	72.54	3.69±0.05^{a}	2.39±0.14^{a}
HPI	41.05±0.25^{a}	82.72±4.36^{a}	88.10	1.97±0.07^{b}	1.16±0.02^{b}

Each value is the mean and standard deviation of triplicate determinations. Different superscript characters (a, b, c, d, e, and f) indicate significant differences at $P < 0.05$ level within a column.

disulfide bonds that are responsible for the stability of the 2S to proteolytic attack (Moreno et al., 2005). House et al. (2010) reported protein digestibility that ranged from 83.50 to 97.50% for hemp products. Similarly, in vitro protein digestibility of hemp protein isolates as measured by nitrogen released was reported to range between 88 and 91% (Wang et al., 2008). These results are similar to the values obtained for hemp seed proteins in the present study. The high protein digestibility of hemp seed proteins is attributed to the absence of protease inhibitors in hemp seed (Wang et al., 2008).

4.4 Total and exposed sulfhydryl (SH)

The results of total and exposed sulfhydryl contents of hemp proteins are presented in Table 4.3. The total (exposed+ buried) and exposed SH contents of 2S are significantly higher than those of 7S, 11S, and HPI. The observed high SH content of 2S could be attributed to the presence of high sulfur-containing amino acids in the protein when compared to other proteins (Table 4.4). The exposure of buried SH has been shown to enhance protein interaction with water (Yildiz et al., 2018; Mundi and Aluko, 2012). The high content of exposed SH of 2S could have contributed to its high solubility. The SH values obtained in the present work is similar to the values previously reported for hemp seed proteins (Tang et al., 2006) and kidney bean proteins (Mundi and Aluko et al., 2012).

4.5 Amino acid composition

The amino acid profiles of HPI, 11S, 7S, and 2S expressed as a percentage of their protein content are presented in Table 4.4. The glutamic and aspartic acid reported in the present study represent glutamic acid plus glutamine and aspartic acid plus asparagine respectively. In the amino acid analysis, standard conditions of acid hydrolysis convert glutamine to glutamic acid

Table 4.4: Percent amino acid composition of HPI and its fractions in *Cannabis sativa* L.

Amino acids	**2S**	**7S**	**11S**	**HPI**	**FAO/WHO suggested requirements (2-5 years)**
Asx	7.50	9.15	11.04	11.60	
Thr	4.14	3.79	3.44	3.49	3.4
Ser	5.01	5.04	5.61	5.36	
Glx	25.63	20.95	18.44	18.13	
Pro	3.93	3.87	3.74	3.64	
Gly	5.75	4.17	4.06	4.18	
Ala	5.86	5.46	5.18	5.19	
Cys	4.88	2.24	1.56	1.22	
Val	3.07	4.87	4.74	5.32	3.5
Met	2.17	2.53	2.48	1.71	
Ile	1.99	3.70	3.81	4.36	2.8
Leu	4.02	6.34	6.61	6.90	6.6
Tyr	2.46	3.14	3.70	3.53	
Phe	1.43	3.67	4.49	4.78	
His	3.20	3.14	2.93	2.91	1.9
Lys	6.36	6.45	3.44	3.28	5.8
Arg	12.45	10.79	13.55	13.24	
Trp	0.18	0.70	1.19	1.14	1.1
HAA	30.86	38.45	40.00	40.80	
AAA	4.07	7.51	9.38	9.45	6.3
NCAA	33.13	30.1	29.04	29.73	
PCAA	22.01	20.38	19.92	19.43	
SCAA	7.05	4.77	4.04	2.93	2.5
EAA	26.56	35.19	33.13	33.89	32.8
BCAA	9.08	14.91	15.16	16.58	
ARG/LYS ratio	1.96	1.67	3.94	4.04	

Asx= aspartic acid + asparagine; Glx= glutamic acid + glutamine; AAA= aromatic amino acids; BCAA= branched- chain amino acids; HAA= hydrophobic amino acids; NCAA= negatively charged amino acids; PCAA = positively charged amino acids; SCAA = sulfur-containing amino acids.

and asparagine to aspartic acid (Wu, 2009). While glutamic acid is the predominant amino acid, hemp proteins are also rich in arginine, aspartic acid, serine, and leucine. This result is in agreement with the previous results reported by Wang et al. (2008) and Tang et al. (2006). High glutamic and aspartic acid contents of hemp proteins are beneficial as these amino acids are the major metabolic fuels for mammalian enterocytes (Wu, 2013). The amino acid profile of HPI is similar to that of 11S and both are limiting in lysine. On the other hand, 7S is rich in lysine but limiting in tryptophan. All the four proteins most especially 2S have a remarkable amount of sulfur-containing amino acids and higher than those reported for hemp proteins by Wang et al (2008). Although 2S is rich in lysine and sulfur-containing amino acids, it is limiting in valine, isoleucine, leucine, and tryptophan. Sulfur-containing amino acids have been shown to have a protective effect against oxidative damage, which could contribute to the antioxidant benefits of hemp seed protein consumption (Girgih et al., 2011). The total essential amino acids of HPI, 11S, and 7S meet the suggested human requirements for 2-5 years old while that of 2S is lower than the suggested requirement. Considering the amino acid profile and the protein digestibility, 2S has lower nutritional value when compared to other fractions and the HPI. Arginine is a sole precursor of nitric oxide, an endothelium-derived relaxing factor that acts as a signal molecule in vasodilation and involves a wide variety of regulatory mechanisms of the cardiovascular system (Boger and Ron, 2005). A growing body of literature suggests L-arginine supplementation as a potential therapeutic approach in hypertension due to its ability to increase the bioavailability of nitric oxide by increasing its production and preventing its quenching by superoxide (Rajapakse and Mattson, 2009). Hence, the consumption of hemp seed proteins with high arginine content could enhance cardiovascular health. The ratio of arginine to lysine was higher in HPI and 11S when compared to 7S and 2S. A higher ratio of arginine to lysine in the diet has been reported to

have a beneficial effect in lowering blood cholesterol and thereby contribute to overall cardiovascular health (Giroux et al., 1999). The arginine to lysine ratio obtained for hemp proteins in this work is in close agreement with 1.74 and 4.37 reported for hemp seed albumin and globulin (Malomo and Aluko, 2015). Branched-chain amino acids (BCAAs) are amino acids with branched aliphatic side chains which include valine, leucine, and isoleucine. They are part of indispensable amino acids not only because they cannot be synthesized in the body but also because they play remarkable metabolic and regulatory roles. About 40% of the total protein required by mammals and 35% of muscle protein essential amino acids are BCAAs (Tamanna and Mahmood, 2014). BCAAs enhance protein synthesis, improve metabolic process, improve immune function, reduce oxidative stress and improve gut health (Ichikawa et al., 2010; Kawaguchi et al., 2011). Hemp seed proteins are rich sources of BCAAs except for 2S, which was limiting in these specific amino acids.

4.6 Electrophoresis (SDS-PAGE)

The SDS-PAGE profiles of protein constituents of HPI, 11S, 7S, and 2S in the presence and absence of 2-ME are presented in Figure 1, and the estimated molecular weight (MW) and relative contents of different subunits under non-reduced and reduced conditions are summarized in Table 4.5 and 4.6. The 2S profile under non-reduced condition has seven polypeptides (163, 148, 114, 80, 19 and 15 kDa) with the 15 kDa in the highest proportion. The 7S profile under non-reduced conditions confirmed the presence of four polypeptides (155, 100, 49, and 15 kDa) with the 15 kDa in the highest proportion. A similar 47 kDa polypeptide was reported for 7S hemp protein fraction by Wang et al. (2008) which corresponds to the β-conglycinin (51 kDa) of 7S soybean protein fraction (Riblett et al., 2001). The non-reduced 11S had five polypeptide

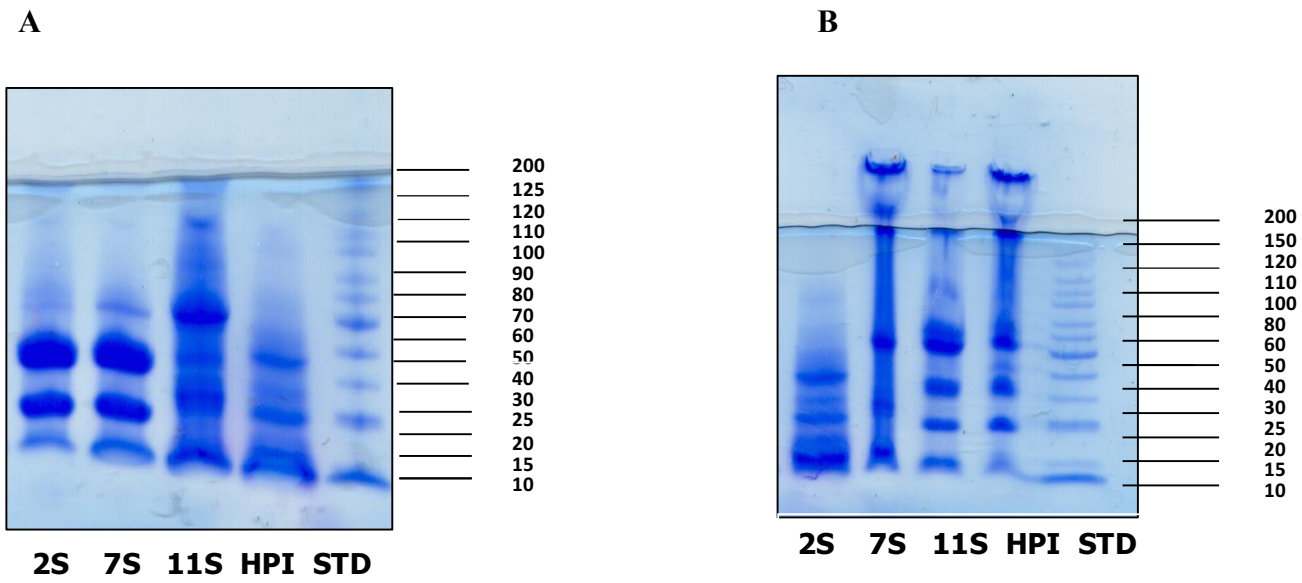

Figure 4.1: SDS-PAGE of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S) under reducing (A) and non-reducing (B) conditions. STD, standard protein molecular weight markers

Table 4.5: Molecular weight (MW) and relative content of the major subunits of hemp seed protein isolate (HPI), 2S, 7S and 11S under non-reduced condition

Samples		**1**	**2**	**3**	**4**	**5**	**6**
2S	MW (kDa)	163.44	148.30	114.14	80.21	19.10	15.67
	Relative content (%)	18.61	7.43	15.55	19.06	10.61	26.82
7S	MW (kDa)	155.20	100.98	49.33	15.68		
	Relative content (%)	11.00	10.77	13.77	64.51		
11S	MW (kDa)	163.44	117.58	83.01	51.35	15.67	
	Relative content (%)	6.32	6.96	15.50	26.77	44.46	
HPI	MW (kDa)	163.44	117.58	80.22	47.36	15.67	
	Relative content (%)	2.16	6.13	7.75	15.24	68.73	

Table 4.6: Molecular weight (MW) and relative content of the major subunits of hemp seed protein isolate (HPI), 2S, 7S and 11S under reduced condition

Samples		**1**	**2**	**3**	**4**	**5**	**6**	**7**
2S	MW (kDa)	126.46	103.42	79.56	18.44	12.65		
	Relative content (%)	3.99	16.26	27.81	16.46	35.48		
7S	MW (kDa)	156.81	106.05	85.00	20.00	15.00		
	Relative content (%)	8.44	15.17	23.83	8.11	44.44		
11S	MW (kDa)	157.81	103.42	82.23	63.94	20.00	15.00	
	Relative content (%)	16.21	16.96	6.93	20.26	8.14	31.49	
HPI	MW (kDa)	186.49	172.56	114.88	101.36	87.85	21.74	15.00
	Relative content (%)	7.00	10.92	11.38	9.52	23.59	10.81	26.69

bands that are similar to non-reduced HPI and contains both basic subunit (18 to 20 kDa) and acidic subunit (30 to 40 kDa). In addition to the acidic and basic subunits, polypeptides with MW of 47, 83, 117 and 163 kDa were present in both non-reduced 11S and HPI. Under reduced condition (A), 2S fraction had five bands (126, 103, 79, 18 and 12 kDa). The disappearance of the 2S 163, 148 and 114 kDa in the presence of mercaptoethanol suggests that these polypeptides contain inter-molecular disulfide bond which is in agreement with its high cysteine and sulfhydryl contents (Table 4.3 and 4.4). The polypeptide profile of 7S under reduced condition showed five polypeptides (156, 106, 85, 20 and 15 kDa). The reduced 11S and HPI showed the presence of basic subunit (18 to 20 kDa) and acidic subunit (30 to 40 kDa) of edestin with less intensity when compared to non-reduced (Figure 4.1). In addition to the acidic and basic subunits, polypeptides with higher molecular weights (186 and 172 kDa) were found in the reduced HPI which were absent in non-reduced. It could be that these proteins were not soluble under non-reduced conditions. Poor solubility of hemp proteins has been attributed to disassociation and /re-association of disulfide bonds between AS and BS of edestin (Tang et al., 2006 and Wang et al., 2008).

4.7 Surface hydrophobicity (So)

Protein surface hydrophobicity (So) is one of the structural characteristics to evaluate the change in protein conformation and is known to be closely related to its functional properties (Arzeni et al., 2012). It is an index of the number of non-polar groups (hydrophobic group) on the surface of a protein molecule in contact with the polar (aqueous) environment (Hu et al., 2013). Figure 4.2 shows the pH-dependent So of HPI and its fractions. The So was obtained at pH 3.0 for all the hemp seed proteins, which decreased with an increase in pH. It has been demonstrated that So measurement using ANS (anionic fluorescent probe) may be influenced by the contribution of

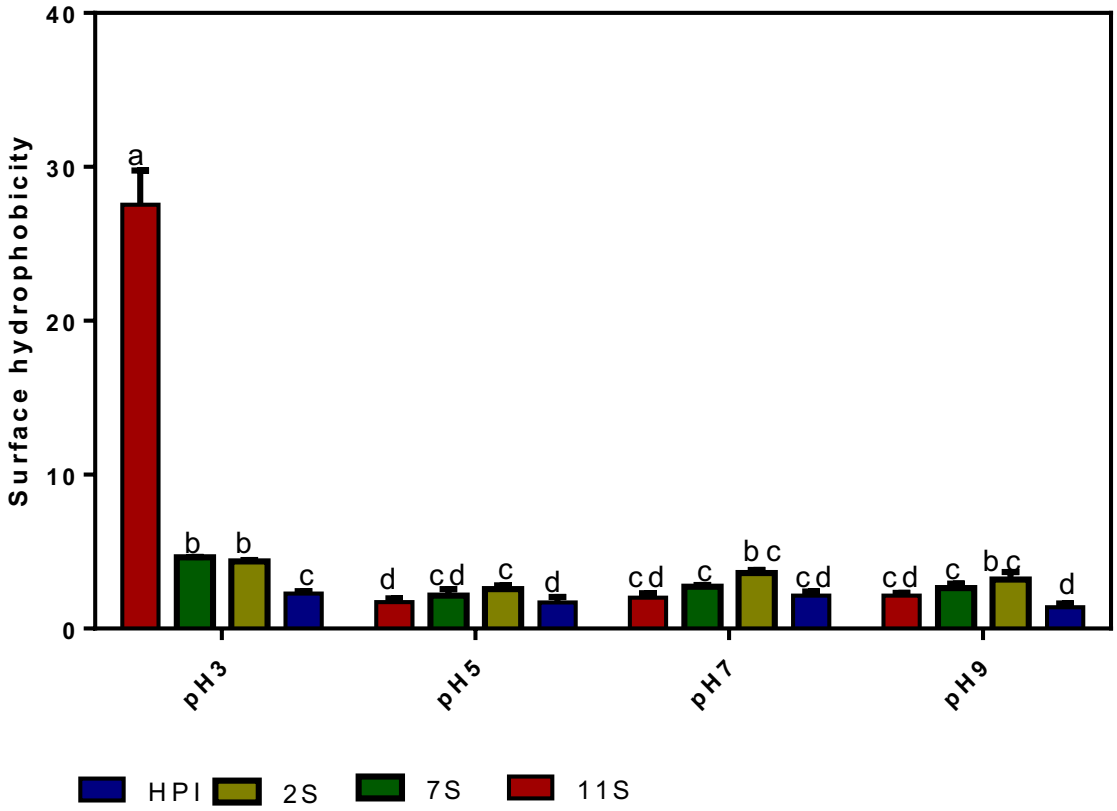

Figure 4.2: pH-dependent surface hydrophobicity of hemp seed protein isolate (HPI) and protein fractions (2S, 7S, and 11S). Different letters (a, b, c) indicate significant differences at $P < 0.05$ level for each pH value.

electrostatic interaction (Alizadeh-Pasdar & Li-Chan, 2000). In addition to hydrophobic interactions between the protein and ANS, at pH 3.0, the anionic probes may interact with positively charged sites on the proteins by electrostatic interaction which will enhance more ANS binding to protein and result in an increase in fluorescence. Hence, the observed high surface hydrophobicity at pH 3.0 may be due to both the contribution of hydrophobic and electrostatic interactions between ANS and protein, since an increase in fluorescence is used as a measure of protein So. On the other hand, at values above pH 3.0, the protein tends to lose its positive charge and at pH above the isoelectric point, it becomes negatively charged, which will result in enhanced repulsion between the ANS and protein and reduced ANS binding (lower So). The present results are in agreement with those reported for flax seed albumin and globulin where ANS hydrophobicity was highest at pH 3.0 but decreased as the pH changed from acidic to basic (Nwachukwu and Aluko, 2018). Similar results were also obtained for whey protein peptides where ANS hydrophobicity was highest at pH 3.0 but decreased at pH 5.0, 7.0, and 9.0 (Alizadeh-Pasdar & Li-Chan, 2000).

4.8 Intrinsic fluorescence emission

Intrinsic fluorescence properties of a protein that contains all three aromatic amino acids depend mainly on tryptophan residues fluorescence intensity (FI) because of the absorbance at the wavelength of excitation and the quantum yield of tryptophan residues are considerably greater than the respective values for tyrosine and phenylalanine (Schmid, 1987). The fluorescence emission of Trp that is buried in a hydrophobic environment is maximum at 330 nm (λmax) while that of the Trp that is located in the polar environment is maximum at >330 nm. In the present study, the Trp residues of all the hemp seed proteins are present in a “polar” environment having their λmax at 330-345 nm at all the pH values (Figure 4.3). The λmax of Trp residue

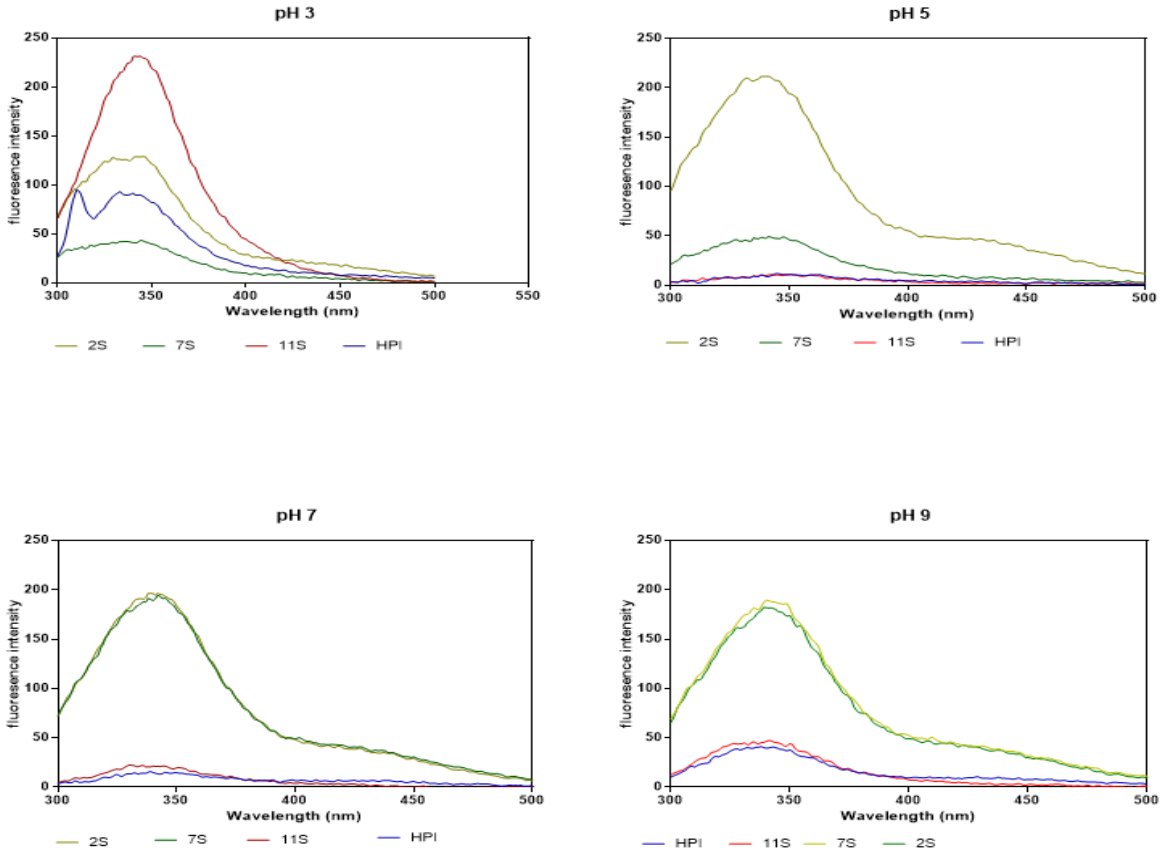

Fig 4.3: Intrinsic fluorescence intensity (arbitrary units) of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S) at different pH values

obtained in this work is lower than the 350-360 nm reported for albumin and globulin of hemp proteins by Malomo and Aluko (2015), although they are both located in polar environments in the two studies. Clara et al. (2007) reported a λmax of 344 and 340 nm for 7S and 11S of soybean proteins, respectively, a result that is similar to those reported in this work. At pH 3.0, the 11S fraction exhibited the highest FI when compared to HPI and other fractions, with a major peak at the λmax of 344 nm. At pH 5.0, which is near to the isoelectric point of the hemp seed proteins, the FI of 11S and HPI reduced drastically while there was an increase in FI of 2S and 7S with 2S having the highest. As the pH moved from the isoelectric point to a more alkaline environment (pH 7.0 and pH 9.0), there was an increase in FI of all the proteins, which was more pronounced in 2S and 7S. The fluorescence data of the proteins at different pH values seem to be related to their pH-dependent solubility. The high solubility of 2S at all the pH values (Figure. 4.3) indicates a good interaction of the protein with the aqueous environment, which will lead to a shift of the aromatic residues to the hydrophobic interior and thus higher FI. At acidic pH (pH 3.0), 11S, 7S, and HPI are relatively soluble, their interaction with the aqueous environment would enhance the shift of the aromatic residue to the hydrophobic environment. This could be attributed to a higher FI obtained for 11S and HPI at pH 3.0 when compared to those of the other pH values. The observed reduction in FI of 11S and HPI at pH 5.0 could be due to high protein-protein aggregation with low solubility that normally occurs at the isoelectric point, which can shield the tryptophan residue from excitation light and thereby result in low FI (Ajibola et al., 2006). As the pH moved from isoelectric point, there would be an increase in net charge due to deprotonation of carboxyl and amine group and the aromatic amino acid residues would tend to move toward the non-polar environment, which favors high FI (Ajibola et al., 2016). The effect of the net charge was more pronounced on FI of 2S and 7S because these two proteins are more

soluble at alkaline pH when compared to HPI and 11S that are sparingly soluble at this pH. The intrinsic fluorescence data obtained for 11S and HPI in this study are in agreement with those reported for HPI by Malomo and Aluko (2014), who observed the quenching of FI of HPI at pH 5.0 and increase in FI at both acidic and alkaline pH.

4.9 Secondary structure conformation

The effect of pH on secondary structures conformation of 2S, 7S, 11S, and HPI are shown in Figure 4.4 and Table 4.7. At pH 3.0, 2S showed a strong secondary structure dominated mostly by the α- helix conformation (80%) as evident in the intense ellipticity between 200 and 220 nm while 7S, 11S, and HPI showed secondary structures that are dominated by β-sheet conformation. As the pH increases, there was a reduction in α- helix proportion of 2S, which was accompanied by an increase in β-sheet and unordered structure. The data suggest that the secondary structure of the 2S might be associated with the net charge on the surface of the protein. Malomo and Aluko (2014) reported a decrease in α-helix conformation of hempseed protein isolate with an increase in pH values, a result that is similar to the present result. For 7S, an increase in pH produced a strong secondary structure that is dominated mostly by β-sheet conformation and less α-helix content. Similarly, as the environment changed from acidic to alkaline, 11S exhibited more of β-sheet secondary conformation with zero α-helix content at pH 9.0. HPI exhibited similar secondary structures at pH 3.0 and 5.0 that are characterized by high β-sheet content. As the pH increased from pH 5.0 to pH 7.0, there was an increase in α-helix content of HPI from 1.85% to 17% accompanied by a reduction in β-sheet content.

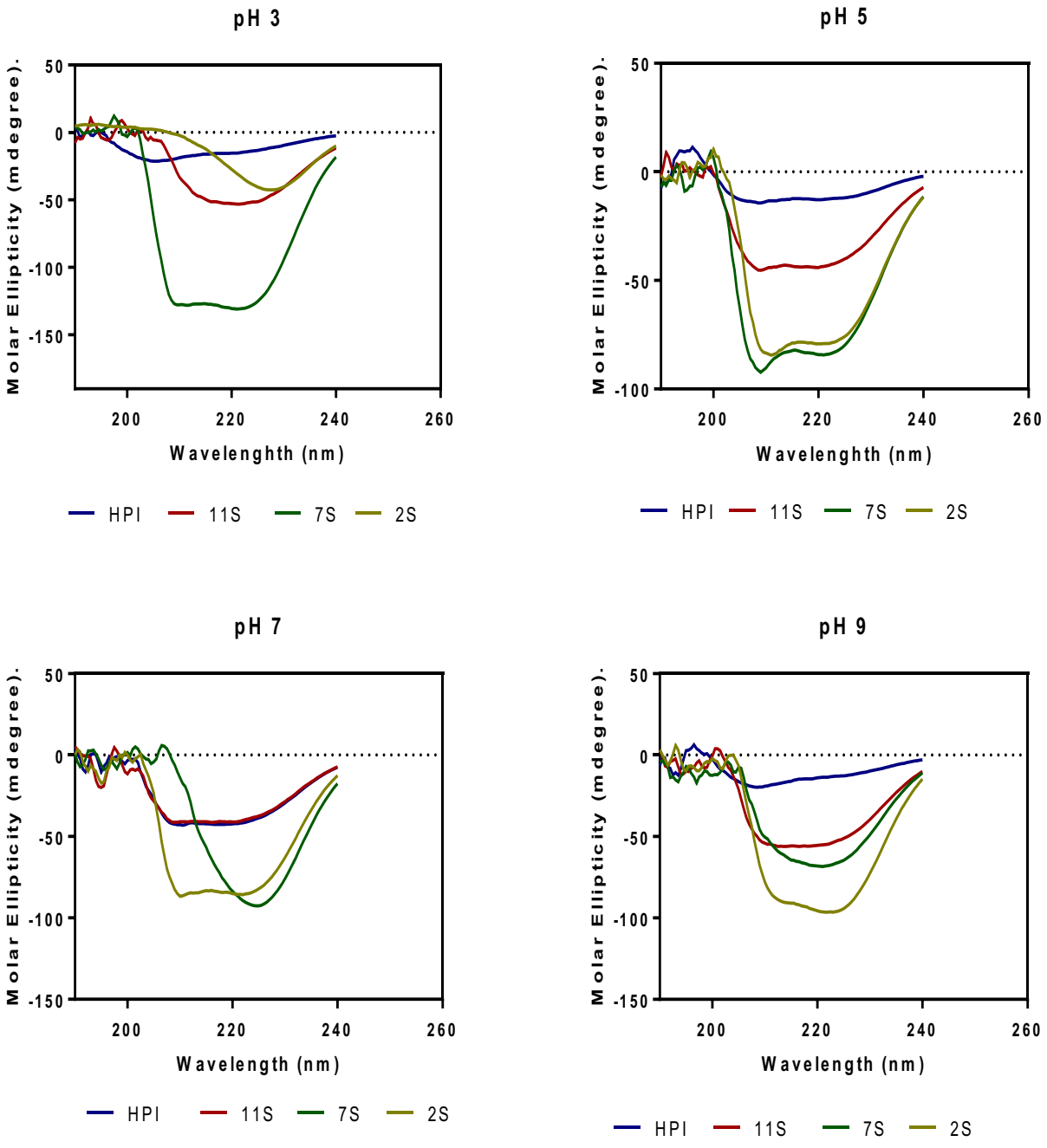

Figure 4.4: Far-UV circular dichroism spectra of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S) at different pH values

Table:4.7: Circular dichroism-derived protein secondary structure composition of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S) at different pH values

pH	Samples	α-Helix (%)	β-Strand (%)	β-Turns (%)	Unordered (%)
pH 3	2S	80.40±0.00	0.70±0.00	5.95±0.01	1.53±0.03
	7S	4.40±0.00	31.18±0.00	17.30±0.00	46.66±0.00
	11S	3.10±0.01	37.45±0.02	17.45±0.01	43.65±0.00
	HPI	2.20±0.01	43.45±0.03	19.45±0.01	34.95±0.00
pH 5	2S	18.60±0.00	3.90±0.01	12.50±0.03	65.05±0.04
	7S	1.40±0.00	39.50±0.00	20.35±0.00	39.05±0.00
	11S	1.55±0.00	43.25±0.02	21.30±0.01	35.50±0.00
	HPI	1.85±0.00	41.50±0.01	20.30±0.00	36.35±0.01
pH 7	2S	1.35±0.01	37.55±0.02	17.55±0.00	47.05±0.01
	7S	2.20±0.00	40.80±0.02	19.05±0.03	38.85±0.03
	11S	3.45±0.00	44.25±0.00	20.30±0.00	32.20±0.00
	HPI	17.40±0.01	26.05±0.01	20.20±0.04	36.15±0.04
pH 9	2S	4.55±0.02	34.30±0.00	17.30±0.00	46.90±0.00
	7S	3.30±0.00	37.10±0.00	17.05±0.00	46.60±0.00
	11S	0.00±0.00	55.45±0.02	23.60±0.01	24.15±0.00
	HPI	0.00±0.00	45.95±0.01	13.70±0.00	39.99±0.03

An increase in α-helix content of HPI produced a stronger secondary structure conformation when compared to the structures obtained at pH 3.0 and 5.0. When the environment was changed to pH 9.0, the helix content of HPI was reduced to zero and accompanied by an increase in β-sheet content. Except for the 2S that has a strong α-helix conformation at pH 3.0, the secondary structures of all the proteins is mostly dominated by β-sheet conformation at all the pH values. The far-UV data obtained for the proteins suggest the pH-dependent variations in electrostatic interaction could change the secondary conformation of the proteins. The present results are in agreement with Choi and Ma (2007) who reported that buckwheat globulin possesses higher contents of β-sheet strands than α-helix at pH 3–11.

4.10 The effect of pH on tertiary structure conformation

The CD spectra in the region 250 to 320 nm (near-UV spectra) arise from the aromatic amino acids such as Trp, Tyr, and Phe. Thus, the actual shape and magnitude of the near-UV spectrum of a protein depend on the number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups, and polarizability) as well as their spatial disposition in the protein (Kelly et al., 2005). The effects of different pH values on tertiary structure conformations of 2S, 7S, 11S, and HPI as analyzed by near-UV CD spectroscopic technique are shown in Figure 4.8. At pH 3.0, albumin showed a more organized tertiary structure when compared to those of 7S, 11S and HPI as evident in the Phe (250-260 nm), Tyr (270 to 280 nm) and Trp (290-305 nm) peaks. Although 7S has a tertiary structure that is similar to that of 2S at pH 3.0 in terms of Phe and Tyr peaks, this protein lacks a prominent Trp peak that was found in 2S. The lack of a distinct tryptophan transition (290-295 nm) in 7S indicates a complete exposure of this residue to a hydrophilic environment or they are not located in an asymmetric environment (Kelly et al., 2005; Ajibola et al., 2017).

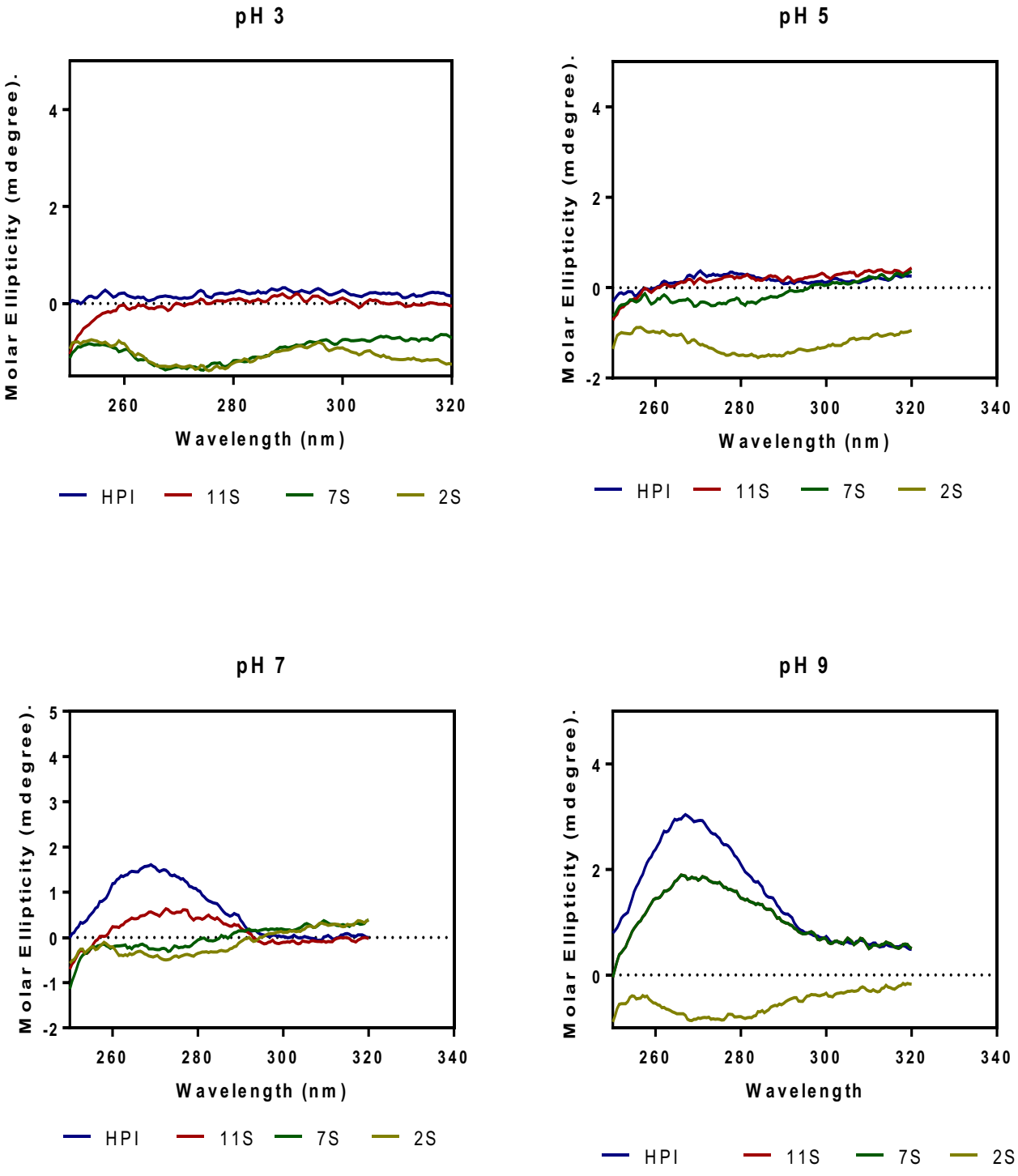

Figure 4.5: Near-UV circular dichroism spectra of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S) at different pH values

At pH 3.0, the 11S showed a weak Phe peak and lacks both the Tyr and Trp peaks, which indicate a less organized structure while the HPI showed almost no organized structure as evident in the almost zero ellipticity values. As pH increases from pH 3.0 to pH 9.0, the 2S showed a structural conformation that lacks CD ellipticity at 290 -295 nm, which indicates a complete exposure of Trp residues to a polar environment. Similar losses in CD ellipticity at 280 nm have been reported for other proteins (Ajibola et al., 2017; Tang et al., 2012; Liu and Tang, 2013). The results suggest that 2S exhibited a more organized tertiary structure at pH 3.0 when compared to other pH values. At pH 5.0 and 7.0, the 7S exhibited a tertiary structure that is similar to those of 2S, although, the Tyr peak intensities were higher than those of 7S. The 7S Tyr peak intensity was smaller at pH 5.0 and 7.0 when compared to that of pH 3.0, which indicates structural rearrangements that pushed tyrosine residues to a more polar environment or simply a more disordered protein conformation. At pH 8.0, the 7S showed a prominent positive Tyr peak at 280-283 nm that was different from that of 2S with a weak Phe peak at 255 nm, which indicates a rigid conformation with Tyr in a hydrophobic pocket. An increase in pH led to the improved structural rigidity of 11S and HPI as evident in increased Tyr peaks, which are more prominent at 9.0. At pH 9.0, the 7S, 11S, and HPI exhibited similar structural conformation with prominent positive Tyr peaks at 280 nm. While 7S and 11S exhibited similar Tyr peak intensity, the intensity of the HPI Tyr peak was higher than those of 7S and 11S. An increase in negative charges as the pH moves towards alkaline pH (pH 9.0) would produce a more hydrophilic environment that favors structural rearrangement that relocates tyrosine into the more hydrophobic environment due to the hydrophobic nature of tyrosine. Although the 2S exhibited tertiary structure that is similar to that of 7S at pH 7.0 and 5.0, the overall results showed that the 2S albumin had tertiary structural conformation in which the Phe structures were

more visible at all the pH values when compared to 11S and HPI. Tang and Wang (2010) reported a similar result in which the albumin fraction of buckwheat protein exhibited a completely different patter of near-UV CD spectrum with more Phe structure when compared to the globulin fraction.

4.11 Protein solubility profiles of hemp seed proteins

The solubility of proteins is the most important factor for their physicochemical properties such as gelation, emulsification, and foaming (Kimura et al., 2008). Figure 4.6 shows the pH-dependent solubility of 11S, 7S, 2S, and HPI. The results show that HPI and 11S had similar solubility profile in which the proteins are fairly soluble at acidic pH value and characterized by minimal solubility around the isoelectric point (pH 4.0 - 5.0) and sparingly soluble at alkaline value. The similar PS profile of the HPI and 11S may be due to the similarity in their amino acid and polypeptide profiles, which is in agreement with the protein yield showing that 11S globulin (Edestin) is the predominant protein in HPI. The observed low solubility of 11S and HPI could be attributed to their high content of hydrophobic amino acids, which enhance protein-protein interaction and result in protein aggregation and weak interactions with the water environment. The results are consistent with previous reports that hemp seed proteins exhibited poor solubility (Tang et al., 2006; Malomo and Aluko, 2014). High surface hydrophobicity can lead to lower solubility due to the attraction of hydrophobic regions of the protein molecules, causing aggregation. However, surface hydrophobicity is not always a good indicator of solubility because the hydrophobicity/hydrophilicity is also an important factor. For instance, the 11S fraction had the highest surface hydrophobicity of 28 at pH 3.0 when compared to other pH values. However, the highest solubility of 60% was obtained for this protein at pH 3.0 when compared to other pH values. At pH 3.0, the 11S may have a high ratio of hydrophilic to

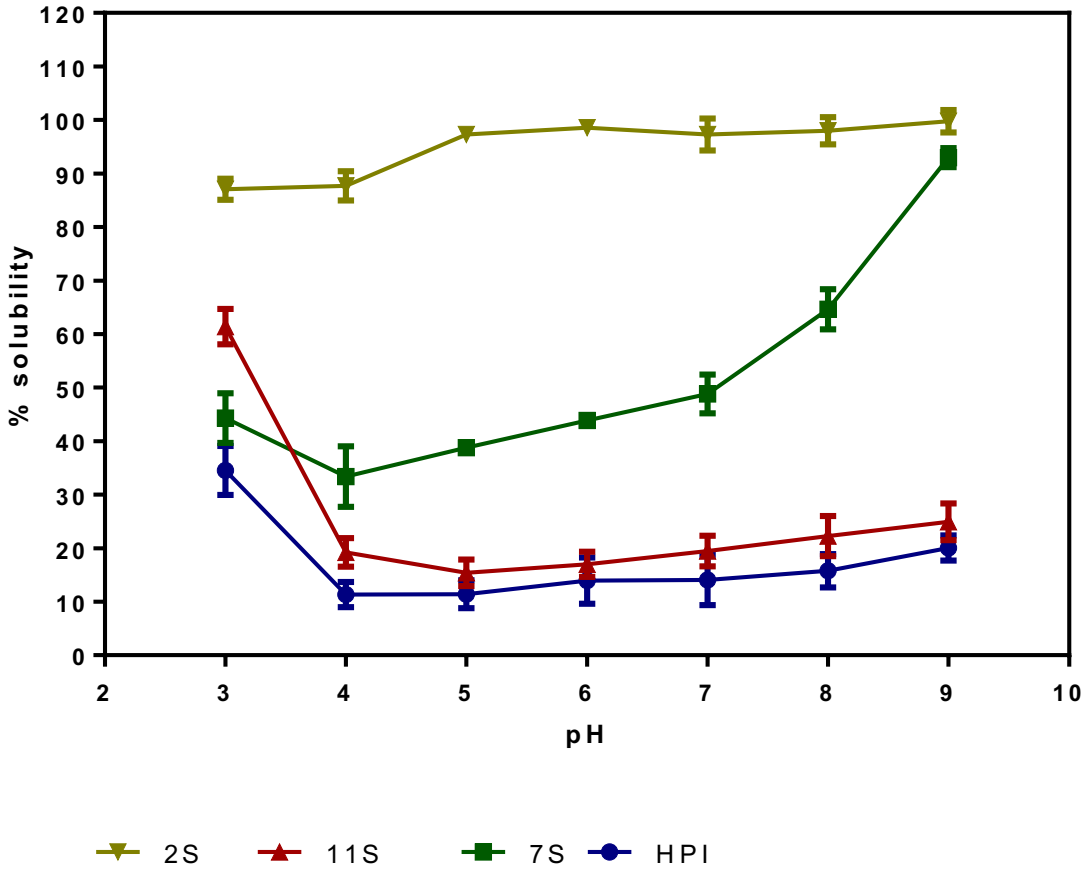

Figure 4.6: Protein solubility profiles of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S) at different pH values

hydrophobic groups on its surface, which enhances its solubility, hence, the high solubility. The PS profile of 7S shows that the protein is fairly soluble at acid pH, characterized by minimal solubility around the isoelectric point and high solubility at alkaline pH. The observed high solubility of 7S at alkaline pH may be associated with its smaller polypeptides, high non-fiber carbohydrate (10%) and increase in charge, which could enhance its structural flexibility and interaction with water environment when compared to 11S and HPI with bigger polypeptides and low non-fiber carbohydrate. The 2S fraction was highly soluble over a wide pH range with value reaching 100 % solubility at alkaline pH. Similar results have been reported for other isolated seed albumins from hemp seed (*Canabis sativa* L) (Malomo & Aluko, 2015), flaxseed (*Linum ussitatissimum*) (Nwachukwu & Aluko, 2018), yellow field pea protein (*Pisum sativum* L) (Adebiyi & Aluko, 2011), African yam bean (*Sphenostylis stenocarpa*) (Ajibola et al., 2016), sunflower (*Heliantus anuu*s L) (Gonzalez-Perez,et al., 2005) and kidney bean **(***Phaseolus vulgaris* L**)** (Mundi & Aluko, 2012). The presence of non-fiber carbohydrates (10% carbohydrate content), low molecular weight polypeptides, low hydrophobic amino acids coupled with high positively and negatively charged amino acids in 2S may have contributed to its high solubility.

4.12 Oil and water absorption capacity

FAC/OAC is defined as the amount of fat/oil that can be absorbed per gram of protein. It is the ability of non-polar side chains of protein to interact with aliphatic chains of oil/fat. Table 4.5 shows similar OHC for the 2S (11.04 g/g) and HPI (10. 32 g/g), which were significantly higher ($p < 0.05$) than the values obtained for 7S (4.93 g/g) and 11S (5.97 g/g). The OHC obtained for HPI in this study is comparable to the values of 13.7 g/g reported by Malomo and Aluko (2014) but higher than the 5.27 g/g reported by Tang et al. (2006) for hemp seed protein isolate. The oil holding capacity of a protein has been reported to be a function of several parameters such as

4.8: Oil and water absorption capacity (g) and least gelation concentration (%) of hemp seed protein isolate (HPI) and fractions (2S, 7S, and 11S)

Samples	**Water absorption capacity (g)**	**Oil absorption capacity (g)**	**Least gelation Concentration (%)**
11S	3.63 ± 0.03^{c}	5.97 ± 0.08^{b}	30.00
7S	4.09 ± 0.21^{b}	4.93 ± 0.51^{b}	10.00
2S		11.04 ± 0.01^{a}	14.00
HPI	5.81 ± 0.01^{a}	10.32 ± 0.01^{a}	22.00

Each value is the mean and standard deviation of triplicate determinations. Different superscript characters (a, b, c) indicate significant differences at $P < 0.05$ level within a column.

physical entrapment of oil, protein surface area, size, charge, and hydrophobicity (Ajibola et al., 2016). As all the four proteins seem to have similar surface hydrophobicity at neutral pH (pH 7.0) (Figure 4.5), the results suggest that besides their surface hydrophobicity, other parameters such as protein surface area, size and charge could have contributed to the high OHC obtained for 2S and HPI in this study. The high OHC of HPI and 2S fraction could be utilized in the food industry for the formulation of meat substitutes, ground meat, baked goods, extenders, and soups (Malomo and Aluko, 2014).

Water holding capacity (WHC) is the ability of proteins to physically hold water against gravity and it is commonly expressed as the amount of water absorbed per gram of protein (Moure et al., 2006). The WHC of HPI (5. 81 g/g) was significantly higher than the values obtained for 7S (4.09 g/g) and 11S (3.63 g/g) (Table 4.). WHC of 2S was not reported due to complete solubility in water. Ajibola et al. (2016) also obtained no value for albumin fraction of African yam bean protein because of its complete solubility in water, a result that is similar to the present work. The complete solubility of 2S could be justified by its high non-fiber carbohydrate content (10% carbohydrate content), low molecular weight polypeptide, low hydrophobic amino acids coupled with high positively and negatively charge amino acids. Proteins with high solubility have been shown to exhibit low WHC (Deng et al., 2011; El- Adawy, 2000). WAC of proteins is attributed to several factors such as the number of charged residues, conformational characteristics, the hydrophilic-hydrophobic balance of amino acids, pH, temperature, ionic strength, and protein concentration. Other factors that have been reported to influence WHC of proteins include experimental procedures (sample preparation), protein structure and denaturation, the presence of hydrophilic carbohydrates and other non-protein components (Toews and Wang, 2013; Withana-Gamage et al., 2011).

4.13 Least gelation concentration (LGC)

The ability of food proteins to form gels on heating is important in the preparation of many foods such as puddings, jellies, pasta, baked goods and in many desserts and meat applications. The protein's ability to form gels is traditionally measured by the LGC, which may be defined as the lowest protein concentration required to form a self-supporting gel that does not slide along the test tube walls in the inverted position (Moure et al., 2006). Hence, lower LGC values indicate the better gelling ability of the protein. The LGC of 2S, 7S, 11S and HPI, were 14, 10, 30 and 22 % (w/v) respectively (Table 4.5). The results show that 7S and 2S fractions have a higher ability to form a protein gel when compared to the 11S and HPI. The ability of the 7S and 2S to form a gel at lower protein concentrations could be associated with their smaller polypeptides and a high percentage of non-protein components (Table 4.1). The ability of a protein to form a gel is not only a function of protein quantity and solubility but could also be related to the type of protein as well as to the non-protein components (Ragab et al., 2004). The poor gelling properties of 11S and HPI could probably be due to their poor solubility after heating at neutral pH. This limits the unfolding of protein to form the tridimensional network and reduce their gel-forming capacity.

4.14 Foaming capacity and stability of hemp seed proteins

Foams are formed when many gas bubbles are trapped in a liquid or a solid (Tay et al., 2006). It is an important protein function that is utilized in the food system for aeration and whipping. The most crucial requirement for foam formation during a whipping is the ability of a surfactant to rapidly reduce the free energy (interfacial tension) and form a continuous and highly viscous film at foam interfaces (Tay et al., 2006). The foaming capacity of HPI and its fraction at different pH values are shown in Figure 4.7. Foaming capacity of 2S fraction was significantly higher ($p < 0.05$) than those of 7S, 11S, and HPI at all the pH values. Tay et al. (2006) reported that the 2S

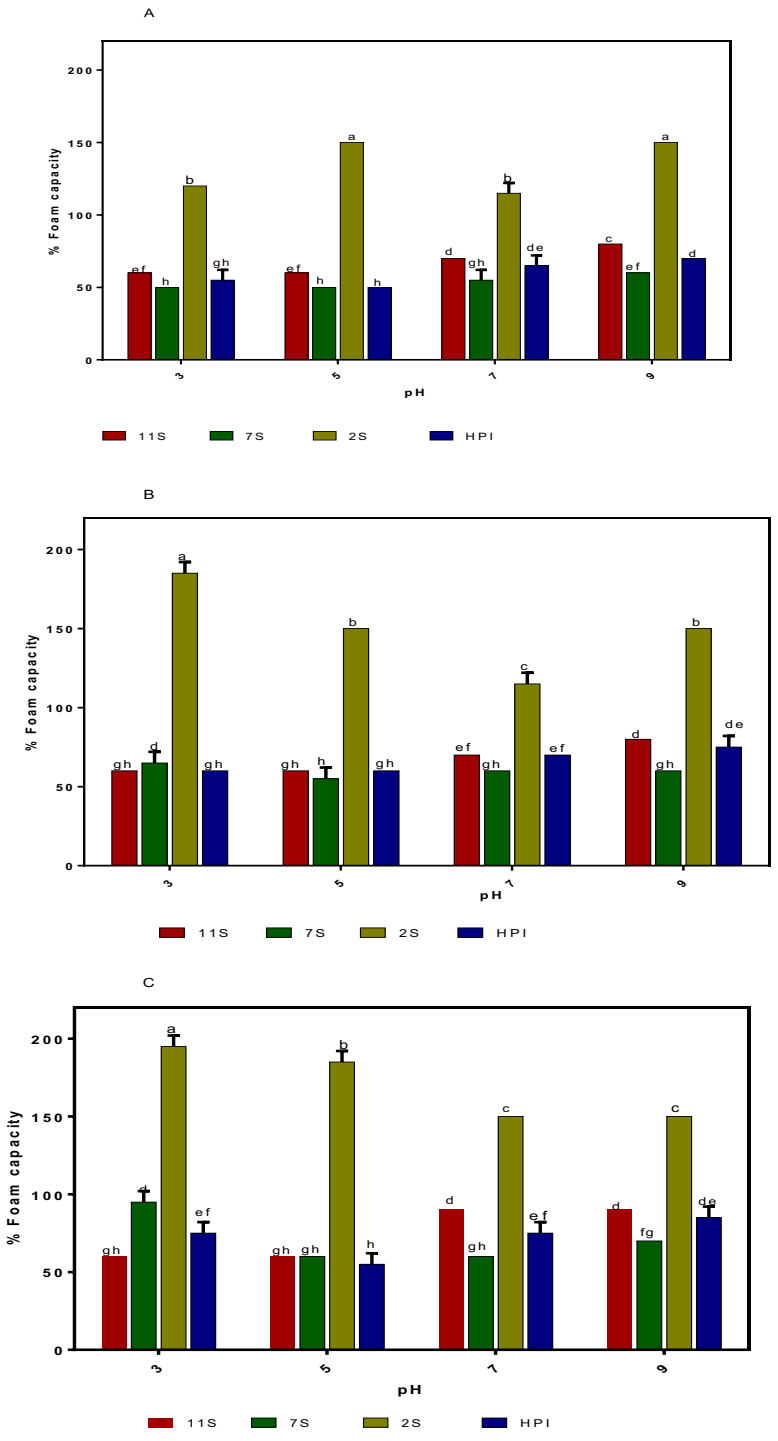

Figure 4.7: Foaming capacity of hemp seed proteins at different pH values: (A) 20 mg/mL, (B) 40 mg/mL, and (C) 60 mg/mL protein concentrations. Different letters (a, b, c) indicate significant differences at $P < 0.05$ level for each pH value.

fraction of soy protein isolates exhibited better foaming properties when compared to those of 7S and 11S of soy protein, a result that is in agreement with the present results. Similar results have been reported for the albumin fraction of commercial yellow field pea protein (Adebiyi and Aluko, 2011) and African yam bean protein (Ajibola et al., 2016). The better foaming capacity observed in 2S fraction might be due to its smaller polypeptides, flexibility and high solubility index when compared to other 7S, 11S, and HPI, which are highly ordered globular proteins. Solubility enhances protein unfolding and the formation of interfacial protein film at the air-water interface, which enhances the encapsulation of air bubbles (Sai-Ut et al., 2009). The foaming capacity of hemp proteins seems to be concentration-dependent, which increased as the concentration of the protein increased. These results are in agreement with the work of Ajibola et al. (2016) and Lawal et al. (2005) who reported an increase in foam capacity of African yam bean protein and African locust bean (*Parkia biglobosa*) respectively as the protein concentration increased. On the other hand, Malomo and Aluko (2015) reported a decrease in foam capacity as protein concentration increased. Ajibola et al. (2016) reported that better foaming observed in African yam bean protein at higher protein concentration might be due to the availability of more protein molecules to encapsulate a higher number of air particles

Figure 4. 8 shows that 2S has lower foaming stability when compared to the 7S, 11S, and HPI. It has been reported that the higher the hydrophobicity of a protein fraction, the more stable the film that forms at the air/water interface (Tay et al., 2006). The observed low foaming stability of 2S might be due to the low content of hydrophobic amino acids, which reduce protein-protein interaction, thus form a weaker interfacial membrane. On the other hand, the high hydrophobic amino acid content of 7S, 11S, and HPI will improve protein-protein interaction and form a strong interfacial membrane that can prevent coalescence of air bubble. Foaming stability seems

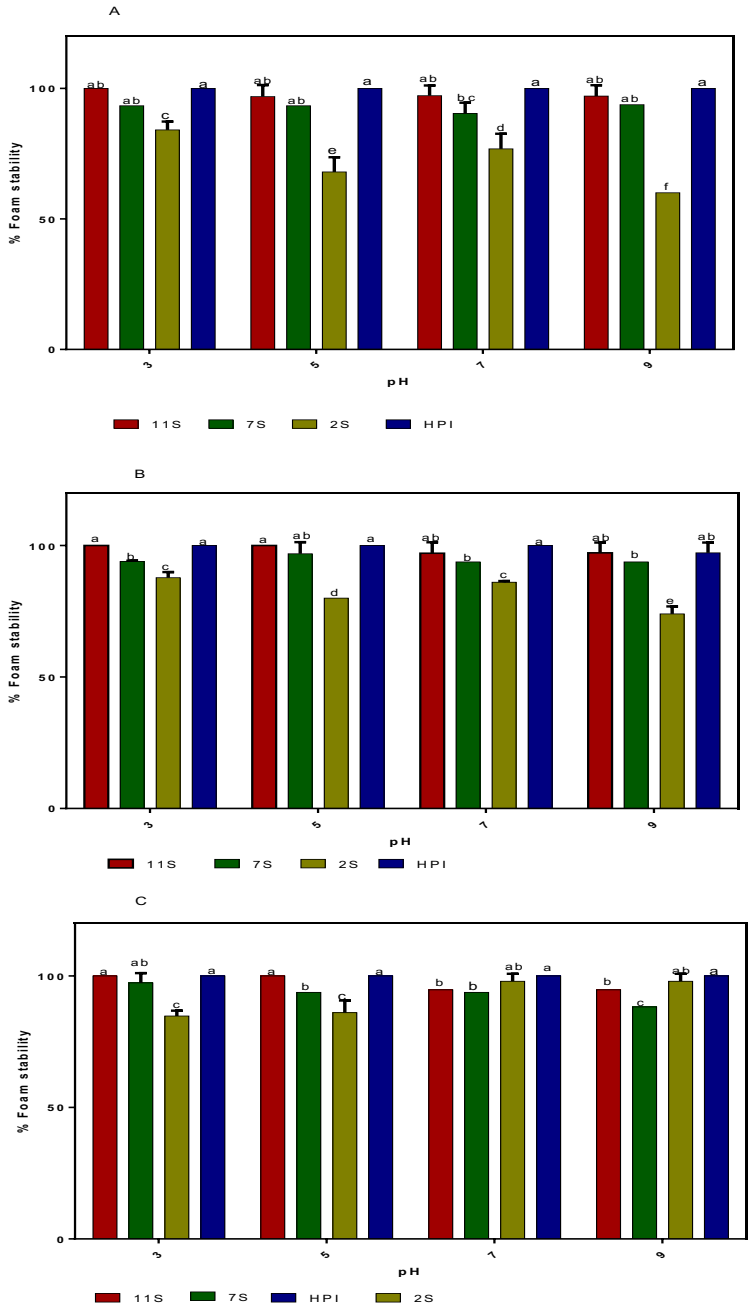

Figure 4.8: Foam stability of hemp seed proteins at different pH values: (A) 20 mg/mL, (B) 40 mg/mL, and (C) 60 mg/mL protein concentrations. Different letters (a, b, c) indicate significant differences at $P < 0.05$ level for each pH value.

to be concentration-dependent which increases as protein concentration increased. An increase in protein concentration would enhance protein-protein interactions, improve solution viscosity and facilitate the formation of multilayer interfacial protein film that is less susceptible to drainage and film rupture (Lawal et al., 2005, Ajibola et al., 2016).

4.15 Emulsion formation and stability of hemp seed proteins

The emulsifying capacity of 2S, 7S 11S, and HPI was analyzed by measuring the oil droplet size of emulsions formed and stabilized by these proteins at different pH and protein concentrations. Emulsion quality is inversely related to oil droplet size because small size (wider surface area) is an indication of the good interfacial property of the emulsifier. The 2S fraction consistently formed smaller oil droplet size at all the pH values and the three concentrations (Figure 4.10). This shows that 2S has better emulsifying properties when compared to 7S, 11S, and HPI that formed bigger oil droplet particle sizes. The results are in agreement with Tay et al. (2006) who showed that the 2S fraction of soybean protein exhibited better emulsifying capacity when compared to 7S and 11S. The mechanism of protein-stabilized emulsion involves rapid diffusion of the protein to the air/water or oil/water interface, adsorption, and reorientation to form a film leading to a reduction in the interfacial tension. The high emulsifying properties observed in 2S could be attributed to its flexibility which enables it to undergo rapid rearrangement at the oil and water interface. In contrast, the low surface-active properties of 7S, 11S, and HPI could be due to emulsifying ability of a protein are surface hydrophobicity, solubility, structural stability that contribute to the balance of hydrophobicity and hydrophilicity and suitable conformational change at the interface between oil and water (Tay et al., 2006). At pH 3.0 and 5.0, the 11S had

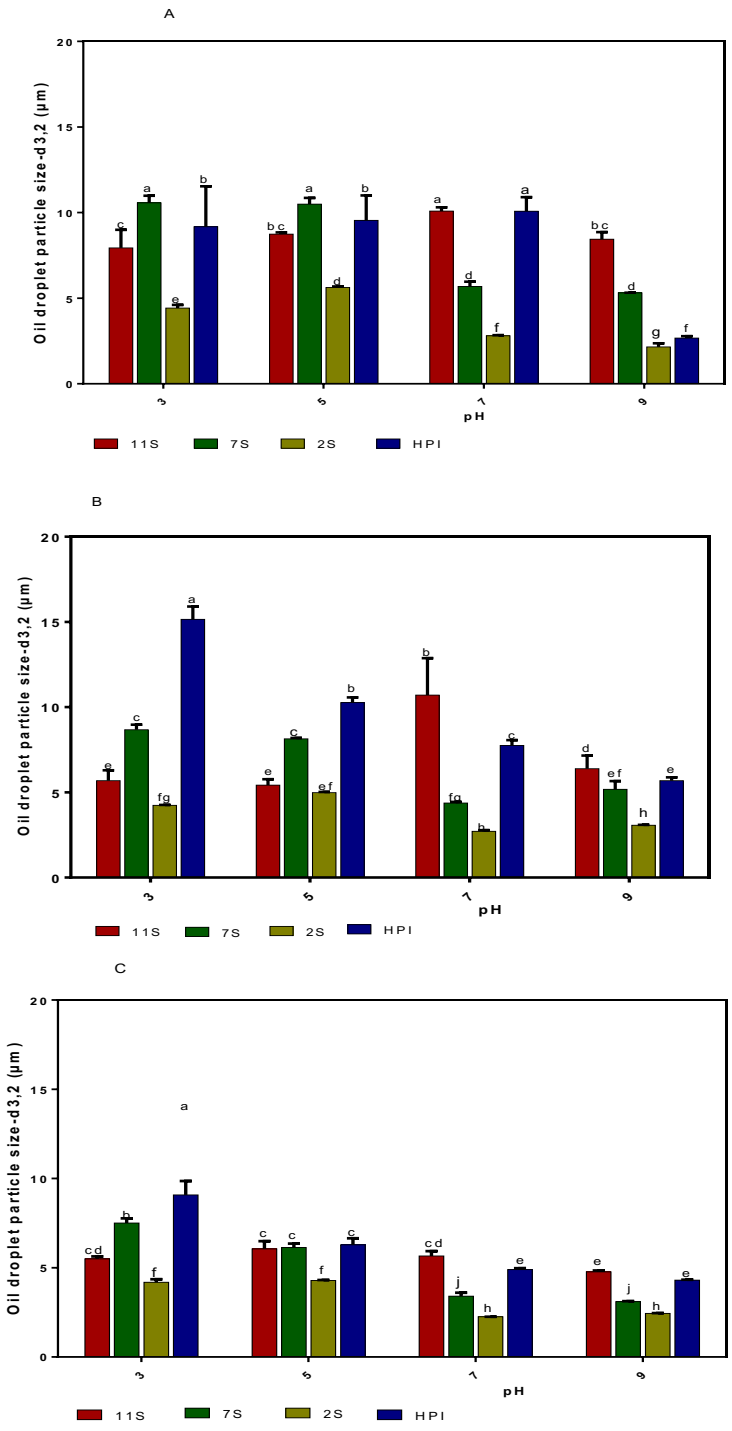

Figure 4.9: Oil droplet sizes of emulsions formed by hemp seed proteins at different pH values: (A) 10 mg/ml, (B) 25 mg/ml, and (C) 50 mg/ml protein concentrations. Different letters (a, b, c) indicate significant differences at $P < 0.05$ level for each pH value.

better emulsifying capacity when compared to 7S and HPI, whereas at pH 7.0 and 9.0, the 7S had better emulsifying capacity when compared to 11S and HPI across the 3 concentrations. The better emulsifying capacity observed in 7S at alkaline pH values suggests increased ability of the protein to unfold and encapsulate oil droplets particles. On the other hand, the 11S fraction has a higher ability at acidic pH values to unfold and encapsulate oil droplets. These results are consistent with their solubility profile, which is a prerequisite for emulsifying capacity. With the exemption of HPI in which emulsifying capacity decreased with an increase in protein concentration at pH 3.0, there was an increase in emulsifying capacity of all the proteins as indicated by a reduction in their oil droplet sizes as the protein concentration increased from 10 to 50 mg/ml at all the pH values. As the protein concentration increases, more protein would be available within the continuous phase to form more interfacial membranes and encapsulate a higher number of oil droplets. A similar decrease in oil droplet size as protein concentration has been reported for hemp seed proteins (Malomo and Aluko, 2015). In contrast, Nwachukwu and Aluko (2018) reported an increase in oil droplet size of emulsion stabilized by flax seed proteins as the protein concentration increased.

Emulsifying stability of emulsion stabilized by 2S, 7S, 11S, and HPI are shown in Figure 4.10. The 2S, 7S and HPI stabilized emulsions exhibited better emulsifying stability at acidic pH values when compared to those of alkaline pH while 11S exhibited better emulsion stability at alkaline pH when compared to acidic pH. All the proteins exhibited better emulsifying stability as the protein concentration increased. An increase in protein concentration from 10 mg to 50 mg/ml would enhance the formation of thicker interfacial membranes and minimize oil droplet coalescence, thus higher emulsifying stability. At all the pH values, the 7S emulsions had monomodal oil droplet size distribution while HPI had bimodal oil droplet size distribution

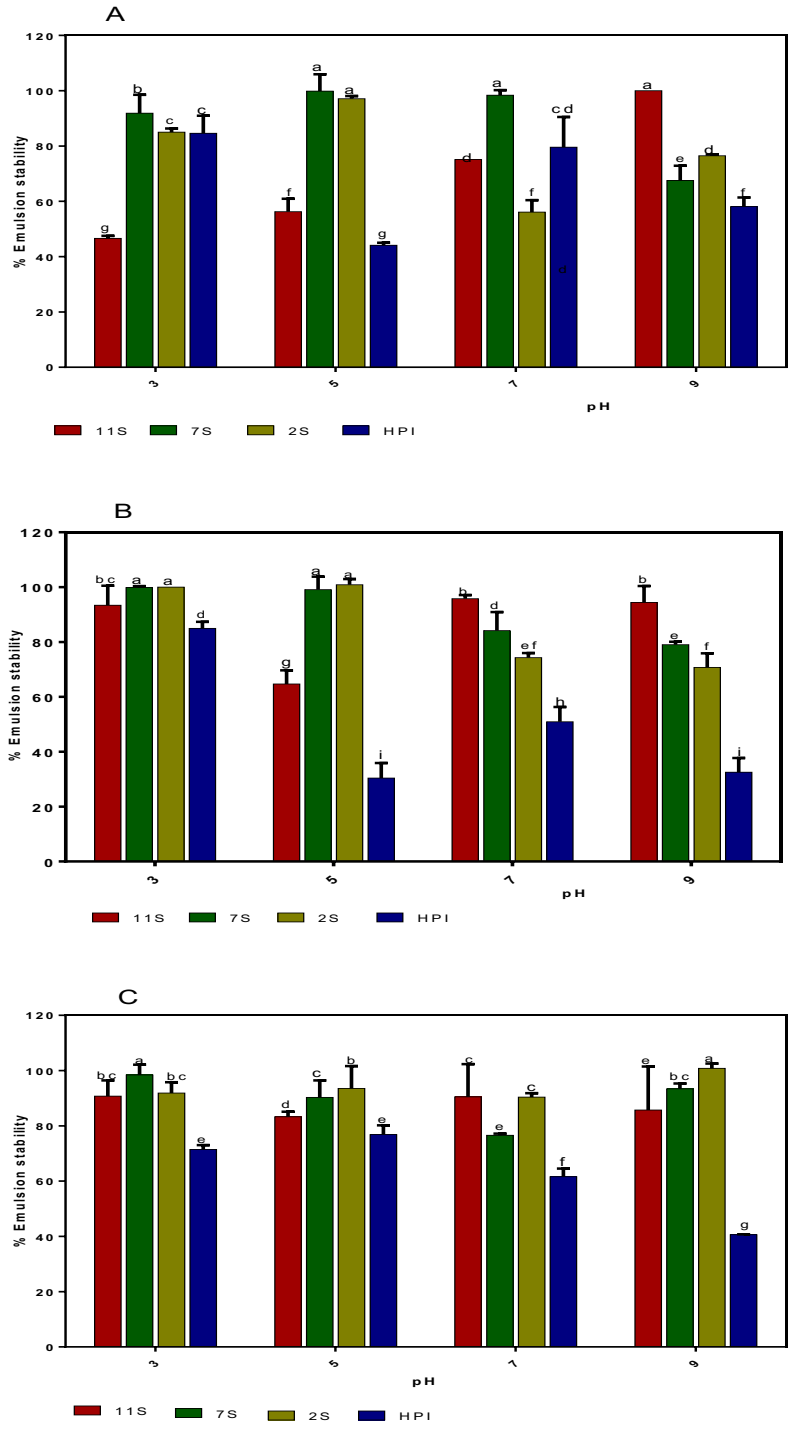

Figure 4.10: Emulsion stability formed by hemp seed proteins at different pH values: (A) 10 mg/ml, (B) 25 mg/ml, and (C) 50 mg/ml protein concentrations. Different letters (a, b, c) indicate significant differences at $P < 0.05$ level for each pH value.

across the 3 concentrations, which indicates uniform distribution. At pH 3.0 and 5.0, the 11S emulsions had monomodal oil droplet size distribution across the 3 concentrations. At pH 7.0 and 10 mg/ml protein concentration, the 11S emulsions had monomodal oil droplet size distribution which became bimodal at 25 and 50 mg/ml protein concentration. At pH 9.0 and 10-25 mg protein concentration, 11S emulsion had bimodal oil droplet size distribution and exhibited monomodal at 50 mg. At pH 3.0-5.0 and 10 mg protein concentrations, the 2S fraction had monomodal oil droplet size distribution and became bimodal at 25-50 mg. At pH 7.0 -9.0 and 10-25 mg protein concentrations, the 2S emulsion had bimodal oil droplet size distribution which became monomodal at 50 mg. Overall, only the 7S emulsion exhibited uniform oil droplet size distribution, which seemed not to be affected by pH and protein concentration while oil droplet size distribution patterns of 2S, 11S and HPI were pH-concentration dependent.

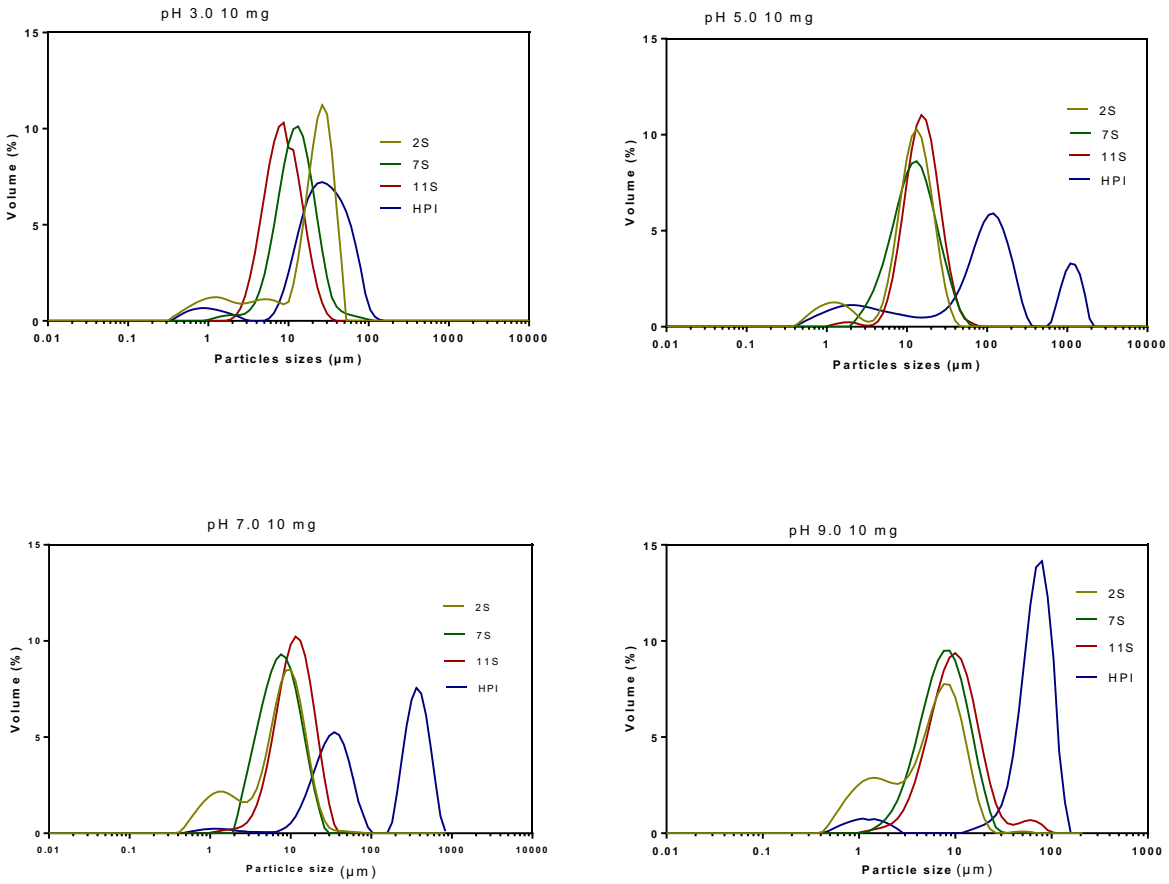

Fig. 4.11: Oil droplet size distribution of emulsions stabilized by 2S, 7S, 11S and HPI of hemp seed at 10 mg/ml at pH 3.0, 5.0, 7.0 and 9.0.

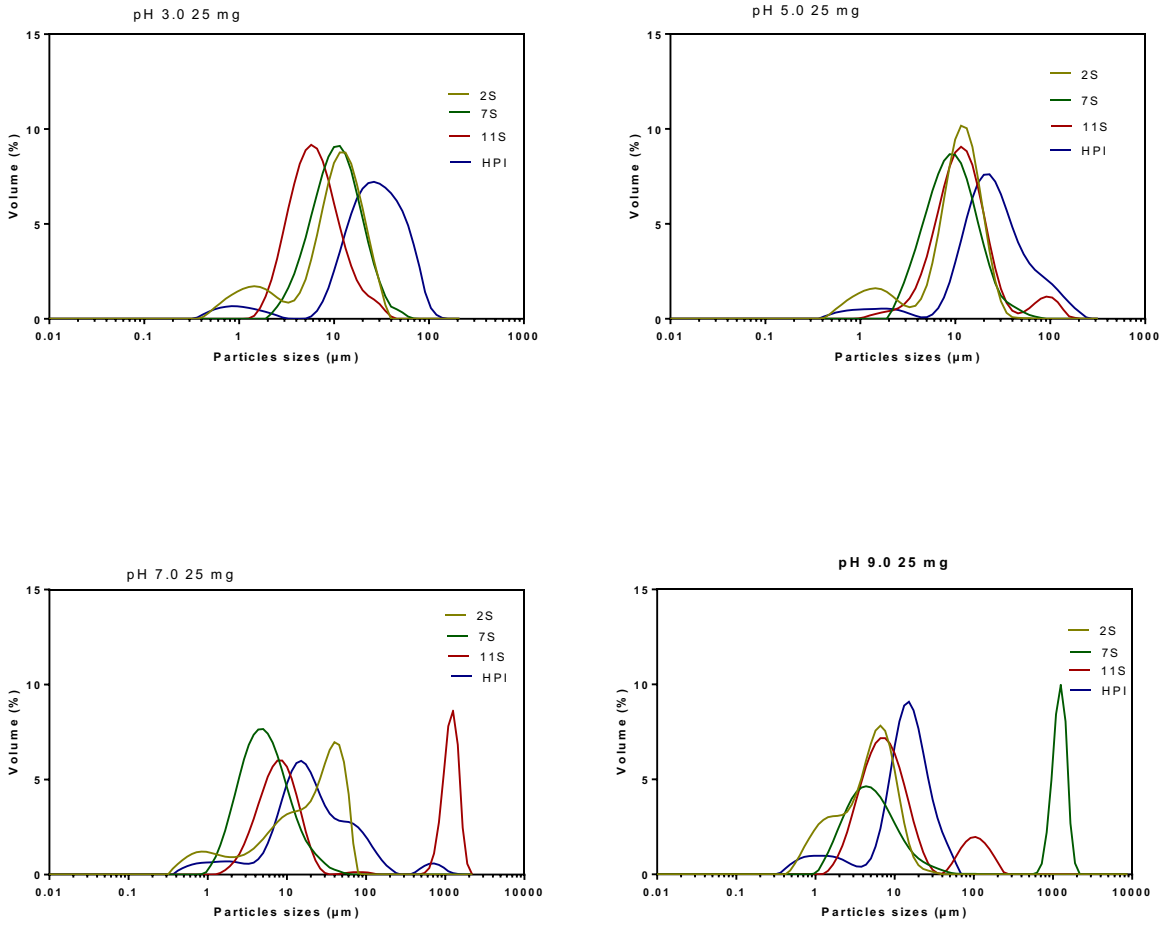

Fig. 4.12: Oil droplet size distribution of emulsions stabilized by 2S, 7S, 11S and HPI of hemp seed at 25 mg/ml at pH3.0, 5.0, 7.0 and 9.0

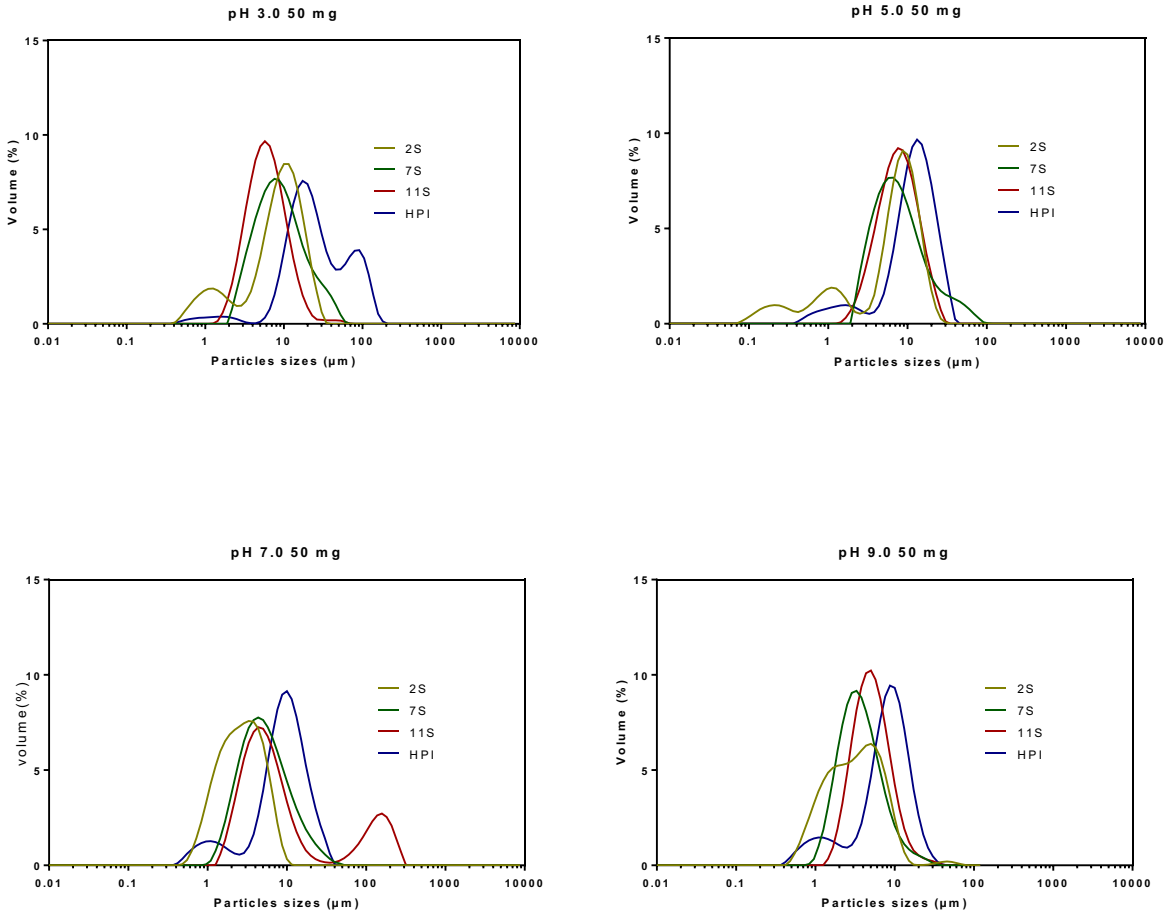

Fig. 4.13: Oil droplet size distribution of emulsions stabilized by 2S, 7S, 11S and HPI of hemp seed at 50 mg/ml at pH 3.0, 5.0, 7.0 and 9.0

CONCLUSIONS

As the hemp seed protein becomes very popular due to its high nutritional quality, this work was designed to examine the potential use of the hemp seed protein fractions as functional ingredients in food formulation by evaluating the structural and functional properties of the 2S, 7S, 11S in relation to the hemp seed protein isolate (HPI).

The study showed that the 11S fraction is the predominant protein in hemp seed and accounted for 72% of the total seed proteins. The 2S and 7S fractions are glycoproteins that contain 10% covalently bound sugar residues. Results of the in vitro protein digestibility gave values, which ranged from 72.54 to 88.28%, showing that the proteins are highly digestible with the 2S having least digestibility. The amino acid profile of hemp seed proteins showed that the proteins are rich in glutamic acid, arginine, aspartic acid, serine, leucine, and sulfur-containing amino acids while limiting in tryptophan and lysine. The total essential amino acids of HPI, 11S, and 7S meet the suggested human requirements for 2-5 year olds while that of 2S is lower than the suggested requirements. Considering the amino acid profile and the protein digestibility, the 2S has a lower nutritional value when compared to other fractions and the HPI. Hemp seed proteins have a high ratio of arginine to lysine which could be beneficial in lowering blood cholesterol and contribute to overall cardiovascular health. Hemp seed proteins are also rich sources of branched-chain amino acids except for 2S, which was limiting in some of these branched-chain amino acids.

The SDS-PAGE profiles of the hemp seed proteins revealed that 7S fraction consists of basic subunits (18 kDa to 20 kDa) and high MW polypeptides (47 kDa and 85 kDa) that correspond to the acidic and basic subunits of soy protein 7S fraction (β-conglycinin). The 11S and HPI fractions had similar polypeptides bands that consist of the basic subunit (18 to 20 kDa), acidic subunit (30 to 40 kDa) and other high MW polypeptides. The 2S profile under reduced

conditions has seven polypeptides (125 kDa, 80, 19 and 15 kDa) with the 15 kDa in the highest proportion. The intrinsic fluorescence and near UV data of the proteins at different pH values seem to be related to their pH-dependent solubility and showed that the aromatic amino acids of the protein samples are more exposed to the polar environment. Except for the 2S that has a strong α- helix conformation at pH 3.0, the far-UV data showed that the secondary structures of all the proteins are mostly dominated by β-sheet conformation at all the pH values. The far-UV data obtained for the proteins suggest the pH-dependent variations in electrostatic interactions could change the secondary conformation of the proteins.

Results of the functional properties of hemp seed proteins showed that changes in the protein environment in terms of pH had significant effects on their functional properties. The 11S fraction and HPI are more soluble at the acidic pH when compared to other pH values. The 7S fraction is more soluble at alkaline pH while 2S fractions are relatively soluble at all the pH values. Thus, the solubility of the 2S fraction over a wide pH range is an important characteristic for food formulation with possible use in the formulation of acidic drinks, desserts, non-acidic beverages, and other liquid beverages while 7S fraction could only be used in the formulation of non-acidic beverages. The 2S and HPI exhibited good fat absorption capacity that is important in the formulation of meat substitutes, ground meat, baked goods, extenders, and soups. The ability 7S fraction to form a good gel at a lower concentration (10%) when compared to other fractions could be used in the preparation of many foods such as puddings, jellies, desserts and meat products. The 2S fraction of hemp protein exhibited higher foaming capacity at all the pH values when compared to other fractions and HPI. Hence 2S fraction of hemp protein is a potential ingredient for food foam formation such as ice-cream. By the virtue of their smaller oil droplet sizes, the 2S, 7S, and 11S fractions formed better emulsions when compared to HPI emulsions

that contained bigger oil droplet sizes. The 11S fraction formed better emulsions at acidic pH values while 7S formed better emulsion at alkaline pH values. The results revealed that protein fractions with suitable emulsion properties can be produced from hemp seed and they may be used as functional ingredients in food formulation such as salad dressings, desserts, and mayonnaise.

Overall, the hemp seed protein fractions differ from one another and HPI in terms of amino acid compositions, polypeptides composition, and protein conformation. Functional properties of the protein fractions differ from one another and are superior to HPI in term of solubility, gelation, foaming capacity and emulsifying capacity.

SIGNIFICANCE OF RESEARCH

This research provides new information on the nutritional value of hemp seed proteins in terms of amino acid composition and digestibility and their potential use as food proteins in human nutrition. This study also provides new information about the structural and functional properties of hemp seed protein fractions (2S, 7S, and 11S) and their potential application as a functional food ingredient in food formulation. This work elucidated the effect of pH-dependent structural conformations on the functional properties of the proteins. The study also provides information on which of the hemp seed protein fractions is best suited for a desirable functional property at a specific pH value. This information could enhance the utilization of hemp seed proteins in the food industry to formulate novel food products. This could increase the value-added utilization of hemp seed proteins and thereby enhance the economic value of the hemp industry.

FUTURE RESEARCH

In this study, we have demonstrated that hemp seed protein fractions differ from one another and are superior to HPI in terms of solubility, gelation, foaming capacity, and emulsifying capacity. However, further work is required to use the individual fractions in actual food formulations such as stabilization of food emulsions (salad dressings and mayonnaise) and replacement of animal fat in meats to manufacture low-fat products. Given the fact that the two protein fractions (2S and 7S) that exhibited the best functional properties in this work had the lowest yield, further work is needed to improve their seed contents through plant biology techniques (traditional breeding or genetic modification). Also, since 11S fraction which is the predominant protein in hemp seed exhibited better emulsifying capacity when compared to the HPI, customized mixtures of 11S with other protein fractions may have superior food functionality than the individual fractions. Further research may need to be conducted to elucidate the structure-function relationship of the customized mixture and their potential use in food formulation.

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