WET FRACTIONATION APPROACHES FOR THE PREPARATION OF FUNCTIONAL ISOLATED GREEN LENTIL SEED PROTEINS

By

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ABSTARCT

Lentil seed proteins isolated using isoelectric precipitation and ultrafiltration, as well as various lentil protein fractions, were investigated for their potential to produce high quality and functional protein products from green lentil seeds. Effect of the defatting and separation processes was apparent, with a protein content of 29% for the defatted lentil flour, and 13% - 94% for the isolated lentil proteins and fractions. GLB which was the predominant protein fraction in the lentil seeds also demonstrated superior solubility (84% - 100%) at acidic and alkaline pH values. However, the membrane isolates from NaCl extraction and GLT were equally most soluble (89%) at the neutral pH. Lentil seed proteins were rich in glutamic acid but generally limiting in valine and methionine. PRL had a particularly high cysteine content which was at least 8-fold the level in other isolated lentil proteins and fractions. Isoelectric pH-precipitated isolate (ISO) and ALB were the most digestible (89%) proteins, and all proteins in this study had 1% or less crude fibre and fat content. GLT had a higher oil holding capacity at a 60 mg/mL concentration, while that of ISO was higher at lower concentrations (20 mg/mL). Similarly, GLT had better water holding capacity at pH 3, 5, and 7, while all protein samples demonstrated poor values at pH 9. Oil-in-water emulsion stability displayed a high variation among the different parameters, with the least stable emulsions formed at pH 7. All the samples produced relatively stable foams (above 50%) at all concentrations and pH used; however, ISO recorded the highest foaming capacity (71.15%) at pH 5. In comparison, ISO and ALB also recorded the highest foam stability (100%) at pH 3 and pH 5. Physicochemical properties such as circular dichroism and intrinsic fluorescence spectra demonstrated the significant impact of change in pH and extraction condition on the structure of each isolated and fractionated protein, which accounts for the observed variations in their functional properties. However, in properties such as denaturation temperature and least gelling

concentration, the results were similar with little to no variation observed irrespective of extraction method.

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FOREWARD

This thesis was compiled using the manuscript format and it consists of two manuscripts, which followed immediately after the general introduction and literature review chapters. Manuscript 1 determined the structural and functional properties of membrane-isolated and isoelectric pH precipitated green lentil protein isolates. Manuscript 2 examined the physicochemical and functional properties of green lentil seed protein fractions. A transition statement is provided after manuscript 1 to link it to the next chapter for a consistent flow. The last chapter provides the overall summary of the study and possible future directions.

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LIST OF ABBREVIATIONS

AAA – Aromatic amino acids

ALB – Albumin lentil protein fraction

BCAA – Branched-chain amino acids

CD – Circular dichroism

DEF – Defatted lentil flour

DSC – Differential scanning calorimetry

EAA – Essential amino acids

EC – Emulsifying capacity

ES – Emulsion stability

FC – Foaming capacity

FI – Fluorescence intensity

FS – Foam stability

GLB – Globulin lentil protein fraction

GLT – Glutelin lentil protein fraction

HAA – Hydrophobic amino acids

HC – Heat coagulability

ISO – Isoelectric pH precipitated lentil seed protein isolate

IVPD – *In-vitro* protein digestibility

LGC – Least gelling concentration

MEM_NaOH – Membrane isolated lentil protein using NaOH solution

MEM_NaCl – Membrane isolated lentil protein using NaCl

MW – Molecular weight

NCAA – Negatively charged amino acids

OHC – Oil holding capacity

PAGE – Polyacrylamide gel electrophoresis

PCAA – Positively charged amino acids

PRL – Prolamin lentil protein fraction

PS -Protein solubility

S_o – Surface hydrophobicity

SCAA – Sulfur-containing amino acids

SDS – Sodium dodecyl sulphate

SEM – Scanning electron microscopy

WHC – Water holding capacity

CHAPTER ONE

1.0 INTRODUCTION

Proteins contain varying amounts of amino acids linked via peptide bonds and are the basic components of living cells, which are essential for biochemical functions in the body (Wu, 2016). Owing to the importance of protein, the Food and Nutrition Board (FNB) of the US Institute of Medicine-National Academy of Sciences recommends a daily intake 0.8 g of protein per kg of body weight for adults between 19 – 50 years old (Institute of Medicine, 2005). The United Nations predicts an increase in the global demand for proteins as a result of the projected 50% rise in population between 2000 and 2050. In addition, the health benefits of proteins, as well as socio-economic changes like increased income and urbanization, are vital contributors to this increased protein demand (Henchion et al., 2017).

Primary dietary sources of protein include meat, and meat products, legumes, dairy products, and cereals. However, the global demand for animal-based protein in developing countries is expected to have doubled by 2050 (Robinson & Pozzi, 2011). In North America, plant and animal-based sources account for about 32% and 68%, respectively, of the total protein in human diets (Wu et al., 2014). The high consumption of animal protein is mostly due to the consumer perception of higher protein content and palatability of animal-based foods in comparison with plant-derived foods (Chadd et al., 2002). Also, plant-based proteins are not only limiting in some essential amino acid profile but also contain antinutrients like tannins and trypsin inhibitors(Jarpa-Parra, 2018). Irrespective of these advantages of animal-based protein sources, several other factors, including religious diet restrictions, ethical concerns affecting meat production, high cost, adverse effects on health, and allergenicity, have led to a gradual shift in the preferred protein source of consumers (Chadd et al., 2002). Another major factor propelling this demand for plant-derived protein sources

is the emerging consumer awareness about the detrimental effects of animal husbandry on the environment. In ruminant animals, emission of gases such as carbon dioxide and methane, which are recognized to be greenhouse gases, has been discovered to play a significant role in global warming (Wu et al., 2014). Unlike animal-based food sources such as cattle, for example, plant-based diets emit about 250 times less greenhouse gases (Tilman & Clark, 2014). Moreover, the consumption of meat has been suggested to have negative impacts on health, including an increased risk of cancer and cardiovascular diseases. Red meat, precisely, has been classified as "probably carcinogenic to humans," possibly as a result of the cytotoxic effects that could be caused by generating lipid oxidation products from catalyzing N-nitroso-compounds (Henchion et al., 2017). In addition, the high level of saturated fat present in red meat could result in an increased low-density lipoprotein cholesterol level, which is a major risk factor for cardiovascular diseases (DiNicolantonio et al., 2016; Williams, 2007).

Plant proteins have high fibre content and phytonutrients with significant health benefits, combined with reduced sodium intake, a healthy fat profile, and an absence of cholesterol (Slavin & Lloyd, 2012). In comparison with animal-based proteins, plant-derived protein sources are more abundant, cost less, and seem to be a healthier dietary choice; however, human consumption of plant protein is still limited. They are instead majorly used as feeds during livestock production, resulting in a low percentage of plant-derived proteins that are directly consumed by humans (Day, 2013). Possible reasons for the under-utilization of plant proteins include the presence of anti-nutritional compounds, limiting amino acid profile, large molecular weight and size, low water solubility, and economic costs associated with the their isolation and recovery (Day, 2013; Henchion et al., 2017).

Plant proteins are known to be less easily digestible than animal proteins, and this is mostly due to the presence of anti-nutritional compounds in plants. Therefore, reducing or eliminating these compounds is one of the ways that is being employed to increase the utilization of plant proteins. Many food processing techniques such as cooking, dehulling, soaking, germination, microwave, irradiation, drying, fermentation and extrusion have been shown to improve the nutritional quality of proteins (Sá et al., 2019). Thermal processes, like cooking, have several benefits on plant proteins, such as improving protein digestibility. This is as a result of protein denaturation, which makes the proteins more susceptible to attack by enzymes. Harmful compounds like protease inhibitors can also be leached out during the cooking process (Fawale et al., 2017). Maillard reaction is a reaction between proteins and reducing sugars, which occurs during cooking at high temperatures (>140 ^{o}C). This reaction has been studied to enhance the solubility and other functional properties of proteins, which could improve their ability to be utilized for various purposes. However, it should also be noted that heat may also have some adverse effects on protein, like the production of Maillard reaction products (Liu et al., 2012).

Microwave heating is another thermal process that is considered useful in enhancing protein quality. The radiation energy of the microwave enables the movement of dissolved ions by disrupting hydrogen bonds. This is similar to the events that occur during extrusion, where there are also changes to the secondary protein structure. These changes improve the oil- and water-holding capacity, emulsifying, and foaming properties of the protein (Pojić et al., 2018). In addition, microwave heating also inactivates protease inhibitors and some other undesirable compounds in some legumes (Vagadia et al., 2017). Irradiation is effective in the removal of some unfavourable compounds; however, it has been discovered to reduce the digestibility of proteins by destroying aromatic and sulphur-containing amino acids (Sá et al., 2019).

Protein fractionation is a modern method of isolating a protein that not only improves the protein qualities like digestibility and functional properties but is also considered ~~as~~ a cost-effective method of isolating and recovering different proteins (Lee, 2017). Wet fractionation is the most commonly used process for industrial protein extraction, including alkaline extraction and isoelectric precipitation (Sá et al., 2019). Unlike the salting-out method, isoelectric precipitation does not require the use of salts. Instead, it can be carried out when the pH of the target protein-rich solution reaches the isoelectric point, at which the protein carries no net charge due to the absence of electrostatic repulsions (Lee, 2017). A more recent method of protein extraction is the membrane separation, like the ultrafiltration method, which involves the use of membrane filters from 1 to 1000 kDa. This method results in an improved protein recovery, as well as improved physicochemical and functional properties of plant proteins like legumes (Saxena et al., 2009). These fractionation methods can produce plant protein ingredients such as flours (20 – 30% protein content), enriched flours (30 – 50%), concentrates (50 – 80 %), and isolates (>90%) (Stone et al., 2019).

Isolated or concentrated proteins from vegetal sources are increasingly being used as food ingredients, as a result of their functional properties, which could improve the nutritive and sensory quality of the food (Carbonaro et al., 2015). Soy and wheat are some of the most currently utilized protein concentrates or isolates in the food industry. However, as a result of possible allergenicity associated with them, consumers and subsequently, the food industry, are in search of alternative plant-protein sources. Due to the low cost, high protein content, and wide acceptability, the production of protein isolates from legumes are emerging in the food sector (Ladjal-Ettoumi et al., 2016).

Legumes are one of the significant plant protein sources that are currently being utilized directly or indirectly for human consumption. Dry legumes or pulses are very ubiquitous edible seeds of leguminous crops (*Leguminosae* or *Fabaceae*), which are globally cultivated. Unlike animal protein, Pulses are inexpensive and low in fat and sodium, which makes it unsurprising that it is often called "poor man's meat" (Shevkani et al., 2019). They are high sources of protein, resistant starch, dietary fibre, vitamins, minerals and other phytochemicals like phenols, carotenoids, tocopherols, saponins, phytic acid, and phytosterols (Zhang et al., 2018). Pulses are second to cereals as essential sources of food, but they contain up to 2 to 3 times more protein than cereals, ranging from 16 to 21% in chickpea and 21% in yellow peas to 23% in green lentils (Ladjal-Ettoumi et al., 2016; Lam et al., 2018; Sharif et al., 2018; Shevkani et al., 2019).

Lentils are extensively cultivated and considered as a staple food in several countries. Lentil seeds are lens-shaped and possess a wide range of colors like green, red, orange, yellow, tan, gray and black (based on either their seed coat color or the color of their cotyledons) (Khazaei et al., 2019). The presence of carotenoids which also indicates the anti-cancer properties of lentils, is responsible for the red, yellow or green color variation in lentil seeds. Similarly, varying concentrations of anthocyanin, pro-anthocyanidin and carotenoid present may explain the other different colors that can be found in lentil seeds (Sanderson et al., 2019). The most commonly grown lentils are the green and red lentils, and they represent the major commercial market classes for lentils (Khazaei et al., 2019). Lentils were first introduced into Manitoba and Saskatchewan in the '70s as a result of its favourable price in comparison with the price of cereals at that time (Thavarajah et al., 2009). Canada now produces about 25% of the total world output and sells to over 100 countries every year, which makes Canada the world's largest lentil exporter (Thavarajah et al., 2009). Like other legume seeds, lentils (*Lens culinaris*) are naturally a rich source of proteins (≈ 26%) and a higher

source of total soluble fibres as well as dietary fibres compared to chickpeas (Khazaei et al., 2019). They are rich in more than half of the total amino acids including, arginine, aspartic acid, glutamic acids, and leucine, while they are limiting in some other essential amino acids like threonine, tryptophan, methionine, phenylalanine, and histidine (WHO & FAO, 2007). However, lentils contain a high amount of tannins (0.49% – 1.28%) phytic acid (0.72% – 4.11%) and possess trypsin inhibitors (2.83%), which could reduce protein digestibility (Hefnawy, 2011; Wang & Daun, 2006)

Nevertheless, the occurrence of anti-nutritional compounds can be reduced by processes like soaking, germination, and cooking of lentil seeds (Shi et al., 2017). Apart from macronutrients, they also contain micronutrients like vitamin B complex, vitamin K, iron, zinc, and various phytochemicals like phytosterols, flavonoids, isoflavones, phytic acid, and saponins (Shevkani et al., 2019). These nutrients can be implicated in various health benefits of lentils, including their antioxidant, anti-inflammatory, and lipid-lowering effects (Zhang et al., 2018). Despite these advantages, effective utilization of lentil proteins in the food industry depends mostly on their functional properties. The functional properties of lentil protein isolates are defined by their physical or chemical properties, which depend on the physicochemical and structural properties of the protein. These properties determine how the protein will behave during processing, storage and consumption (Shevkani et al., 2019). Several studies have investigated the solubility, foaming, and emulsification properties of lentil proteins (Boye et al., 2010a; Jarpa-Parra, 2018; Karaca et al., 2011; Ko et al., 2017; Ladjal-Ettoumi et al., 2016), with the lowest solubilities occurring between pH 2 to pH 4, while the maximum solubility, emulsifying, and foaming properties occurring at pH 7 or pH 8. Apart from whole protein isolates, albumin, globulin, glutelin and prolamin lentil fractions, which are water-soluble, salt-soluble, alkali-soluble, and ethanol-soluble respectively,

can also be extracted. Albumins and globulins are the most abundant and contain 10 – 20% of the total protein (Jarpa-Parra, 2018).

Although some studies have addressed the functional properties of lentil protein isolates and concentrates, there is still a research gap between the influence of the lentil protein structure, their physicochemical properties, and their potential use in food applications (Jarpa-Parra, 2018). In addition, there are currently no studies addressing the physicochemical and functional properties of the various lentil protein fractions (Ladjal-Ettoumi et al., 2016). Therefore, the overall aim of this research is to determine the physicochemical and structural properties of lentil protein isolates and lentil protein fractions.

1.1 Hypotheses

a) Defatting lentil seed flour using acetone will have marginal effect on the native protein structure but will have a positive impact on the extraction and functionality of the protein.

b) Ultrafiltration membrane-assisted removal of non-protein materials will have an improved food functionality in comparison with isolated proteins from isoelectric precipitation due to its more intact structural conformation.

c) The various protein fractions obtained will have more superior functional properties than those of the isolated lentil seed proteins.

d) The membrane protein isolate will have higher contents of native structural conformations and produce higher level of food functionality when compared to protein materials produced by isoelectric precipitation.

1.2 Objectives

- The overall aim of this research is to determine the physicochemical and structural properties of lentil protein isolates and lentil protein fractions.

- Therefore, the specific objectives of the proposed study are to:
    a) To isolate various types of lentil seed proteins using isoelectric precipitation, enzyme-assisted hydrolysis and membrane separation using NaOH solution, and membrane separation using NaCl solution.
    b) To isolate and separate the lentil seed protein into albumin, globulin, glutelin and prolamin fractions.
    c) Investigate and compare the food formulation-related functional properties of each isolated lentil seed proteins and fractions including, protein solubility, heat coagulability, emulsion, foaming, and least gelation properties, water holding capacity, and in vitro protein digestibility.
    d) Determine and compare the physicochemical properties (proximate composition, amino acid composition, gel electrophoresis, intrinsic fluorescence, surface hydrophobicity, circular dichroism, and differential scanning calorimetry) of each isolated lentil seed proteins and fractions.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lentil

Lentil (*Lens culinaris*) is an edible fruit of pod-bearing plant belonging to the legume family. It ranks fifth in total worldwide production of edible pulses with an average yield of 850–1100 kg ha^{-1}, and a global production of 5.734 million metric tonnes (*FAOSTAT*, 2019). The shallow root of the lentil plants allows them to adapt to dry climates with limited rainfall, and this is responsible for their ability to be grown anywhere in the world (Janzen et al., 2014). Canada is the top producer of lentils, followed by India, Turkey, Australia, The United States and Nepal, together making up to 57.5 % of the global lentil production (*FAOSTAT*, 2019). Western provinces like Saskatchewan, Manitoba and Alberta, produce most of the lentils in Canada, with Saskatchewan being responsible for up to 70% (*Pulse Canada*, 2019).

Lentil varieties can be distinguished by seed pattern and size, but the most distinguishing properties are their colours. Green Lentils are most prominent in the United States and Canada, while Red Lentils are more common in South Asian countries like India and Nepal (Alexander, 2015). They are usually planted in the fall and harvested at the end of summer. Some of the common food processes that take place after harvesting lentils include threshing, drying, storage and splitting (for red lentils). Green lentils are recommended to have a moisture content of 16-18% during harvest in order to maximize yield for the producers. After drying, the moisture content of the lentils should be less than 13% in order to prolong their shelf life. At the optimum storage moisture, lentils can be stored for more than 15 weeks at regular room temperature (less than 30 ^{o}C) (Chelladurai & Erkinbaev, 2020).

2.2 Composition and Nutritional Value of Lentils

The Latin word *lens* descriptively refers to the lens-like shape of the lentil seeds. Lentil seeds mainly consist of the embryo, seed coat, and cotyledons, which make up 2, 8 and 90% respectively, of the dry seed weight. Cotyledons are made up of parenchymatous cells, which contain mostly starch granules dispersed in the protein bodies (Joshi et al., 2017). Proximate composition has been done by several researchers to determine the chemical composition of lentil seeds as shown in Table 1. Lentils are generally higher in total soluble fibre and dietary fibre when compared to other pulses like peas, beans and chickpeas (Brummer et al., 2015). In addition, they are an abundant source of protein, low in fat, and excellent sources of minerals and phytochemicals such as prebiotics, polyphenols, carotenoids, and saponins (Zhang et al., 2018). Since cotyledons are the major component of lentil seeds as well as the nutrient reservoir, removing the seed coat during dehulling will not have a significant effect on macronutrient value (Chelladurai & Erkinbaev, 2020). However, it has been reported by several researchers including Pal et al. (2017) that a high percentage of polyphenols contained in lentils are present in the seed coat. As a result, the variety of these seed coat colours can be in part attributed to polyphenols like anthocyanins, flavonoids, glycosides, and tannins (Singh et al., 2017).

Table 1. Nutritional facts of lentils per 100 g or ½ cup serving*

Nutrients	Unit	Raw	Sprouted	Cooked
Water	g	6.98–9.97	51.85–67.34	69.64–137.89
Energy	kcal	343–356	82–106	116–226
Protein	g	18.98–28.3	6.9–8.96	9.02–17.86
Total lipid (fat)	g	0.8–2	0.42–0.55	0.38–0.75
Carbohydrate	g	60–64.44	17.05–22.14	20.13–38.69
Total dietary fiber	g	10.7–31.4	-	7.9–15.6
Total sugars	g	2.03–2.86	-	1.80–3.56
Minerals				
Calcium	mg	35–57	19–25	19–38
Iron	mg	6.51–7.71	2.47–3.21	3.33–6.59
Magnesium	mg	47–69	28–37	36–71
Phosphorus	mg	281–335	133–173	180–356
Potassium	mg	677–943	248–322	369–731
Sodium	mg	3–6	8–11	123–471
Zinc	mg	3.27–5.89	1.16–1.51	1.27–2.51
Vitamins				
Vitamin C	mg	3.4–4.5	12.7–16.5	1.5–3.0
Thiamin	mg	0.756–0.873	0.176–0.228	0.169–0.335
Riboflavin	mg	0.189–0.211	0.099–0.128	0.073–0.0145
Niacin	mg	2.605–3.459	0.869–1.128	1.060–2.099
Vitamin B6	mg	0.540–0.698	0.146–0.190	0.178–0.352
Folate	µg	479–555	77–100	181–358
Vitamin B12	µg	0.00	0.00	0.00
Vitamin A, RAE	µg	2.0–2.5	1.8–2.0	0
Vitamin A, IU	IU	32–39	35–45	8–16
Vitamin E	mg	0.49–0.55	0	0.11–0.22
Vitamin K	µg	4.2–5.0	0	1.7–3.4

Nutrients	Unit	Raw	Sprouted	Cooked
Lipids				
Total saturated fatty acids	g	0.154–0.198	0.044–0.057	0.053–0.105
Total monounsaturated fatty acids	g	0.0179–0.193	0.08–0.104	0.064–0.127
Total polyunsaturated fatty acids	g	0.469–0.526	0.169–0.219	0.175–0.346

*Adapted from the United States Department of Agriculture (USDA) nutrient data sheet (*FoodData Central*, 2019) with open access permission

Pulses like lentils initially gained recognition as a result of the health benefits associated with their high protein content as well as the presence of other macro and micronutrients. Nonetheless, sufficient evidence has emerged over time suggesting that the bioactive compounds present in lentils also play a significant role in promoting human health (Singh et al., 2017). Ferulic acid for example, is a phytochemical which is most abundant in pulses like lentils. This polyphenol in addition to other bioactive compounds present in lentils, is believed to be responsible for exerting antioxidant and anti-inflammatory activities, which according to epidemiological studies, are inversely associated with the occurrence of cardiovascular diseases (CVD), cancer, and type II diabetes mellitus (T2DM) (Ganesan & Xu, 2017).

2.3 Constraints in the use of Lentils

Although lentils are valuable dietary sources, their consumption in developed countries like North America, Europe, and Australia is still limited. Some of the factors limiting the utilization of lentils are their slow cooking rate, often unpleasant flavour, presence of antinutritional compounds, allergens, poor protein digestibility, and lack of varied dish options (Joshi et al., 2017). The occurrence of trypsin inhibitors (0.28%), condensed tannins (0.49% – 1.28%), and lectins (10.9 – 11.1 HU/mg dry mater) in lentils could inhibit the activity of proteolytic enzymes, crosslink with proteins which in turn reduces the availability of amino acids or carbohydrate by making them

unavailable during digestion. In addition, the presence of phytic acid (0.72% – 4.11%) could specifically lead to a reduction in the bioavailability of minerals in lentils through several ways (Hefnawy, 2011; Shi et al., 2018; Wang & Daun, 2006). This includes the ability of phytic acid to either decrease the solubility or form stable complexes with minerals, thereby preventing intestinal uptake and making them unavailable for absorption (Jarpa-Parra, 2018). Another growing concern put into consideration when consuming lentils are the occurrence of allergens. Although lentils are defined as non-priority legume allergens in Canada, IgE-mediated hypersensitivity reactions caused by lentils, have been commonly reported in children in many countries. In Spain, lentils are the fourth most common food allergen with an estimated occurrence frequency of around 20% (Hildebrand et al., 2020; Sackesen et al., 2020).

The slow cooking of lentil could also be a deterrence to its consumption. Since lentil is primarily consumed in its cooked form, its cooking time is another factor usually taken into consideration when choosing legumes or other plant-based food sources, which also determines the cooking quality (Theologidou et al., 2018). The cooking time of lentil, which is typically between 30 to 70 min, could be affected by harvesting process and cooking condition in addition to genetic, physicochemical, and environmental properties (Joshi et al., 2017). Resulting from the knowledge that lentil seed cultivars with a faster hydration rate had a faster cooking time, it can then be assumed that the cotyledon, which is responsible for the hydration behaviour of lentil seeds, plays a significant role in the cooking time (Theologidou et al., 2018).

2.4 Processing Techniques Used to Maximize the Utilization of Lentil

Lentil seeds usually need to be processed to a more accessible and palatable form before they can be consumed. These could be traditional processing techniques like dehulling, splitting, sprouting,

and soaking, or food preparation methods involving the application of heat in the form of cooking or roasting (Joshi et al., 2017).

2.4.1 Dehulling

Dehulling is performed to remove the seed coat, also known as the "hull", from the lentil seed. The seed coat may be rich in polyphenols, but they are often indigestible, occasionally have a somewhat bitter taste, and contain antinutritional compounds. Dehulling has been found to not just reduce the hydration and cooking time of lentil seeds, but also enhance their taste, appearance, and palatability (Chelladurai & Erkinbaev, 2020; Joshi et al., 2017). In addition, removing the seed coat of lentil can add value, reduce the antinutritional compounds, and increase the nutritional value of the resulting lentil seeds. This was reported in a study carried out by Pal et al. (2017), which states that the total soluble sugar, starch and lipid contents significantly improved after the lentil seed hulls were removed. Their study also reported a significant reduction in the level of tannins, phytic acid, and trypsin inhibitors present in the dehulled seeds.

2.4.2 Soaking and Sprouting

The practice of soaking, or prolonged soaking, which results in sprouted seeds, is one of the most simple and common techniques used reducing the cooking time of lentils during preparation. In addition, the concentration of some soluble antinutritional compounds like phytic acids and tannins can be reduced when the soaking solution is discarded after prolonged hydration of the lentil seeds (Joshi et al., 2017). Sprouting has also been known to increase the nutritive value of the sprouted seeds, and also cause a significant increase in their protein, riboflavin, crude fibre, hemicellulose, and free amino acid contents (Fouad & Rehab, 2015).

2.4.3 Cooking

Cooking, which could involve boiling, roasting, frying, or microwaving is a way in which lentils are usually prepared before consumption (Chelladurai & Erkinbaev, 2020). Boiling is the most common method used, and it has been found to not only enhance food flavour, palatability, and nutritional value, but also digestibility and bioavailability by reducing the concentration of heat-sensitive non-nutritional and volatile compounds present (Nosworthy et al., 2018; Pal et al., 2017). Cooking of lentil seeds has also been reported to significantly increase the concentration of resistant starch and other low digestible carbohydrates present (Siva et al., 2018). More importantly, autoclaving, which could be referred to as an extreme form of cooking, was also reported to have a significant decreasing effect on the IgE binding capacity of lentil seed allergens (Cabanillas & Novak, 2019). Despite the various traditional processing techniques used to maximize the consumption of lentils, the per capita global consumption is still on the decline (Chelladurai & Erkinbaev, 2020). Thus, finding diverse and novel applications of the seeds and creating new demand is critical to the success of the lentil food industry.

2.5 Lentil Total Protein Extraction and Isolation of Lentil Protein Fractions

The extraction and isolation of useful nutrients like proteins from the protein-rich seeds for use as higher value food ingredients is becoming another popular way in which lentils can be utilised. Lentil seed proteins, either as flour fractions, isolates or concentrates, can be used as ingredients to improve the protein quality and sensory characteristics of formulated food products (Ladjal-Ettoumi et al., 2016). The lentil seeds, which could either be whole or dehulled, are first milled to obtain a fine grind, then are either defatted using organic solvents (e.g. acetone, ethanol, hexane, and isopropanol) or used directly for the protein extraction processes (Arise et al., 2017a; Ko et al., 2017). This defatting process also serves as one of the cost-effective ways that can be used in

reducing the amount of aldehydes, alcohols, acryloyl, and ketones responsible for the somewhat unpleasant beany-flavor of lentils when used as ingredients for various foods (Roland et al., 2017). Separation or isolation of lentil seed proteins can be carried out using wet or dry processes. Dry processes include air classification, while the more common wet separation processes are alkaline extraction followed by isoelectric pH precipitation or membrane filtration, salt and water extraction, acid extraction, enzymatic digestion with membrane filtration, and organic solvent extraction (Boye et al., 2010). These preparation methods are thought to contribute to the nutritional quality as well as functional and physicochemical properties. In addition, removing the main non-protein components present in the lentil seeds is usually necessary for the study of protein properties (Jarpa-Parra, 2018).

2.5.1 Air Classification

During dry processing such as air classification, the lentil seeds are ground into fine powders, then differentiated into fractions of starch and protein based on their shape, size, and densities (Khazaei et al., 2019). Air classification separates the ground seed powders into a light to fine fraction (the protein concentrate) and a heavy or coarse fraction (the starch granules) through a spiral air stream (Pelgrom et al., 2015). The milling process used must be able to produce a very fine powder to enable a complete disruption of the cells without a substantial damage to the starch granules, which would result in a more efficient separation. To further increase the purification of the obtained fractions, the air classification process can be repeated as many times as required (Boye et al., 2010). Although the air classification process is a fairly simple separation method, the protein concentration obtained is generally not as high, in comparison with other isolation methods (Pelgrom et al., 2015).

2.5.2 Alkaline Extraction

2.5.2.1 Alkaline Extraction Coupled with Isoelectric Point Precipitation

Legume proteins can be extracted by alkaline solubilisation followed by acid precipitation at the isoelectric pH, which involves separating the solubilised protein from insoluble materials and precipitating the dissolved protein (Joshi et al., 2011). The protein flour is first dispersed in water at pH 8-10, then the dispersion is stirred while keeping pH and temperature constant (Boye et al., 2010; Ladjal-Ettoumi et al., 2016). The processing conditions used during extraction can also affect yield, purity, and functionality of the obtained isolate. Although extraction is commonly carried out at room temperature, higher temperatures are also often used to increase solubilisation and protein recovery Boye et al., 2010). After alkaline extraction, the mixture is centrifuged to separate the insoluble materials, then the protein present in the supernatant is precipitated by adjusting it to pH 4.5 (Ladjal-Ettoumi et al., 2016). This pH, which is also known as the isoelectric point, is the pH at which most of the lentil proteins are least soluble. The resultant protein concentrate or isolate is then dried using either freeze, spray or other drying methods (Joshi et al., 2011). For example, Boye et al. (2010) carried out an extraction of lentil seed protein using the isoelectric point precipitation method, which involved solubilisation at pH 9 with dilute sodium hydroxide solution. This extraction was carried out at room temperature (25 ^{o}C) with a 1:10 solid-to-solvent ratio, and they obtained concentrates with 78.2-79.1% protein contents. Ladjal-Ettoumi et al. (2016) also prepared lentil seed protein isolates according to the method used by Papalamprou et al. (2010) for pea protein isolates, which also included alkaline solubilisation at pH 8 followed by precipitation at pH 4.5. The resultant protein content of the lentil protein isolate (84.8%) was comparable with the 81.7–83.5% protein content that was previously reported from another study (Kaur et al., 2007). Although these pH values have been reported to be the optimum extraction parameters by several authors, the yield and quality of protein is impacted by cultivar-type, particle

size of the flour, type of solubilizing agent and temperature of the extracting solution (Joshi et al., 2017).

2.5.2.2 Alkaline Extraction with Ultrafiltration

Membrane separation after alkaline solubilisation is another commonly used substitute for isoelectric point precipitation. In this separation process, instead of the precipitating the solubilised proteins, they are subjected to diafiltration/ultrafiltration (Boye et al., 2010). This method of isolating proteins has been known to produce more functional proteins and a higher protein content due to its ability to eliminate solvents, minerals, and other low molecular weight compounds that are not are not required (Alonso-Miravalles et al., 2019). Ultrafiltration is a pressure-driven membrane filtration, employed to separate various components of a solution by means of a semipermeable membrane (Tiwari & Singh, 2012). Ultrafiltration membranes are usually made of ceramic or polymeric materials like cellulose and polyethersulfone. Maintaining a maximum temperature of 50 ^{o}C and a maximum pressure of 70 psi (483 kPa) is recommended during ultrafiltration (Millipore Sigma, 2011). Their pore sizes range from 0.001 µm to 0.1 µm and can concentrate solutions containing solutes of molecular weight between 1 kDa to 10 kDa (Tiwari & Singh, 2012). Solutes with a molecular weight higher than that of the membrane used are retained, and therefore known as the retentate. The term permeate, is then used to describe the solution that is able to pass through the membrane, including water, monovalent ions, multivalent ions, and lower molecular weight solutes (Wang et al., 2013). Although the ultrafiltration method has been used in several studies for protein purification, its use in the extraction and purification of lentil seed protein is not very common. Boye et al. (2010) carried out an extraction of lentil seed proteins using this method. The aqueous solution containing solubilised protein at pH 6.0 was filtered using a 50 kDa molecular weight cut-off (MWCO) membrane and protein contents of 82.7 and 88.6%

were obtained for red and green lentils, respectively. Alonso-Miravalles et al. (2019) on the other hand, using a 10 kDa MWCO membrane and an extraction temperature of 50 ^{o}C produced a lentil isolate with a protein content of 93.7%.

2.5.3 Enzyme-Assisted Extraction with Ultrafiltration/Diafiltration

Enzyme-assisted extraction (EAE) coupled with ultrafiltration/diafiltration is another extraction method being used to enhance the quality of isolated seed proteins. Some plant proteins have inaccessible amino acid residues, which are buried in the protein core and also tend to possess a higher proportion of carbohydrates. As a result, enzymes can be used to expose the buried hydrophobic amino acid residues, and also degrade the rigid cell wall in order to release cellular proteins before further purification with ultrafiltration (Gong et al., 2015; Tiwari & Singh, 2012). EAE can be optimized to make use of proteases, carbohydrases (e.g. cellulase, amylase, hemicellulose, xylanase, pectinase), phytase, or their combinations (Hanmoungjai et al., 2002). Proteases, for example, are able to release bound cellular proteins from the polysaccharide matrix. After release, the proteins are further broken down into smaller polypeptides and amino acids by the cleaving action of the proteases on the peptide bonds within the protein. Proteolysis not only leads to a higher protein yield, but it also has been known to improve various functional properties of the isolated protein (Zhan et al., 2019). Similar advantages have also been found with the use of carbohydrases like α-amylase, which breaks the α-1,4 linkage of starch or cellulase and xylanase, which degrade the β-1,4 linkage of cellulose and xylose, respectively (Malomo & Aluko, 2015b; Tiwari & Singh, 2012). After phytase treatment of soybean flour, Wang et al. (2014) reported higher protein contents (91 - 93%) and better in vitro digestibility when compared to the control (soybean protein isolates that were not processed with phytase). However, there was a slight decrease in solubility of the soybean protein isolates treated with phytase when compared to

the control. In the same vein, Tirgar et al. (2017) also made a comparison between the composition and functional properties of cellulase-treated flaxseed protein (CFP) concentrates and flaxseed protein concentrates obtained through isoelectric point precipitation (AFP). CFP had a higher protein content and a reduced fat content when compared to AFP. The solubility profiles of both samples were similar, apart from an increased solubility displayed in CFP between pH 5 to pH 8. Malomo & Aluko (2015) prepared hemp seed protein concentrates (mHPC) by digesting with a combination of cellulase, hemicellulose, xylanase, and phytase, before subjecting the sample to ultrafiltration/diafiltration. When compared to the sample produced using the traditional isoelectric point precipitation method (iHPI), it was found that although mHPC had a lower protein content, it had a significantly higher solubility profile (>75%) than iHPI (<25%).

2.5.4 Salt Extraction/Micellization

Although proteins can be extracted together as isolates or concentrates, each protein can also be separated based on their solubility in aqueous solutions. This process of separating storage proteins into their albumin and globulin fractions is referred to as salt extraction or micellization. This extraction method is based on the salting in and salting out phenomenon, which is heavily dependent on the ionic strength of the solution used. At low salt concentrations, solubility of proteins increases because of the increased protein-water interaction brought about by improved stability of the various charged groups on a protein molecule (Singhal et al., 2016). This is commonly known as salting-in. The salting out process on the other hand, describes the precipitation that occurs when the water can no longer solubilise the protein molecules due to the presence of salt. This occurs when the concentration of salt in a solution is increased to the point that there are insufficient water molecules interacting with proteins in the solution, thereby promoting protein-protein aggregation (Tiwari & Singh, 2012).

A variety of salts have been used for protein precipitation and purification through the salting-out process, including sodium chloride, ammonium sulfate, potassium chloride, magnesium sulfate, and calcium chloride (Singhal et al., 2016). However, ammonium sulfate and sodium chloride are the most commonly used salts for protein research purposes. Ammonium sulfate, especially, is highly soluble in water, has no negative effect on proteins, and are fairly inexpensive (Ajibola et al., 2016; Karaca et al., 2011; Tiwari & Singh, 2012). Once the proteins have been salted out, the solution is centrifuged to separate insoluble materials, then diluted to induce precipitation through dialysis. Centrifugation of the dialysed solution produces a supernatant and precipitate, which contain the albumin and globulin fractions, respectively (Mundi & Aluko, 2012). Although the isolation of various protein components of other storage proteins and their functionality are well documented (Adebiyi & Aluko, 2011; Ajibola et al., 2016; Malomo & Aluko, 2015a; Stone et al., 2015), there is scant information about the properties of the lentil seed protein fractions. The isolation of globulins in lentil seeds was first reported by Osborne & Campbell (1898), then later by Bora (2002). In a subsequent study carried out by Karaca et al. (2011), it was reported that while the lentil protein isolate obtained via isoelectric point precipitation was significantly more soluble, the emulsifying capacity was lower in comparison to the lentil seed protein fractions.

2.6 Functional Properties of Food Proteins

As has been reported by various studies, the roles of proteins in food products go beyond providing amino acids required for nutrition. Proteins are known to improve the non-nutritive properties of the food, and this function determines how they will be utilised in various food systems (Shevkani et al., 2019). The use of proteins as functional food ingredients influences the overall quality of foods, including their behaviour during processing, shelf life, appearance, and sensory characteristics. Some of the functional properties of proteins that potentially improve the quality

of these foods are hydration-related properties like solubility, surface properties like water absorption, oil holding capacity, emulsion, foaming behaviours in addition to rheological properties like gelation (Boye et al., 2010). Further information about the functionality of food proteins and their various applications in food products is illustrated in **Table 2**. In addition, though various studies commonly use similar methods for testing the functionality of proteins with just slight modifications, there are no standard methods being used by researchers. Keeping these possible causes for varied results in mind, directly comparing data from different studies may be difficult (Lam et al., 2018).

Table 2. Factors influencing protein functionality

Intrinsic factors	**Extrinsic factors**	**Processing factors**
Amino acid composition	pH	Heating/cooling
Conformation	Salts	Pressure
Surface charge	Ionic strength	Shear forces
Isoelectric point	Temperature	Storage time
Reactive sites	Solvent-type	Freezing
Conjugated subunits	Interaction with other food constituents	Chemical or enzymatic modification
Molecular size/flexibility		Concentrating
Surface hydrophobicity		Drying

(Alfaro-Diaz et al., 2021; Arise et al., 2017; Boye et al., 2010; Malomo & Aluko, 2015b; Singhal et al., 2016)

2.6.1 Protein Solubility

Solubility of the food protein isolate or concentrate is generally the primary functional property determined when developing new protein ingredients for some food and beverages. It is an important property because of its influence on other properties such as emulsifying, foaming, water or oil binding, and gelation (Shevkani et al., 2019; Zayas, 1997). In comparison with other pulses like chickpea and soybean protein, lentil protein solubility was significantly higher as reported by Aydemir & Yemenicioğlu (2013). Protein solubility can simply be said to be the amount of protein in a sample that dissolves in a solvent (Shevkani et al., 2019; Zayas, 1997). The solvent used in solubilisation is usually either water or buffers. Since the degree of protein solubility is dependent on the protein-protein and protein-solvent interactions, it could further be defined as the equilibrium between hydrophilic and hydrophobic residues in the structure. Some other terms like "nitrogen solubility" and "protein dispersibility" are also used to describe the solubility of proteins (Lam et al., 2018). A liquid system is considered to have a high protein solubility when there is a high breakdown of protein aggregates, which leads to the formation of uniformly dispersed colloidal systems. However, increase in the amount of proteins dissolved in a solution leads to a certain protein concentration of a solution, above which the soluble protein content decreases and a solid phase appears (Zayas, 1997).

The solubility of pulse protein isolates is dependent on factors including their protein structure, surface charge, and environmental factors (pH, temperature, pressure, and salt type) (Aryee et al., 2018). The pH of the solution especially has a significant impact on the solubility of pulse proteins. At acidic and alkaline pH values below or above the isoelectric point (pI) there is increased protein-solvent interaction due to the electrostatic repulsion exhibited by the charges on the protein surface (Singhal et al., 2016). Lentil seed proteins specifically, are sparingly soluble at the pH around their

pI, which is typically between pH 4.0 and 5.0 because there is no net surface charge, leading to reduced electrostatic repulsive forces, aggregation, and subsequently precipitation (Boye et al., 2010; Jarpa-Parra et al., 2014)

Salts present could impact the solubility of proteins in a solution by either reducing the thickness of the electric double layer surrounding individual proteins or influence protein hydration. At low salt concentration, salts like perchlorates, thiocyanates, and barium increase the solubility of proteins by stabilising the structure of the hydration layer around the proteins and promoting protein-solvent interactions (salting-in). Conversely, 'salting out' salts like potassium and ammonium salts reduce the solubility and subsequently cause aggregation of proteins (Lam et al., 2018). The salting out process occurs because the salts reduce the thickness of the diffuse layer by screening the electric double layer, resulting in the exposure of hydrophobic patches, which leads to the promotion of protein-protein interactions over protein-water interactions (Tiwari & Singh, 2012).

Modifications in the solubility of proteins in a solution can also be achieved through the conformation changes to the protein structure brought about by heating. Heating a solution below the protein's denaturation point enhances the protein solubility. However, at temperatures between 40 – 50 ^{o}C, solubility of proteins is significantly reduced (Zayas, 1997). This decrease in protein solubility can be ascribed to the irreversible changes that occur when higher temperatures disrupt stabilizing hydrogen bonding, causing proteins to unfold and expose buried hydrophobic groups. These exposed groups interact and lead to the aggregation and subsequent precipitation of proteins (Lam et al., 2018). Solubility profile of the protein can, therefore, be an indicator of the degree of protein denaturation during extraction and processing. In addition, the disulfide-sulfhydryl

exchange that occurs during heating also contributes to the decrease in protein solubility through changes in the aggregation state of proteins (Tiwari & Singh, 2012).

2.6.2. Water Binding or Retention Ability

The ability of proteins to hold water is an important determinant of consumer acceptability when formulating certain foods because it affects the texture, juiciness, and mouthfeel of the food product (Singhal et al., 2016). Water held by a protein structure can be classified as absorbed or retained depending on whether the water is no longer available as a solvent and bound to the protein or if it is trapped by the protein matrix. Several terms including water holding, water retention, water binding, water adsorption and other terms, have been used interchangeably to describe the interactions of proteins with water (Zayas, 1997). The water holding or retention capacity of foods can be defined as the ability of a protein to imbibe water and retain it against gravitational forces within a protein matrix. Water hydration, binding, or absorption capacity on the other hand, is defined as the total grams of water bound per gram of protein (Boye et al., 2010). Although there are no standard terminologies used to describe these protein-water interactions, a noticeable difference between absorbed and retained water is that bound water is not freezable at -40 ^{o}C and cannot be separated by centrifugation (Zayas, 1997). The capacity of proteins to absorb and retain depends on the availability and type of exposed polar groups within the protein polypeptide chain. Other important factors that influence the water holding capacity of proteins include the concentration and structure of the protein, as well as extrinsic properties like pH, presence of salts, temperature, and processing conditions. As a result of the effect of processing and extraction conditions, the water holding capacity of isolates was found to be higher when compared with whole grain flours (Tiwari & Singh, 2012). Lentil proteins were also reported to

have a similar behaviour in which the water holding capacity of the cooked lentil seed proteins and isolates was higher than that of the flour (Aryee & Boye, 2017).

2.6.3. Fat or Oil Holding Capacity

The importance of the oil holding capacity of proteins in food formulations is similar to that of the water holding capacity, with added significance of its effects on the emulsifying capacity of proteins (Aryee & Boye, 2017). Oil holding capacity is the ability of a matrix of proteins to physically entrap large amounts of fat, which is calculated as the amount of oil that can be absorbed per gram of protein (Aryee et al., 2018). This functionality is typically governed by the pore size of the protein, the type of fat and the droplet size of the fat. Since the nonpolar amino acid side chains are important in binding the lipid hydrocarbon chains, the amount of hydrophobic amino acids present in proteins is also a determinant of fat holding capacity (Tiwari & Singh, 2012). Although processing conditions have been reported to make varied impacts on the oil holding capacity of pulse proteins (Boye et al., 2010), Aryee & Boye (2017) reported that isolated lentil seed proteins had a higher oil holding capacity than the cooked or raw flour.

2.6.4. Emulsifying Ability

The ability of food proteins to form and stabilise emulsions has a significant effect on the sensory properties and shelf-life of formulated food products. An emulsion can be defined as a dispersion of immiscible liquids created by mechanical agitation such that one is dispersed as small droplets (the dispersed phase) in the other (the continuous phase) (Avramenko et al., 2013). Food emulsions are generally either oil-in-water emulsions as seen in salad dressings or water-in-oil emulsions like in margarine. Due to interfacial surface tension resulting from the mixture of hydrophobic and hydrophilic molecules, emulsions are thermodynamically unstable and will eventually separate into the respective constituent layers with time unless emulsifiers or surfactants are added to

system (Singhal et al., 2016). An emulsifier acts as a stabilizer by forming a physical barrier around each oil droplet in order to lower the interfacial tension and subsequently impedes coalescence. Proteins are good emulsifiers because of their surface active properties with both hydrophilic and hydrophobic amino acids, as well as their ability to unfold and form elastic films around the emulsion droplets (Karaca et al., 2011). Emulsion capacity, therefore, is a measure of the maximum amount of oil that can be used within a protein solution under a specific set of conditions. Emulsion activity index (EAI) and emulsion stability index (ESI) are two indices often used to evaluate the emulsifying properties of proteins. EAI describes the ability of a protein to form an emulsion by providing an estimate of the amount of oil that can be emulsified per unit protein, while ESI measures the stability of the emulsion over a defined period of time (Aryee et al., 2018). The emulsifying ability of legume proteins depend on several intrinsic, and extrinsic factors, including the type of legume. For example, Joshi et al. (2011) reported that the emulsion stability of lentil seed protein isolate was significantly higher than that of soybean protein isolate but lower than casein and whey protein isolates. An increased emulsion stability was attributed to the disulfide bonds formed within the absorbed proteins while less stable emulsion were credited to the intermolecular disulfide bonds formed as a result of electrostatic repulsion (Tcholakova et al., 2006). Better and more stable emulsions were formed at the more acidic or alkaline regions (away from the isoelectric point), showing that the pH of the system also plays a significant role in the resultant capacity and stability of emulsions (Alfaro-Diaz et al., 2021). In addition, Nwachukwu & Aluko (2018) demonstrated that an increase in protein concentration also led to a higher emulsifying capacity

2.6.5. Foaming Ability

Foams are similar to oil-in-water emulsions because they are dispersions of immiscible gas bubbles in hydrophilic liquids and require energy to facilitate their formation. They are formed when dispersed proteins adhere to the gas-liquid interface and create a viscoelastic film around the gas bubbles in order to keep them in suspension and prevent collapse and fusion (Shevkani et al., 2019). In the food industry, foams can enhance the appearance and appeal of a food while also providing a soft and velvety taste. For a protein to be considered as an effective foaming agent, the protein must rapidly conform and be absorbed to the gas-liquid interface during foam formation (Damodaran, 2017). Quantitative properties of foams are often measured using the following indices: foam expansion (FE), foam capacity (FC) and foam stability (FS). FC or FE of a protein is its ability to generate foams under a specific condition (i.e., concentration, temperature, pH), and this is expressed as the percentage increase in foam volume after homogenization. FS on the other hand, indicates the ability of the protein to retain its foam structure over a specified period, usually 0-30 min (Boye et al., 2010). Although it has been reported that processing and extraction had a negative effect on the foaming abilities of the protein (Stone et al., 2015), a previous work of Boye et al. (2010) found no significant difference when comparing the foaming capacity of pea and lentil concentrates that were extracted using either isoelectric precipitation or ultrafiltration techniques. The foaming properties of a proteins were also directly related to their solubility according to the results from Malomo & Aluko (2015) and Malomo et al. (2014), indicating that higher solubility resulted in better capacity to form foams.

2.6.6. Gelation Properties (Least Gelation Concentration)

When referring to foods, a gel can be defined as a material with a continuous, well-defined and three-dimensional network, made up of a solid phase (proteins, for example) which is embedded within a liquid phase (aqueous solvent like water) (Gaonkar & McPherson, 2016). A gel is an intermediate between the solid and liquid state, which explains why it can behave like solids yet possesses many characteristics of a fluid. The gelling capacity of a protein is commonly defined by measuring the lowest concentration of protein required to form a self-supporting gel. This is known as the least gelation concentration (LGC), in which a lower LGC value indicates a protein with a more superior gelling ability (Boye et al., 2010). Gelation of proteins can either be induced physically through application of heat or pressure (Malomo et al., 2014) or by chemical (Vasbinder et al., 2004) and enzymatic treatments (Tarhan et al., 2016). Although all the aforementioned processes would induce a gel network, the interactions and mechanism involved could vary depending on the method used. The most common food gels are produced through heat-induced gelation. Formation of gels through this method first involves unfolding of the protein molecules as a result of partial denaturation. This leads to the exposure of the buried hydrophobic sites or reactive amino acid residues. The exposed amino acids interact with each other and facilitate the association and irreversible aggregation of proteins, resulting in a three-dimensional cross-linked gel network. These gels are thermally irreversible and are usually formed via linkages like disulfide bridges, hydrogen bonds, and ionic reactions (Aryee et al., 2018; Joshi et al., 2017). However, subjecting proteins to excessive heat (≥100 ^{o}C) may cause the breakage of the primary peptide bonds in the protein and prevent gelation (Joshi et al., 2017).

Table 3. Functional properties of food proteins and their various applications in food products

Functional property	Description	Considerations	Application
Solubility	Amount of protein that dissolves in a solution	pH, salts, temperature	Beverages (high sol); sports bars (low sol); Meat analogues (low sol)
Oil holding capacity	Flavour interaction/flavour binding and fat retention	Level of denaturation, hydrophobicity	Meat analogues
Water holding capacity	Protein interaction in water, water binding and swelling	Level of denaturation, hydrophilicity	Meat analogues, surimi, yogurt, cheese
Foaming	Protein adsorption at the interface, coating of air cells	pH, salt, continuous phase viscosity, sugars, shear	Meringues, whipped toppings, baked goods
Emulsification	Protein adsorption at the interface, coating of oil droplets	pH, salts, lipid, hydrophilic-hydrophobic balance, shear	Salad dressings, sauces, mayonnaise, encapsulation
Gelation	Gel formation	pH, salts, protein aggregation, temp., fixatives	Meat analogs, yogurt, tofu, surimi, encapsulation
Nutrition		Amino acids, digestibility	Nutritional supplement products

Table Adapted from Proteins in Food Processing (Aryee et al., 2018) with additions some additions (Boye et al., 2010; Shevkani et al., 2019)

Table 4: Functional properties of different types of isolated lentil and pea proteins

Functional property	Extraction method		Author
Solubility	Isoelectric precipitation: lentil	Min: 2%, 4%, (pH 5), <10% (pH 4.0), 121 mg/g (pH 4.5) Max: 82%, 73%, (pH 10), 50%, 92% (pH 9) pH 7: 91%, 78%, 90%	(Alonso-Miravalles et al., 2019; Aryee & Boye, 2017; Boye et al., 2010; Johnston et al., 2015; Karaca et al., 2011; Ladjal-Ettoumi et al., 2016)
	Isoelectric precipitation: pea	Min: 2 – 4%, 3.6% (pH 5), 210mg/g (pH 4.5) Max: 69 – 95% (pH 9) pH 7: 62 – 64%, 64 – 80%	(Ladjal-Ettoumi et al., 2016; Shevkani, Singh, et al., 2015; Stone et al., 2015)
	Membrane isolate using NaOH solution	Min: 5% (pH 5), Max: 89%, 83% (pH 10), 83% (pH 2), 54.7% (pH 9) pH 7: 43%	(Alonso-Miravalles et al., 2019; Boye et al., 2010)
Oil holding capacity	Isoelectric precipitation: (lentil) Membrane isolate using NaOH solution	6.9 – 10.4 g/g, 91% 120%, 125%, 2.1g/g 175%, 225%, 2.2 g/g (N.M)	(Aryee & Boye, 2017; Aydemir & Yemenicioğlu, 2013; Boye et al., 2010)
Water holding capacity	Isoelectric precipitation: (lentil)	pH 7: 1.1 – 1.5 g/g, 0.48 g/g 3.8 mL/g, 2.60 g/g (N.M)	(Alonso-Miravalles et al., 2019; Aydemir & Yemenicioğlu, 2013; Boye et al., 2010; Matina Joshi et al., 2011)
	Membrane isolate using NaOH solution	4.2 mL/g, 3.4 mL/g 3.96 g/g (N.M),	

Foaming capacity	Isoelectric precipitation: lentils	pH 7: 79%, 63%, 37%, 42% 0.1 – 3% (w/v): 19 – 57 %	(Alonso-Miravalles et al., 2019; Boye et al., 2010; Shevkani et al., 2015)
	Isoelectric precipitation: pea	pH 7: 87 – 105%	
	Membrane isolate using NaOH solution	pH 7: 69%, 63%, 38, 36% 0.1 – 3% (w/v): 9 -70% (N/M)	
Foaming stability	Isoelectric precipitation: (lentils)	0.1 – 3% (w/v): 0 - 39	(Alonso-Miravalles et al., 2019; Boye et al., 2010; Shevkani et al., 2015)
	Isoelectric precipitation: pea	pH 7: 94 – 96%	
	Membrane isolate using NaOH solution	0.1 – 3% (w/v): 0 – 45%	
Emulsion activity (m^2/g)	Isoelectric precipitation: (lentils)	pH 7: 44, 90, 44, 5, 16.5	(Alonso-Miravalles et al., 2019; Boye et al., 2010; M. Joshi et al., 2012; Karaca et al., 2011; Shevkani et al., 2015)
	Isoelectric precipitation: pea	pH 7: 12 – 14, 43,	
	Membrane isolate using NaOH solution	pH 7: 5.1, 5.9, 14.3	
Emulsion stability (min)	Isoelectric precipitation: (lentils)	87, 18, 51	(Alonso-Miravalles et al., 2019; Boye et al., 2010; Karaca et al., 2011; Shevkani et al., 2015)
	Isoelectric precipitation: pea	pH 7: 53 – 95, 12	
	Membrane isolate using NaOH solution	pH 7: 19, 64	
Least gelation	Isoelectric precipitation: (lentils)	pH 7: 12 -14 g/100g, 16% 11%, 12%	(Adebiyi & Aluko, 2011; Alonso-

	Isoelectric precipitation: Pea	20% (N.M)	Miravalles et al., 2019; Aydemir &
	Membrane isolate using NaOH solution	10% (N.M), 8%, pH 7: 11%	Yemenicioğlu, 2013; Boye et al., 2010; Matina Joshi et al., 2011)

N.M: pH value not mentioned or not adjusted.

Table 5: Functional properties of lentil and pea protein fractions

Functional property	Description		Author
Solubility	Albumins	pH 7: 90% (lentil), 86 – 91%, 38% (pea)	(Karaca et al., 2011; Stone et al., 2015)
Oil holding capacity	Albumins	pH 7: 1.8 g/g (lentil), 5.2 -5.4 (pea)	(Ghumman et al., 2016; Stone et al., 2015)
	Globulin	pH 7: 1.6 g/g	
Water holding capacity	Albumins	pH 7: 0.4 g/g (lentil), 0.3 – 2.6 g/g (pea)	(Ghumman et al., 2016; Stone et al., 2015)
	Globulin	pH 7: 1.2g/g	
Foaming capacity	Albumins	pH 7: 79% (lentil), 153 – 258% (pea)	(Ghumman et al., 2016; Jarpa-Parra et al., 2015; Stone et al., 2015)
	Globulin	pH 7: 8.9%, 410%	
Foaming stability	Albumins	pH 7: 55% (lentil), 49 - 70% (pea)	(Ghumman et al., 2016; Jarpa-Parra et al., 2015; Stone et al., 2015)
	Globulin	pH 7: 4.4%, 84 min	
Emulsifying activity (m^2/g)	Albumins	pH 7: 6.5, 37 (lentil), 43 (pea)	(Ghumman et al., 2016; Karaca et al., 2011; Stone et al., 2015)
	Globulin	pH 7: 7.9%	
Emulsifying stability (min)	Albumins	pH 7: 10, 11 (lentil), 11 (pea)	(Ghumman et al., 2016; Karaca et al., 2011; Stone et al., 2015)
	Globulin	pH 7: 17.2% (lentil)	
Gelation	Albumins	10% (N.M)	(Adebiyi & Aluko, 2011)

N.M: pH value not mentioned or not adjusted.

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CHAPTER THREE

3.0 COMPARATIVE STUDY OF THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF LENTIL PROTEIN ISOLATES

3.1 Introduction

Interest in plant proteins has significantly grown over the past decade (Joshi et al., 2017; Shevkani et al., 2019). Good proportion of amino acids in plant proteins makes them viable alternatives to animal protein sources which might be needed due to various dietary restrictions including related allergenicity, halal consideration, vegetarianism and so on (Carbonaro et al., 2015; Duranti, 2006). Plant proteins from various sources including peas, beans, chickpeas, and lentils, have been considered for their versatility, high protein content, low cost, and good nutritional profile (Ladjal-Ettoumi et al., 2016; Mundi & Aluko, 2012). Lentils are among the most cultivated legumes in the world, with Canada being the second largest producer and world's largest lentil exporter (*FAOSTAT*, 2019; Thavarajah et al., 2009). Like other legumes, they are rich in more than half of the total amino acids including, arginine, aspartic acid, glutamic acids, and leucine, while they are limiting in some other essential amino acids like threonine, tryptophan, methionine, phenylalanine, and histidine (WHO & FAO, 2007). However, despite the high protein component of legumes, their quality as ingredients to be used in food formulation is dependent on their functional properties and how it affects their behavior during processing, storage and consumption of the food product (Kinsella & Melachouris, 1976). As a result, recent research efforts have been geared towards the production of protein concentrates or isolates with good solubility, emulsion, foaming, gelation, and other functional properties which also contribute to the sensory qualities and consumer acceptability of the food product (Johnston et al., 2015; Khazaei et al., 2019).

Various techniques involving wet or dry extraction are used to isolate and concentrate proteins obtained from legumes. However, isolation of pulse proteins is easier using wet processes because of their high solubility in both acidic and alkaline conditions (Boye, Zare, et al., 2010). Isoelectric precipitation is one of the most commonly used methods of isolating proteins which involves alkaline solubilisation followed by isoelectric precipitation (Malomo et al., 2014). However, this method has denaturing effects on proteins and negatively impacts their functional properties. For example, (Alonso-Miravalles et al., 2019) investigated lentil proteins isolated using membrane filtration and isoelectric precipitation method. They concluded that the isolated protein obtained from membrane filtration had better functional properties as expected, since membrane filtration concentrates proteins and removes non-protein materials without the harsh extraction and potentially denaturing conditions used during isoelectric precipitation. Nevertheless, the protein isolate obtained from isoelectric precipitation had a significantly higher protein content in comparison. Results obtained from this study clearly indicates the effect of the isolation method on the protein composition as well as the functional properties of the resulting protein isolate or concentrate. In addition, external factors such as pH, ionic strength, temperature, and the presence of enzymes could also alter the structural conformation and functionality of the isolated protein (Mundi & Aluko, 2013).

The present study would provide information on the physicochemical and functional properties of isolated lentil proteins obtained using various extraction techniques and conditions. The information would be useful in effectively identifying the applications of these lentil proteins as ingredients in the food industry. Therefore, the objectives of the study were to: a) Establish possible relationships between the physicochemical and functional properties of isolated green lentil seed proteins and b) to study and compare the properties of the different isolated proteins.

3.2 Materials and Methods

3.2.1 Raw materials and sample preparation

Dehulled green lentils were purchased from a local grocery store (Winnipeg, MB, Canada) and stored at -20 ^{o}C. The whole seeds were ground into flour (≈ 302 μm) with a Coffee Grinder PC2770, then defatted using acetone (1:10, w/v, flour:acetone ratio) for 2 h to remove lipids and polyphenolic compounds and reduce green colour taint of the isolated proteins. The mixture was then filtered through a cheesecloth (grade 90, 40 x 36 thread count), and dried overnight in a fume hood to remove the acetone. The defatting process was repeated twice, and the dried flour was milled again to obtain a fine flour. The defatted flour was packed in plastic bags, sealed, and stored at -20 ^{o}C. Unstained markers for SDS-PAGE (10-200 kDa) and native electrophoresis were purchased from Fisher Scientific (Oakville, ON, Canada). Double-distilled water (DDW) produced from Millipore milli-Q^{TM} water purification system (Millipore Corp., Milford, MA, USA) was used during this research, and analytical grade chemicals used were purchased from either Fisher Scientific or Sigma Aldrich (St. Louis, MO, USA).

3.2.2 Isoelectric precipitation of lentil protein

Lentil flour was mixed with DDW at a 5:100 (w/v) ratio and adjusted to pH 10 using 1 M NaOH to solubilize the proteins. The mixture was continuously stirred for 1 h followed by centrifugation at 5600 x *g* for 30 min. The supernatant was collected, filtered with cheesecloth (grade 90, 40 x 36 thread count), adjusted to pH 4.5 with 1 M HCl, stirred for 30 min, and then centrifuged. The resulting precipitate was washed with water to remove contaminating materials and centrifuged again to get the final precipitate, which was adjusted to pH 7.0 before freeze-drying to obtain the lentil protein isolate.

3.2.3 Preparation of lentil protein concentrates using the membrane isolation method

Lentil flour was mixed with DDW at a 5:100 (w/v) ratio and adjusted to pH 10 using 1 M NaOH. The mixture was continuously stirred for 1 h, after which it was centrifuged at 5600 x *g* for 30 min. The supernatant was collected, filtered with cheesecloth (grade 90, 40 x 36 thread count), adjusted to pH 5.5 with 1 M HCl, then digested with 1% cellulase + 1% α-amylase at 50 ^{o}C for 1 h. After digestion, pH of the solution was adjusted to pH 7.0, then cooled down to room temperature. The NaCl extraction on the other hand, involved a 5:100 (w/v) ratio mixture of lentil flour and a 0.1 M NaCl solution, which was also mixed for 1 hr and similarly centrifuged to obtain a supernatant. The alkaline and NaCl extracts were then separately filtered using a 5-kDa ultrafiltration membrane and diluted periodically with DDW until the permeate was clear. Once a clear permeate was obtained, the retentate was collected and then freeze-dried to obtain the membrane alkaline-soluble protein or salt-soluble protein concentrates.

3.2.4 Physicochemical and functional properties of isolated lentil proteins

3.2.4.1 Proximate composition analysis

Relevant Association of Official Analytical Chemists' methods were used to analyze the moisture, dry matter, crude protein, and ash contents of the lentil protein isolates (Horwitz & Chemists, 2010). Crude fibre and fat contents were determined using the methods outlined by the American Oil Chemists' Society (Mehlenbacher et al., 2010).

3.2.4.2 Amino acid composition

Amino acid profile of the isolated lentil proteins was analyzed using an HPLC system with a pico-tag column after being digested with 6 M HCl for 24 h (Bidlingmeyer et al., 1984). The Sulphur containing amino acids (cysteine and methionine), as well as the tryptophan were then determined

after performic acid oxidation and alkaline hydrolysis, respectively (Gehrke et al., 1985; Landry & Delhaye, 1992).

3.2.4.3 Gel electrophoresis

Reducing (with mercaptoethanol), non-reducing (without mercaptoethanol), and native (without SDS or mercaptoethanol) polyacrylamide gel electrophoresis (PAGE) of the lentil proteins were determined using the method outlined by Aluko & McIntosh (2004) with minor modifications. Solutions containing Tris-HCl buffer, SDS, and bromophenol blue with (reduced) or without (non-reduced) β-mercaptoethanol, were used in the dissolution of each isolated protein to give a final concentration of 1% (w/v). The samples for the native gel electrophoresis were dispersed in a Tris-HCl buffer containing L-alanine and bromophenol blue. All prepared mixtures were separately heated at 100 ^{o}C for 10 min, cooled to room temperature, then centrifuged for 10 min at 10,000 x *g* A 1 µL aliquot of the supernatant obtained from each sample was separately loaded onto PhastGel® 8–25% gradient gels and electrophoresis performed with a Phastsystem Separation and Development unit according to the manufacturer's instructions (GE Health Sciences, Montréal, Canada). Gels were stained with Coomassie brilliant blue followed by de-staining in water:methanol:acetic acid solution (ratio), and finally preserved using a glycerol solution.

3.2.4.4 Intrinsic fluorescence

The Intrinsic fluorescence measurements were carried out according to the procedure outlined by Ajibola et al. (2016). Stock solutions were prepared by dispersing 10 mg of each protein sample (based on protein weight) in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and Tris-HCl (pH 9) buffers, followed by centrifuging and collecting the supernatant. The supernatants were diluted to 0.002% (w/v) using the appropriate buffers and the emission spectra was recorded at 25 ^{o}C in a spectrofluorometer (Jasco FP-6300, Tokyo, Japan) equipped with a 1 cm path length quartz

microcuvette (150 µL capacity). Protein samples were excited at 280 nm (tyrosine and tryptophan) with emissions recorded from 300 to 500 nm; emissions of the buffer blanks were subtracted from those of the respective samples to obtain the fluorescence spectra. The fluorescence intensity (FI) was expressed in arbitrary units. Fmax is the maximum FI obtained during the wavelength scan, while λmax is the wavelength at Fmax.

3.2.4.5 Surface hydrophobicity

Surface hydrophobicity of protein isolates was determined as described by Karaca et al (2011), using 1-anilino-8-naphthalenesulfonate (ANS) as the probe. In this method, the hydrophobic groups in the protein are determined due to fluorescent nature of ANS when bound to hydrophobic sites on the protein's surface. Stock solutions of each protein isolates were prepared by mixing 10 mg/mL sample dispersions (based on protein weight) in a 0.1 M sodium phosphate buffer phosphate buffer (pH 7.0) for 1 h, followed by centrifugation 10,000 x *g* for 10 min. The collected supernatants were each diluted to final concentrations of 50, 100, 150, 200, and 250 µL/mL. For each protein concentration, A 20 µL aliquot of 0.8 M an ANS solution prepared in 0.1 M sodium phosphate buffer phosphate buffer (pH 7.0) was added. Thereafter, the fluorescence intensity (FI) of each mixture was measured at excitation and emission wavelengths of 390 nm and 470 nm, respectively, using the Jasco FP-6300 spectrofluorometer. Fluorescence intensity values for the mixtures without ANS were subtracted from the fluorescence intensity of the protein solutions containing ANS. The initial slope (S_0) of the plot of the fluorescence intensity versus protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

3.2.4.6 Protein secondary and tertiary structure measurements by circular dichroism (CD)

The far- and near-UV spectra providing information about secondary and tertiary structure of the proteins were obtained following the methods described by Agboola and Aluko (2009). The protein isolates were solubilized in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and Tris-HCl (pH 9) buffers, then centrifuged at 10000 x *g* for 30 min. The supernatants were then diluted to 2 and 4 mg/mL concentrations for far- and near-UV spectra measurements, respectively. The far-UV spectra were measured at 190 nm – 240 nm using a cuvette with a 0.05 cm path length, while 250 – 320 nm range was used for near-UV spectra in a cuvette of 0.1 cm path length. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of respective buffer spectra.

3.2.4.7 Differential scanning calorimetry

Calorimetric measurements were taken using the TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE). Each protein isolate was dispersed in 0.1 M phosphate buffer (pH 7) to a concentration of 100 mg/mL for the wet analysis of the samples. Both dry and wet samples were accurately weighed in an aluminum pan, hermetically sealed, then heated from 40 °C to 140 °C at a rate of 10 ^{o} C/min. A sealed empty pan was used as a reference and all experiments were conducted in duplicates. Onset temperature (T_o), denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed using the instrument's software (Universal Analysis 2000, Version 4.5).

3.2.4.8 Scanning electron microscopy (SEM)

Gels were formed using the least gelling concentration obtained for each sample according to the method of Malomo et al. (2014) and stored at -20 ^{o} C. A thin layer of each of the gels was deposited

on a double-sided adhesive carbon tape mounted on an aluminum stub and then coated with a thin gold layer with the help of gold sputter. Micrographs of the samples were observed with a scanning electron microscope (FEI Quanta E-SEM) at an accelerating potential of 10 kV.

3.2.4.9 Lentil protein solubility

Solubility of the protein isolates was determined using the protocol earlier described by Malomo, He, and Aluko (2014) with some modifications. In summary, 10 mg of each sample was dispersed in 5 mL of 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris buffers (pH 9) of pH 3, 5, 7 and 9 on a 1% w/v, protein weight basis. The resulting mixture was vortexed thoroughly, hydrated for 1 hr, then centrifuged at 5600 x *g* for 30 min. The protein content of the supernatants were then determined using the modified Lowry method (Markwell et al., 1978). The total protein content of the samples was also determined with the same method after dissolving the samples in 0.1 M NaOH solution. The total protein content and solubility was determined by reading absorbance at 660 nm using a UV spectrophotometer, and then calculated as:

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

3.2.4.10 Heat coagulability

Heat coagulability was determined according to the method outlined by Malomo et al. (2014), which was slightly modified as follows. Protein solutions (10 mg/mL) were prepared in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and Tris-HCl (pH 9) buffers. The mixtures were heated in a boiling water bath (100 ^{o} C) for 15 min, cooled to room temperature, and centrifuged at 5600 x *g* for 30 min. The protein contents of the supernatants were determined using the modified Lowry method (Markwell et al., 1978). Heat coagulability was then obtained by calculating the percentage difference between the protein content of the supernatant and that of the sample.

3.2.4.11 Emulsion formation & stability

Emulsions were prepared by adding 1 mL of pure canola oil to centrifuge tubes containing aqueous dispersions of the isolated proteins in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and Tris-HCl (pH 9) buffers. The sample/oil mixtures containing varying protein concentrations of 10, 15 or 20 mg/mL, were homogenized at 20,000 rpm for 1 min, using the 12 mm non-foaming shaft on a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The oil droplet size ($d_{3,2}$) and volume weighted mean ($d_{4,3}$) of the emulsions were determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as dispersant (Chao et al., 2018). Using a transfer pipet, emulsions were carefully removed into about 100 mL of constantly sheared, ultrapure water contained in the Hydro 2000S wet sample dispersion component, until the sufficient level of obscuration was achieved. After taking triplicate readings, the mean values were computed as an indicator of their emulsion capacity. Emulsion stability was determined by repeating the measurements to determine the oil drop size ($d_{3,2}$) 30 min after emulsion formation.

3.2.4.12 Foam formation and stability

Foaming properties for each isolated protein were determined according to the method of Chao et al. (2018) with slight modifications. Protein solutions of various concentrations (10, 15 and 20 mg/mL) were prepared with 0.1 M acetate, phosphate, or Tris-HCl buffer solutions, vortexed thoroughly and left to hydrate for 30 min. Samples 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 foam was formed in a 50 mL graduated centrifuge tube to determine the volume of foam formed (foaming capacity). The volume of foam remaining after 30 min at room temperature was expressed as a percent value of original foam volume to obtain foam stability.

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

3.2.4.13 Water and oil holding capacity

The water and oil holding capacity was determined using the method of Malomo et al. (2014) with some modifications. Aqueous protein solutions of 20, 40, or 60 mg/mL of protein isolates were prepared in pre-weighed 15 mL centrifuge tubes containing 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and Tris-HCl (pH 9) buffers. To determine oil holding capacity, similar sample concentrations were prepared using pure canola oil instead of buffers. The sample dispersion (water or oil) was vortexed thoroughly, then allowed to stand for 30 min at room temperature. The mixtures were centrifuged at 5600 x *g* for 30 min and the supernatant containing the excess water or oil was drained for 15 mins, after which the centrifuge tubes were reweighed to determine the amount of oil retained per gram of protein.

3.2.4.14 Least gelation concentration (LGC)

The LGC of the isolated proteins were determined using the method of Malomo et al. (2014). A series of aqueous protein solutions was prepared in 0.1 M phosphate buffer of concentrations between 2% to 20% w/v (based on protein weight). The mixtures were placed into test tubes, vortexed for 5 min, then heated in a water bath at 95 °C for 1 h. The tubes were immediately cooled to room temperature and stored at 4 °C for 14 h. LGC was determined as the minimum concentration at which the gel did not slip when the tube was inverted.

3.2.4.15 In vitro protein digestibility

The in vitro digestibility of the isolated proteins was analysed using the protocols previously outlined by Hsu et al. (1977) with some modifications. The protein samples were suspended in an aqueous solution containing DDW and adjusted to pH 8 with 0.1 M NaOH while stirring at 37 ^{o}

C. A 3 mL aliquot of an enzyme solution (containing 1.6 mg trypsin, 3.1 mg chymotrypsin and 1.3 mg peptidase/mL) was taken from the enzymatic solution maintained in an ice bath and added to 30 mL of each protein suspension. The drop in pH of the mixture was recorded every 30 s over a 10 min period using a pH meter and the analysis was repeated to obtain duplicate results. The per cent protein digestibility of each protein sample was calculated using the regression equation of Hsu et al. (1977) as follows:

% Protein digestibility (Y) = 210.46 – 18.10Xf

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

3.2.4.16 Statistical analysis

Except where indicated, all data was reported as mean ± standard deviation from triplicates. Statistical analysis including one-way ANOVA and the Duncan's multiple-range tests (accepted at $p<0.05$) were performed using IBM SPSS version 26.

3.3 Results and discussions

3.3.1 Chemical composition and protein yield

The proximate composition and protein content of DEF, ISO, MEM_NaOH and MEM_NaCl are shown in Table 1a. ISO had a significantly higher protein content when compared to the membrane protein isolates. The protein contents of the lentil isolates in this work are comparable to the 85.10 – 93.7% values that have been reported in scientific literature (Alonso-Miravalles et al., 2019; Avramenko et al., 2013; Joshi et al., 2011). The protein content of the defatted lentil flour was the lowest compared to those of the isolates and is similar to the protein contents of red (29.37%) and green (26.59 – 27.70%) lentil flours as reported by Barbana & Boye (2013) and Ghumman et al. (2016), respectively. The significantly higher amount of protein in the isolates compared to the defatted flour emphasises the effectiveness and importance of the various isolation processes used

for protein extraction. In terms of protein yield (Table 1b), ISO had a significantly higher protein yield (48%) than MEM_NaOH (35%) and MEM_NaCl (13%). Fat content was generally low for the defatted flour and protein isolates (<2%), while the carbohydrate content varied significantly with MEM_NaCl having the lowest and DEF having the highest. A correlation of the carbohydrate content with the amount of crude proteins present is apparent, with an increasing trend of non-fibre carbohydrates corresponding to a reduced crude protein content. The impact of a higher amount of carbohydrates or other non-protein constituents like ash, or fat being co-extracted with proteins is also apparent in the results obtained by Johnston et al. (2015) for faba bean, lentil, and soy protein isolates. These proportional differences in the composition of the various isolates play a significant role in predicting some of their physicochemical and functional properties such as their water and oil holding capacities. ISO also had the highest protein yield from the protein yield presented in Table 1b.

The amino acid composition data of the isoelectric protein isolate (ISO), alkaline protein concentrate (MEM_NaOH) and salt protein concentrate (MEM_NaCl) were similar with very few notable differences (Table 2). The most abundant amino acids present were glutamic acid (15 – 17%) and aspartic acid (12%), which included glutamine and asparagine, respectively. In general, the results are similar to those obtained for glutamic (13% – 17%) and aspartic acid (9% – 11 %) reported for lentil, pea, and Bambara proteins (Arise et al., 2017b; Stone et al., 2015; Wang & Daun, 2006). The human body tissues store glutamine and asparagine obtained from foods and this is used by the intestines as the preferable energy supply during the body's metabolic processes (Blanco & Blanco, 2017), hence the high levels are good for maintaining optimal human health. The next most abundant amino acids are arginine and leucine, as is common with legumes (Arise et al., 2017b; Stone et al., 2015). Arginine is a conditionally essential amino acid that generates

nitric oxide, which promotes normal endothelial function, insulin secretion, and pancreatic beta cell protection. Arginine has also been known to have a positive impact on type II diabetes Mellitus by intensifying energy expenditure, improving glucose homeostasis, maintaining lean body mass, and lowering blood lipids as well as blood pressure. Dietary requirement is the amount and quality of amino acids needed to adequately fuel metabolic processes and maintain appropriate body composition and growth. Therefore, the amino acid profile of the protein is pertinent to its perceived nutritive value. All lentil protein isolates were limiting in methionine when compared with the standard amino acid requirements (WHO & FAO, 2007). With the exception of methionine, all other essential amino acids including tryptophan, threonine, cysteine, valine, isoleucine, histidine, and lysine were found to be higher than the FAO/WHO requirements for adults. This makes lentil proteins nutritionally superior, especially when paired with cereal-based foods, which are deficient in lysine and high in methionine (Gómez et al., 2008). Besides, branched-chained amino acids have beneficial effects on the human immune function, protein synthesis, and gut health (Kawaguchi et al., 2011).

The lentil protein cysteine content, especially, was notably higher than the proposed dietary requirements (amino acid score >226%), which is in direct contrast with the past studies that have concluded that legumes are generally limiting in this amino acid (Stone et al., 2015). Cysteine serves as a substrate for the biosynthesis of glutathione, which is a powerful antioxidant that protects the body against oxidative damage, and has been known to promote fertility in males (Meletis & Barker, 2005; Uneyama et al., 2017). Valine content of lentil proteins was also found to be significantly higher (5.1% - 5.4%) when compared with soybean (4.7%), peas (4.5%), or chickpea (4.0%) (El-Beltagi et al., 2017; Grela et al., 2017; Sotak-Peper et al., 2017). The isolated lentil proteins all had slightly higher amino acids but were still similar to previously reported data

in literature (Grela et al., 2017; Pirman et al., 2001; ZIA-UL-HAQ et al., 2011). Two notable differences were the serine and glycine. Serine in all the isolated proteins were significantly higher (>5.1%) than previously documented results (≈ 4.7%), while glycine in ISO (3.4 %) and MEM_NaOH (3.8 %) was significantly lower (4.1%).

Table 1: a) Proximate composition of defatted lentil four (DEF), isoelectric pH-precipitated isolate (ISO), and membrane isolates from alkaline (MEM_NaOH) and salt (MEM_NaCl) solution extractions; b) protein yield of isoelectric pH-precipitated isolate (ISO), and membrane isolates from alkaline (MEM_NaOH) and salt (MEM_NaCl) solution extractions.

(a)

	DEF	ISO	MEM_NaOH	MEM_NaCl
Moisture content	7.12 ± 0.54^{a}	5.24 ± 0.72^{b}	8.59 ± 0.52^{a}	2.64 ± 0.83^{c}
Crude fibre	4.41 ± 0.25^{a}	0.10 ± 0.07^{b}	0.02 ± 0.02^{b}	0.02 ± 0.01^{b}
Fat	1.36 ± 0.66^{ab}	0.35 ± 0.13^{b}	1.57 ± 0.23^{a}	0.39 ± 0.15^{b}
Ash	2.96 ± 0.04^{d}	6.00 ± 0.21^{a}	3.72 ± 0.17^{c}	4.75 ± 0.05^{b}
Non-Fibre Carbohydrates	60.99 ± 1.32^{a}	6.48 ± 1.43^{c}	11.24 ± 0.95^{b}	3.61 ± 0.76^{c}
Crude protein	29.35 ± 0.45^{d}	86.13 ± 1.02^{b}	82.55 ± 0.54^{c}	90.28 ± 0.67^{a}

(b)

	ISO	MEM_NaOH	MEM_NaCl
Protein yield	48.81 ± 0.76^{a}	35.05 ± 1.63^{b}	13.35 ± 0.05^{c}

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

Table 2: Percent amino acid composition of lentil protein isolates

AA	ISO[a]	MEM_NaOH[b]	MEM_NaCl[c]	Amino acid score (%)
Asx	12.29 ± 0.17	12.89 ± 0.12	11.79 ± 0.38	
Thr[1]	3.78 ± 0.00	4.06 ± 0.02	4.76 ± 0.06	118[a], 108[b], 140[c]
Ser	5.40 ± 0.08	5.38 ± 0.14	5.12 ± 0.09	
Glx	18.01 ± 0.19	17.71 ± 0.09	15.77 ± 0.11	
Pro	4.19 ± 0.06	4.69 ± 0.00	4.21 ± 0.04	
Gly	3.47 ± 0.13	3.79 ± 0.04	4.02 ± 0.07	
Ala	3.84 ± 0.06	3.85 ± 0.09	4.84 ± 0.19	
Cys[1]	0.96 ± 0.56	1.41 ± 0.13	1.38 ± 0.13	163[a], 153[b], 164[c]
Val[1]	5.21 ± 0.10	5.33 ± 0.01	5.49 ± 0.05	95[a], 83[b], 94[c]
Met[1]	1.01 ± 0.04	1.31 ± 0.06	1.33 ± 0.02	47[a], 52[b], 56[c]
Ile[1]	4.52 ± 0.42	4.78 ± 0.49	4.26 ± 0.41	115[a], 104[b], 102[c]
Leu[1]	8.43 ± 0.04	8.18 ± 0.09	7.31 ± 0.05	103[a], 85[b], 83[c]
Tyr	3.55 ± 0.02	3.56 ± 0.16	4.18 ± 0.40	
Phe	5.81 ± 0.06	5.74 ± 0.02	5.08 ± 0.02	
His[1]	2.32 ± 0.25	2.41 ± 0.35	2.36 ± 0.21	120[a], 108[b], 112[c]
Lys[1]	6.75 ± 0.18	6.54 ± 0.13	8.78 ± 0.39	110[a], 90[b], 135[c]
Arg	8.94 ± 0.25	6.92 ± 0.11	6.58 ± 0.06	
Trp[1]	0.85 ± 0.06	1.01 ± 0.05	1.20 ± 0.03	107[a], 106[b], 136[c]
AAA	10.21 ± 0.01	10.31 ± 0.10	10.46 ± 0.40	
BCAA	18.16 ± 0.37	18.30 ± 0.60	17.06 ± 0.41	
HAA	38.38 ± 0.95	39.87 ± 0.78	39.28 ± 0.32	
PCAA	18.01 ± 0.68	15.87 ± 0.59	17.72 ± 0.54	
NCAA	30.30 ± 0.02	30.61 ± 0.21	27.56 ± 0.49	
SCAA	1.97 ± 0.60	2.72 ± 0.19	2.70 ± 0.15	

ISO (isoelectric precipitation); membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solutions; 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; [1]Essential amino acids; amino acid score calculated with EAA requirements for adults according to WHO & FAO (2007)

3.3.2 In vitro protein digestibility

Protein quality not only depends on the pattern and number of amino acids but also on the amount that will be made available for absorption after digestion. Protein digestibility is, therefore, also an important parameter when evaluating the nutritive value of protein food products. The presence of antinutritional compounds like phytic acid, and tannins, as well as the large size and folded conformation of plant storage proteins, lower the digestibility of raw lentil flours (Martín-Cabrejas et al., 2009). To improve their digestibility and nutritional quality, the effects on these antinutrients can be reduced by various processing methods including the extraction and fractionation of lentil protein. In vitro protein digestibility (IVPD) values of the isolated lentil proteins are presented in Figure 1. The protein digestibility of ISO (89.8%) can be seen to be significantly higher than those of the membrane isolates, while that of MEM_NaOH (77.9%) and MEM_NaCl (77.6%) were identical. The results obtained for ISO was significantly higher than 83.2% and 85.4% recorded by Barbana & Boye (2013) and Aryee & Boye (2016) who also used the isoelectric precipitation method to prepare their lentil protein isolates. Although the membrane isolates had significantly lower digestibility in comparison, they were comparable or even higher then that of quinoa protein isolate (78.4%) and other plant protein products such as flaxseed (68.0%), barley (77.5%), chickpea (77.0%), and common bean (73.5%) (Carbonaro et al., 2012; Elsohaimy et al., 2015; Marambe et al., 2013). ISO on the other hand, performed admirably when compared with animal protein products that are usually known to have higher digestibility. According to Carbonaro et al (2012) chicken meat was slightly more digestible (92.0%) whereas ISO had a higher digestibility than mozzarella cheese (87.0%) and pasteurized milk (84.0%). The lower digestibility of the membrane isolated proteins indicates presence in a more native conformation

(folded) when compared to the ISO, which may have been denatured (greater unfolding) during acid precipitation.

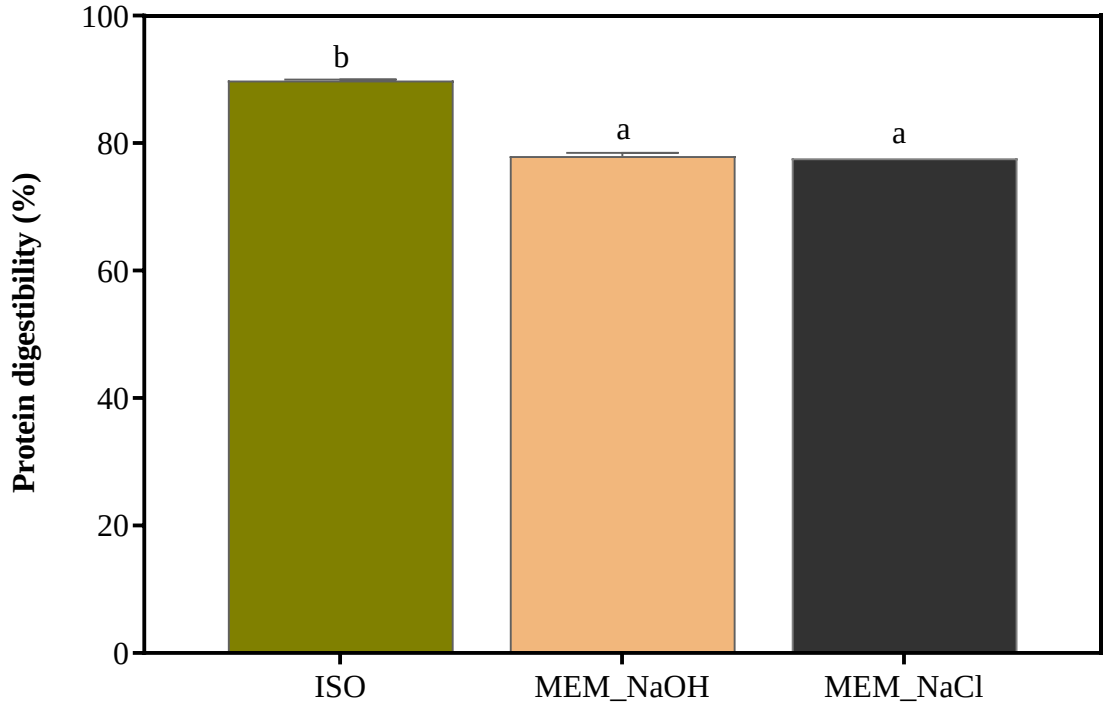

Figure 1. In vitro protein digestibility of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl) solutions

3.3.3 Intrinsic fluorescence

Aromatic amino acids like tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) can emit a fluorescence spectrum with a maximum range of 310 – 360 nm when they are excited in the visible region. These emissions reveal the conformational changes, which also reflects the level of exposure of the aromatic amino acids to the hydrophilic environment (Eftink, 2000). Since this gives more information about how the proteins are folded, it can also be used to predict other functional properties of the protein such as solubility, which involves a protein-solvent interactions (Malomo et al., 2014). Trp and Tyr residues of the lentil protein isolates were excited at a

wavelength of 280 nm and showed a single fluorescence peak (Figure 2). The maximum fluorescence intensity (FI) occurred at less than 350 nm for all samples. An exception was MEM_NaOH at pH 9, which had a maximum FI (λmax) of 353 nm, showing about 10 nm red shift compared to ISO and MEM-NaOH at the same pH. This indicates that the indole group in the Trp residues of MEM_NaOH at pH 9 were positioned in a more polar environment, while the Trp residues of ISO, MEM_NaCl were in a more hydrophobic environment. In general, MEM_NaCl was the only sample that had a fluorescence peak at each pH, and except for pH 9, its FI also remained the highest in comparison. This could be attributed to the higher content of Trp and Tyr residues in MEM_NaCl but also indicates a more folded conformation when compared to ISO and MEM_NaOH. MEM_NaOH on the other hand, only had a peak at pH 9, signifying that there were no Trp residues in a hydrophobic environment at pH 3, 5 or 7, which is similar to ISO at pH 3 and 5. In both cases, since there was no distinct Trp fluorescence, the lower emission wavelength detected indicated extensive interactions with the hydrophilic environment (Eftink, 2000). The results obtained were generally comparable to λmax of 322 – 332 nm, and 333 nm reported for lentil protein isolate and Bambara proteins, respectively (Arise et al., 2017b; Avramenko et al., 2013).

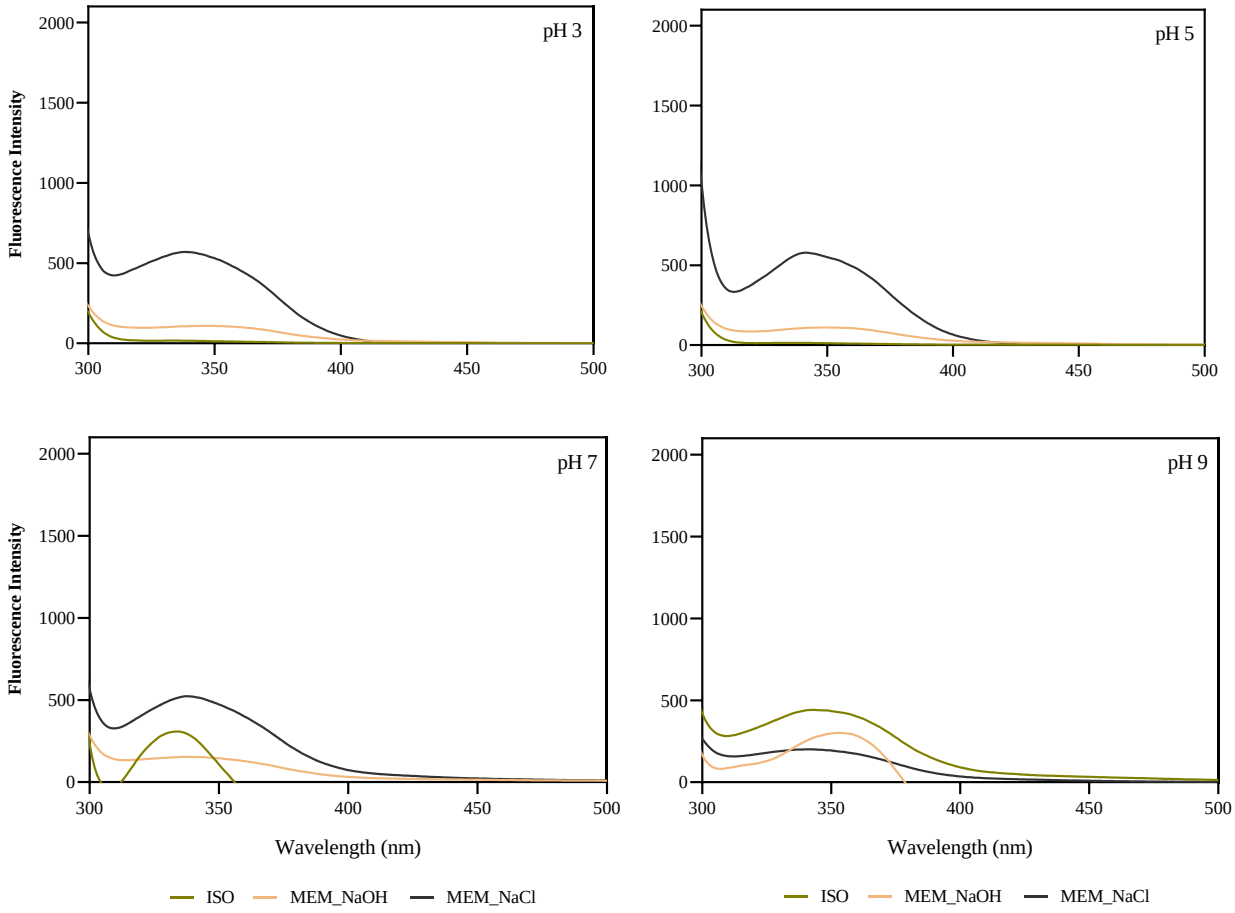

Figure 2. Intrinsic fluorescence intensity of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl) solutions.

3.3.4 Surface hydrophobicity

Surface hydrophobicity (S_o) is an important means of characterizing proteins and evaluating their surface related functional properties. It reflects the change in protein conformation resulting from the extent of intermolecular protein interactions (Arogundade et al., 2016). As shown in Figure 3. the S_o of MEM_NaCl (5.8) was higher than that of ISO (5.1) and MEM_NaOH (1.0), implying that there were more exposed hydrophobic clusters in MEM_NaCl and ISO than in MEM_NaOH. Variation in the S_o of the different proteins could be attributed to their varied amino acid compositions, resulting in a higher or lower exposure of aromatic and aliphatic amino acid residues (Jahaniaval et al., 2000). Denaturation caused by alkaline isoelectric precipitation of ISO compared to the milder membrane filtration process used in MEM_NaOH could also account for the significantly lower S_o of MEM_NaOH, since denaturation increases the exposure of buried hydrophobic groups (Mine, 1997). Similarly, the salt used in the isolation process of MEM_NaCl could have a denaturing effect by disrupting non-covalent electrostatic bonds and also reduce the solvation of the protein molecules. The possible influence of the degree of denaturation (caused by pH) on hydrophobicity was also in agreement with the result of Arogundade et al. (2016), illustrating that the S_o of their African yam bean protein isolate obtained from ultrafiltration was lower than that of isoelectric precipitation. Alonso-Miravalles et al. (2019) concluded that the method of extraction had no major impact on their S_o but noted, however, that there was a

significant difference between the hydrophobicity of both lentil protein isolates.

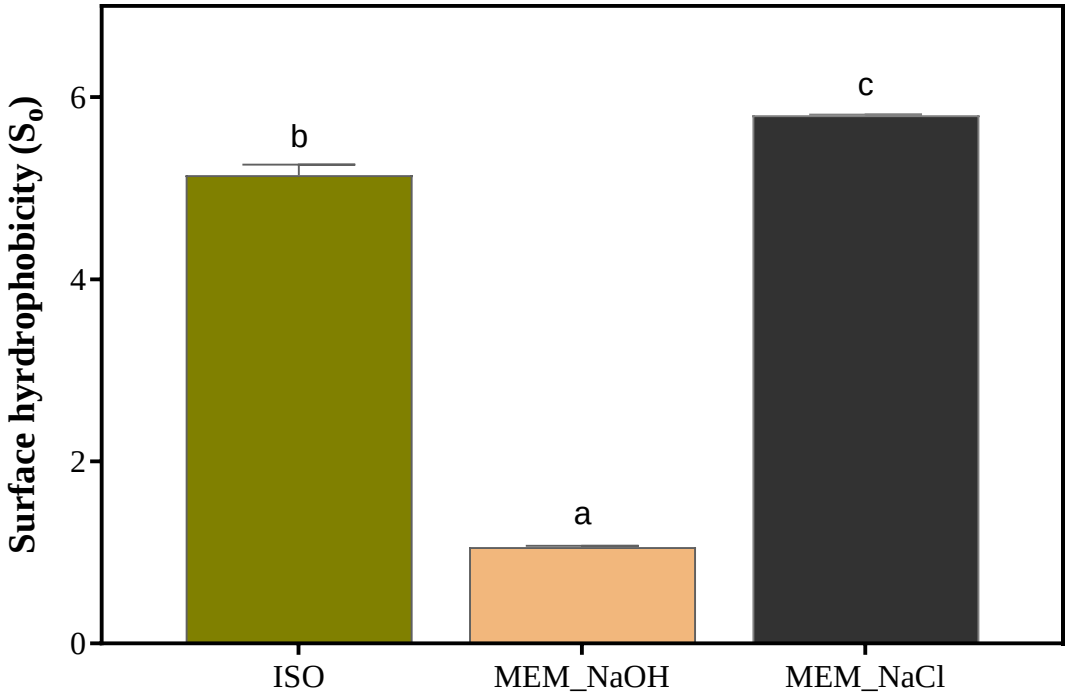

Figure 3. Surface hydrophobicity of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl) solutions

3.3.5 Circular dichroism (CD)

The effect of pH on secondary structure conformations of ISO, MEM_NaOH, and MEM_NaCl was investigated using far-UV CD as shown in Figure 4. Generally, more transitions were observed in all samples when shifting from acidic to alkaline mediums, suggesting less compact protein structures at pH 3 and 5 (Schmid, 1990). At acidic pH, helix 1, which had a more coiled structure was noticeably absent or greatly reduced in the samples, with the exception of MEM_NaCl. The spectra of ISO, for example, is seen to have reduced and almost zero ellipticity at pH 3 and 5, but higher degrees of α-helix conformation characterised by the double minimum occurring at 205 and 225 nm were observed at pH 7 and 9. On the other hand, increasing intensity of ISO across pH 3, 5, 7 and 9 also most likely negatively correlates with the presence of β-sheets. A similar trend of increasing α-helix but decreasing β-sheet conformations when moving from acidic to alkaline pH ranges can also be observed in the MEM_NaOH spectra. In contrast with the patterns of helical structures present in ISO and MEM_NaOH, the MEM_NaCl had higher degrees of helical conformation at the acidic pH range, which was evident from the intense peaks present at about 210 nm in pH 3 and 5. The results also show that β-sheet structures of MEM_NaCl were greatly reduced in comparison with the other samples. However, all the protein samples had more β-sheet than helical conformations. The negative helical peak appearing at 200 – 210 nm was similarly found in the spectra of lentil protein isolates as previously reported by Alonso-Miravalles et al. (2019) and Aryee & Boye (2017).

Less intense peaks occurring at 190 – 195 nm at pH 7 and 9 for all samples also indicate the presence of more random coils at neutral and basic pH range compared to acidic pH values. The presence of high ratio of random coils indicates highly disordered and less compact protein structures at pH 7 and 9. Loss of the compact nature of the protein structures could be associated

with a higher protein solubility as shown by the improved solubility values reported at pH 7 and 9.

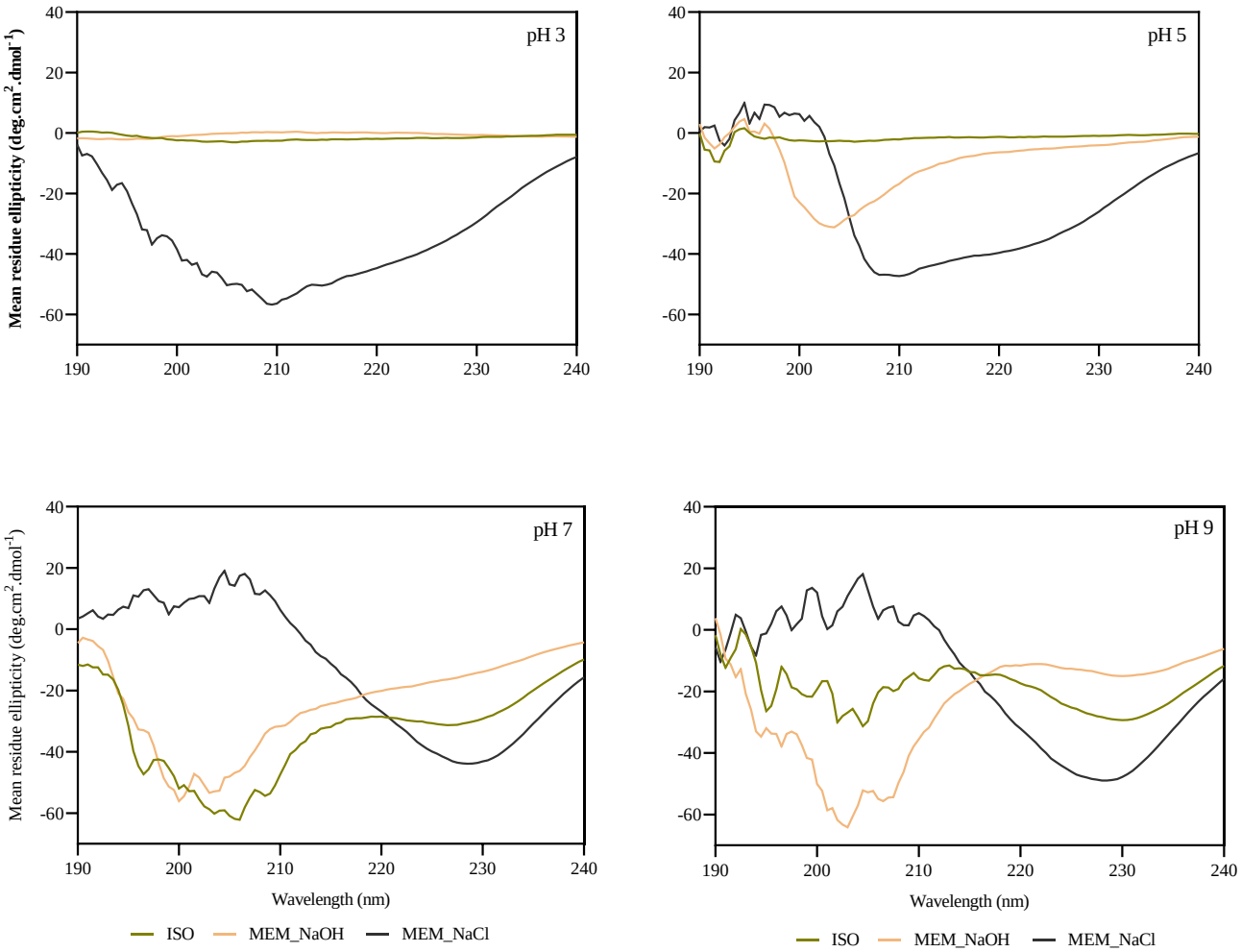

Figure 4. Far-UV circular dichroism spectra of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl) solutions at different pH values at different pH values.

Table 3: Circular dichroism-derived protein secondary structure composition of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl) solutions at different pH values at different pH values.

ISO				
pH	3	5	7	9
α-helix	2.37 ± 0.03	2.52 ± 0.03	7.65 ± 0.28	6.05 ± 0.28
β-sheet	17.60 ± 0.07	17.00 ± 0.14	9.75 ± 0.64	12.38 ± 0.39
Turns	18.90 ± 0.00	18.85 ± 0.21	14.20 ± 0.28	13.65 ± 0.64
Unordered	41.25 ± 0.07	42.10 ± 0.14	50.95 ± 1.48	49.55 ± 1.34
Total	100.10 ± 0.00	100.00 ± 0.00	99.95 ± 0.07	100.05 ± 0.07
	100.10 ± 0.28	100.00 ± 0.07	99.95 ± 3.61	100.05 ± 3.32

MEM_NaOH				
pH	3	5	7	9
α-helix	2.45 ± 0.00	3.35 ± 0.14	3.92 ± 0.25	5.65 ± 0.64
β-sheet	17.57 ± 0.03	14.87 ± 0.39	13.55 ± 0.64	12.00 ± 0.21
Turns	18.50 ± 0.00	17.80 ± 0.28	16.80 ± 1.27	14.40 ± 0.28
Unordered	41.50 ± 0.00	45.75 ± 1.20	48.25 ± 2.19	50.30 ± 0.14
Total	100.05 ± 0.07	100.00 ± 0.14	100.00 ± 0.14	100.00 ± 0.00
	100.05 ± 0.07	100.00 ± 2.54	100.00 ± 5.23	100.00 ± 2.12

MEM_NaCl				
pH	3	5	7	9
α-helix	6.20 ± 0.28	7.78 ± 0.60	5.08 ± 0.25	6.13 ± 0.32
β-sheet	11.85 ± 0.85	13.25 ± 0.28	13.17 ± 0.39	12.20 ± 0.49
Turns	17.40 ± 0.42	17.95 ± 0.35	13.35 ± 0.92	12.55 ± 0.49
Unordered	46.50 ± 0.71	40.00 ± 2.12	50.20 ± 1.27	50.75 ± 0.21
Total	100 ± 0.00	100.00 ± 0.00	100.05 ± 0.07	99.95 ± 0.07
	100 ± 3.39	100.00 ± 4.24	100.05 ± 3.46	99.95 ± 2.33

The effect of pH on the tertiary structures of ISO, MEM_NaOH, and MEM_NaCl, which are dependent on the location of aromatic amino acid residues (Phe, Tyr, and Trp), was investigated using near-UV (250-320 nm) as shown in Figure 5. The CD signal of each protein depends on the number and proximity of the aromatic amino acid residues, degree of H-bonding, polar groups present, disulfide bonds, and nature of the chromophores (Kelly et al., 2005).

Similar to what was observed in their secondary structure conformations, the near-UV spectra for ISO and MEM_NaOH were similar but relatively different from that of MEM_NaCl (except pH 9). Nevertheless, change in pH resulted in variations in the tertiary structures of all protein samples. At pH 3, the lentil proteins had a positive peak ellipticity at 255 – 261 nm, which is consistent with Phe residues within a hydrophobic atmosphere. Another peak indicating the presence of Trp can also be seen at 290 – 305 nm for ISO and MEM_NaCl. Similar Phe and Trp peaks were also found at pH 5 but transitioned into more intense Phe peaks at pH 7 and 9 for all samples, indicating a degree of denaturation had occurred at that pH. More intense positive peaks and lack of distinct Trp transition at pH 7 and 9 also suggested that a shift into a more hydrophilic exterior had occurred in the location of aromatic residues within the protein conformation. However, the MEM_NaOH protein had the least tertiary structure as evident in the almost zero CD values at all the pH values, which suggests substantial protein denaturation during alkaline extraction. It is also interesting to note that all the proteins show extensive denaturation at pH 5, which is close to the isoelectric point and indicate substantial structural disorganization. In contrast, the ISO and MEM-NaCl proteins regained more compact structural conformations at pH 7 and 9 than at pH 3 and 5. The results suggest that for ISO and MEM-NaCl proteins, compact structures were formed at pH 7 and

9, probably as a result of increased net charges that resulted in translocation of the aromatic amino acids away from the hydrophilic surface and into the hydrophobic core.

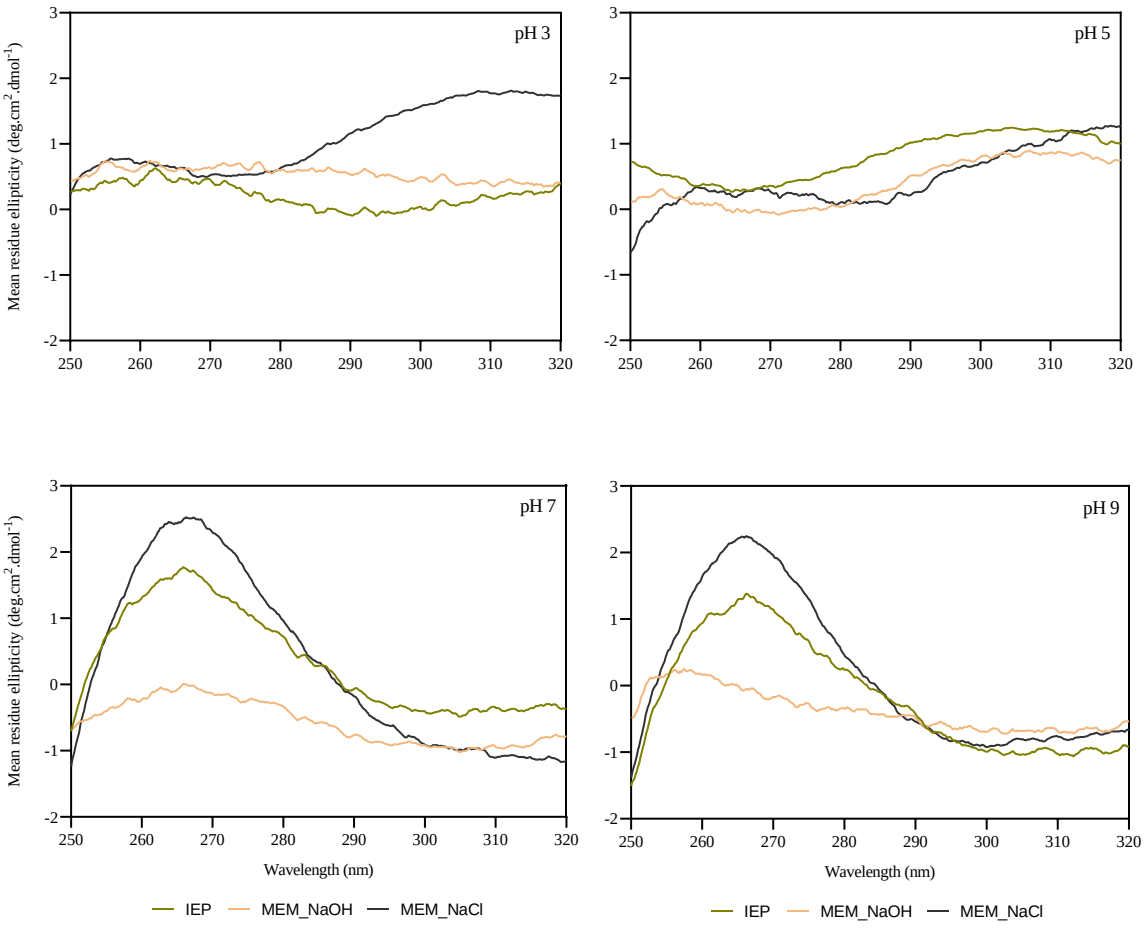

Figure 5. Near-UV circular dichroism spectra of of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl) solutions at different pH values.

3.3.6 Differential scanning calorimetry (DSC)

Thermal properties of the isolated lentil proteins were analysed using DSC and the thermograms along with thermal transition parameters are displayed in Table 4, respectively. The peak denaturation temperature was observed at around 90 ± 0.12 ^{o} C while the calculated thermal enthalpy ranged from 0.16 – 034 J/g for the proteins. The denaturation temperature obtained in this work is significantly lower than the 106.5 ^{o} C and 118.0 ^{o} C single peak denaturation temperatures reported by Ladjal-Ettoumi et al. (2016) and Joshi et al. (2011) for freeze-dried lentil protein isolates. Since a similar method of preparation was used, the difference in peak denaturation temperatures might be as a result of difference in cultivars or concentration of proteins used for testing. Differences could also indicate that the proteins used in this work suffered a higher degree of structural disorganization (increased denaturation level) than those previously reported in literature. A higher denaturation temperature indicates a more thermally stable protein, which is an important property to consider when studying other functional properties like gelation. While the denaturation temperature signifies the thermal stability of proteins, change in enthalpy characterises the extent of protein aggregation and other exothermic and endothermic effects (Barbana & Boye, 2013). Therefore, the MEM_NaCl protein has a more ordered structure (higher ΔH), which is consistent with data reported in preceding sections for the fluorescence and CD measurements.

Table 4: Thermal properties obtained using differential scanning calorimetry.

Sample	Onset T_o (ºC)	Maximum Tp (ºC)	Area ΔH (J/g of sample)
ISO	86.43 ± 0.49	90.66 ± 0.10	0.16 ± 0.09
MEM_NaOH	86.19 ± 0.13	90.72 ± 0.12	0.19 ± 0.04
MEM_NaCl	85.86 ± 0.34	90.90 ± 0.06	0.34 ± 0.00

- ISO (isoelectric precipitation); membrane isolates using alkaline (MEM_NaOH) and salt (MEM_NaCl).

3.3.7 Molecular weight (MW) analysis

The non-reduced and reduced SDS-PAGE of the ISO, MEM_NaOH, and MEM_NaCl proteins are presented in Figure 6. Under non-reduced condition, the ISO profile has eight polypeptides (90, 65, 52, 30, 25, 23, 19, and 15 kDa) with 90 kDa having the highest band intensity (Table 5). The other isolates under the same condition had different protein profiles, emphasizing the effect of extraction method on their molecular weight distribution. MEM_NaOH had nine polypeptide bands (70, 47, 40, 28, 23, 22, 20, 18, and 15 kDa), while MEM_NaCl had five (57, 31, 23, 18, and 15 kDa) with the highest proportion being 70 kDa and 57 kDa, respectively. All protein isolates in this study showed some of the typical bands consistent with legumin and vicilin-like proteins. Legumins are hexamers made up of acidic (∼40 kDa) and basic (∼20 kDa) subunits linked together by a disulfide bond. MEM_NaOH were primarily made up of MW bands of <47 kDa, indicating that legumins are the major polypeptides present (Berrazaga et al., 2020; Ladjal-Ettoumi et al., 2016; Singhal et al., 2016). Similarly, all isolates had the similar recurring bands at ∼15 kDa, ∼19 kDa, and ∼23 kDa, corresponding to the β- subunit of legumin. Vicilin is a trimer consisting of α, β and γ- polypeptide subunits with molecular weights between 50 – 67 kDa and can be found in MEM_NaCl and ISO (Jarpa-Parra, 2018; Ladjal-Ettoumi et al., 2016). Subunits of 70 kDa and 90 kDa present in MEM_NaOH and ISO on the other hand, correspond to convicilins. These results are similar to those reported in literature for other isolated lentil proteins, which also had corresponding bands between 14 – 75 kDa (Alonso-Miravalles et al., 2019; Aryee & Boye, 2016; Joehnke et al., 2021). When β-mercaptoethanol was added, there was a change in the major polypeptide bands of all isolates including the breakdown of the β- legumin subunits to yield low MW polypeptides <14 kDa. This supports the implication that the legumins were previously stabilised by disulphide bonds (Table 6). This is in contrast with previously reported literature data

that indicated only subtle differences in the protein bands of reduced and non-reduced conditions (Zang et al., 2019). In contrast, absence of significant changes in the band intensity of vicilins in ISO and MEM_NaCl under reducing condition prove that the 55 – 65 kDa polypeptides lack of disulfide bonds.

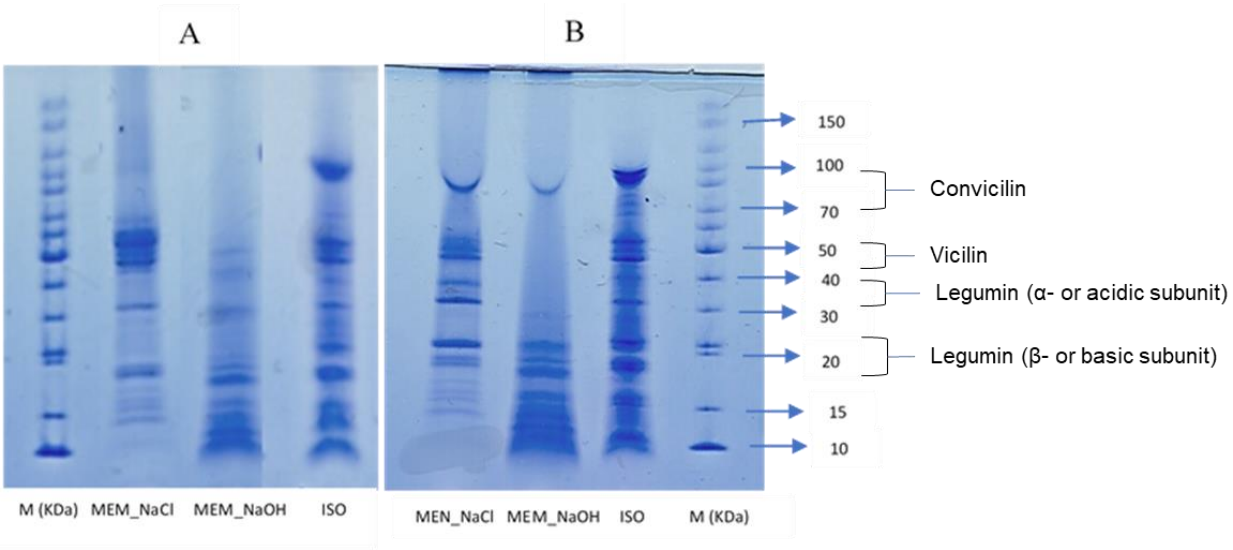

Figure 6. SDS-PAGE of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt solution (MEN_NaCl) under non-reducing (A) and reducing (B) conditions.

Table 5: Protein Composition of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solutions.

	Non-reduced Condition			
	MW (kDa)	ISO	MEM_NaOH	MEN_NaCl
Convicilin	65 – 70	8.77	19.77	-
Vicilin	25 – 31	15.6	11.89	8.48
	47 – 57	16.89	12.91	44.19
Legumin α	40		9.50	-
Legumin β	15 – 23	39.83	46.25	47.32
V/L		0.82	0.44	1.11
V + C/L		1.03	0.80	1.11
	Reduced Condition			
Convicilin	63	17.77	-	-
Vicilin	9 – 14	21.04	21.95	26.25
	26 – 27	16.68	-	18.67
	55	-	10.79	17.65
Legumin β	15 – 23	17.55	28.22	10.79
V/L		2.15	1.16	4.16
V + C/L		4.11	-	-

3.3.8 Scanning electron microscopy (SEM)

Microstructures of the isolated lentil proteins determined using SEM at 500 µm are presented in Figure 7. The SEM micrographs depict clumped structures for all lentil protein isolates, especially MEM_NaOH and MEM_NaCl which indicate molecular aggregation of the polypeptides. In addition, the membrane isolates (MEM_NaOH and MEM_NaCl) appear to have a more uniform particle size distribution compared to ISO. Generally, the appearance of smooth but shrivelled surfaces was common with ISO, MEM_NaOH and MEM_NaCl.

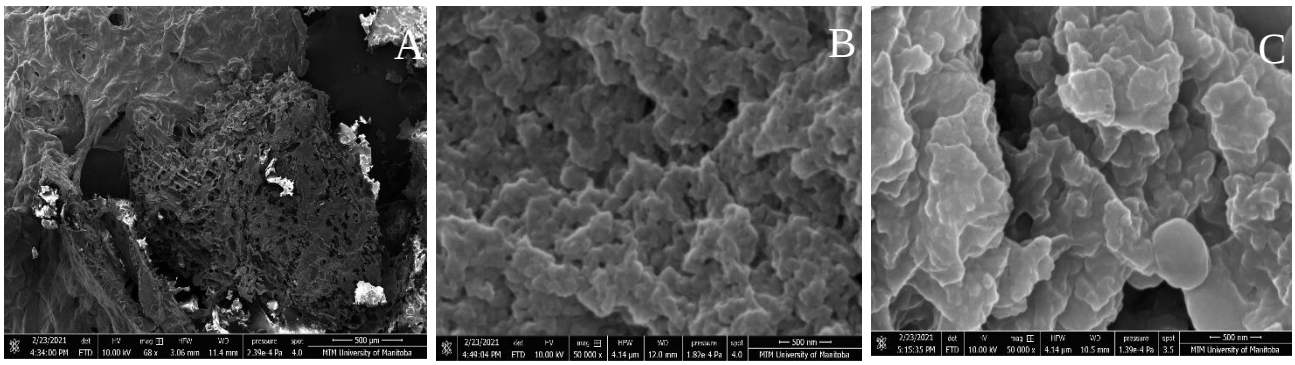

Figure 7. Scanning electron micrographs at 500 µm of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solutions; A, B, and C, respectively.

3.3.9 Solubility as a function of pH

Solubility is primarily dependent on the equilibrium between protein-solvent and protein-protein interactions, which influence other functional properties (Shevkani et al., 2019). The solubility profile at various pH levels can, therefore, serve as a useful indicator of the protein's efficiency in food systems and the degree of protein denaturation induced by the extraction process (Jarpa-Parra, 2018). The solubility of the lentil protein isolates was investigated at pH values ranging from 3 to 9 as presented in Figure 8. Their solubility profiles indicate the highest solubility for ISO and MEM_NaOH at pH 9 (100% and 100%, respectively), with the highest being pH 8 for MEM_NaCl (92 %). This is inline with the solubility of most pulse proteins, which are most soluble at high alkaline pH values but deviates from the trend as a result of their low solubility at pH $\leq$3-4, which is also characteristic of pulse proteins (Ladjal-Ettoumi et al., 2016; Rahmati et al., 2018). However, the significant decrease in the solubility of the lentil proteins at pH 4 and pH 5 (<28%) corresponds with the low solubility observed in literature at the isoelectric point of pulse proteins, which is at between pH 4 and pH 6. At the isoelectric point, there is a reduction in the electrostatic repulsion due to weakly charged proteins and this leads to aggregation of proteins as well as lower solubility (Karaca et al., 2011). The isoelectric point of the proteins also reveal that their major proteins are acidic in nature, which is evident from high amount of acidic amino acids (glutamic and aspartic) present in their amino acid composition.

The higher solubility of ISO compared with MEM_NaOH at pH ranges above its isoelectric point is consistent with the findings of Karaca et al (2011) and (Boye et al., 2010) for pea and lentil proteins. Also similar to their findings are the characteristically higher solubility at neutral pH for concentrates obtained though ultrafiltration when compared to isoelectric precipitation. The difference in solubility is usually attributed to an inverse correlation with their surface

hydrophobicity (Karaca et al., 2011; Malomo & Aluko, 2015c), but that was not the case with the present study. For instance, MEM_NaOH had a significantly low surface hydrophobicity compared to ISO and MEM_NaCl but was still not the most soluble of the three samples at any pH recorded. This emphasizes the degree of influence of other surface and structural characteristics on protein solubility, especially conformation and level of protein-protein interactions.

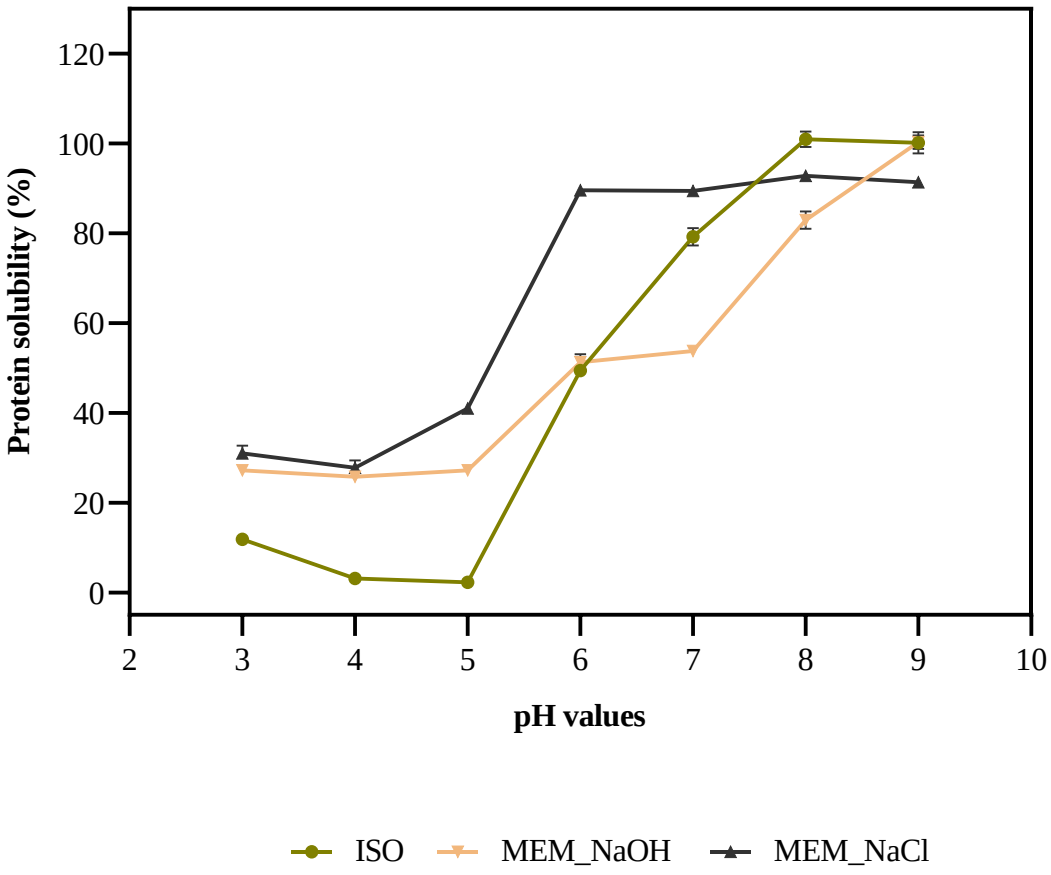

Fig 8. Percentage solubility of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl) from pH 3.0 - 9.0.

3.3.10 Emulsion formation and stability

Emulsions are generally formed when immiscible liquids are mixed to produce a continuous phase with one liquid dispersed as small droplets in the other liquid. Due to the high surface tension that prevents mixing of the two immiscible liquids and the high level of thermodynamical instability of the emulsified product, emulsifiers such as lentil proteins can be used to enhance formation and stability of emulsions (Singhal et al., 2016). During the emulsification process, proteins migrate to the oil-water interface where they are adsorbed and form viscoelastic films around the oil droplets such that the hydrophobic and hydrophilic groups of the protein are positioned towards the oil and aqueous phases, respectively (Johnston et al., 2015; Singhal et al., 2016). Changes in the stability and droplet size of the emulsion formed using the isolated lentil proteins were measured at 10 mg/mL, 15 mg/mL, and 20 mg/mL, for pH 3, 5, 7 and 9 (Fig. 9 – 11). MEM_NaOH protein showed a consistent reduction in oil droplet size ($d_{3,2}$ and $d_{4,3}$) as pH increased, indicating that better emulsions were formed, irrespective of the protein concentration. The surface area and droplet size of the oil in the emulsions formed using MEM_NaCl protein remained relatively unchanged across various concentrations and pH range, suggesting minimal effect of pH on protein unfolding. ISO and MEM_NaOH proteins formed emulsions with higher oil droplet sizes and wider surface areas at acidic pH (3 and 5) when compared to emulsions formed by MEM_NaCl protein. The oil droplet size of ISO, however, doubled for all concentrations when the pH of the emulsion was increased from pH 3 to pH 5 but was drastically reduced at pH 7 and pH 9. This is a clear indication because ISO was near its isoelectric point, there was a net neutral charge on the protein's surface and therefore was no electrostatic repulsion present to prevent aggregation of the droplets. As a result, most emulsions had the lowest oil droplet size at pH 9. Overall, pH change had more of an effect on emulsion properties than protein concentration. The results obtained are comparable to previous

reports by Chang et al. (2015), Ladjal-Ettoumi et al. (2016), and Shevkani et al. (2015) for pea and lentil proteins.

Changes in the oil droplet size of the emulsions were measured after a 30-minute interval to estimate stability. As seen in Figure 12, the emulsion stability (ES) of the lentil proteins varied considerably with changes in pH and concentration of protein. At 10 mg/mL for example, ISO demonstrated the highest emulsion stability at pH 7 and pH 9 (75% and 117%), while the reverse was the case for pH 3 and pH 5 using the same concentration (41% and 83%). This is consistent with the bigger oil droplet sizes at acidic pH and increased solubility at pH 7 and pH 9 for ISO, which emphasizes the inverse relationship between oil droplet size and emulsion stability. Similarly, MEM_NaCl protein had lower solubility and formed emulsions with bigger oil droplets and least stability at pH 7 and 9 when compared to the other isolated proteins. Following this trend, only minimal changes to stability should have been observed for emulsions formed by MEM_NaCl protein based on the relatively unchanged oil droplet size recorded. That was however not the case for MEM_NaCl protein-stabilized emulsions, suggesting that other physicochemical properties such as protein size, surface hydrophobicity and structural conformation contribute to the hydrophilic-lipophilic balance of emulsified oil droplets.

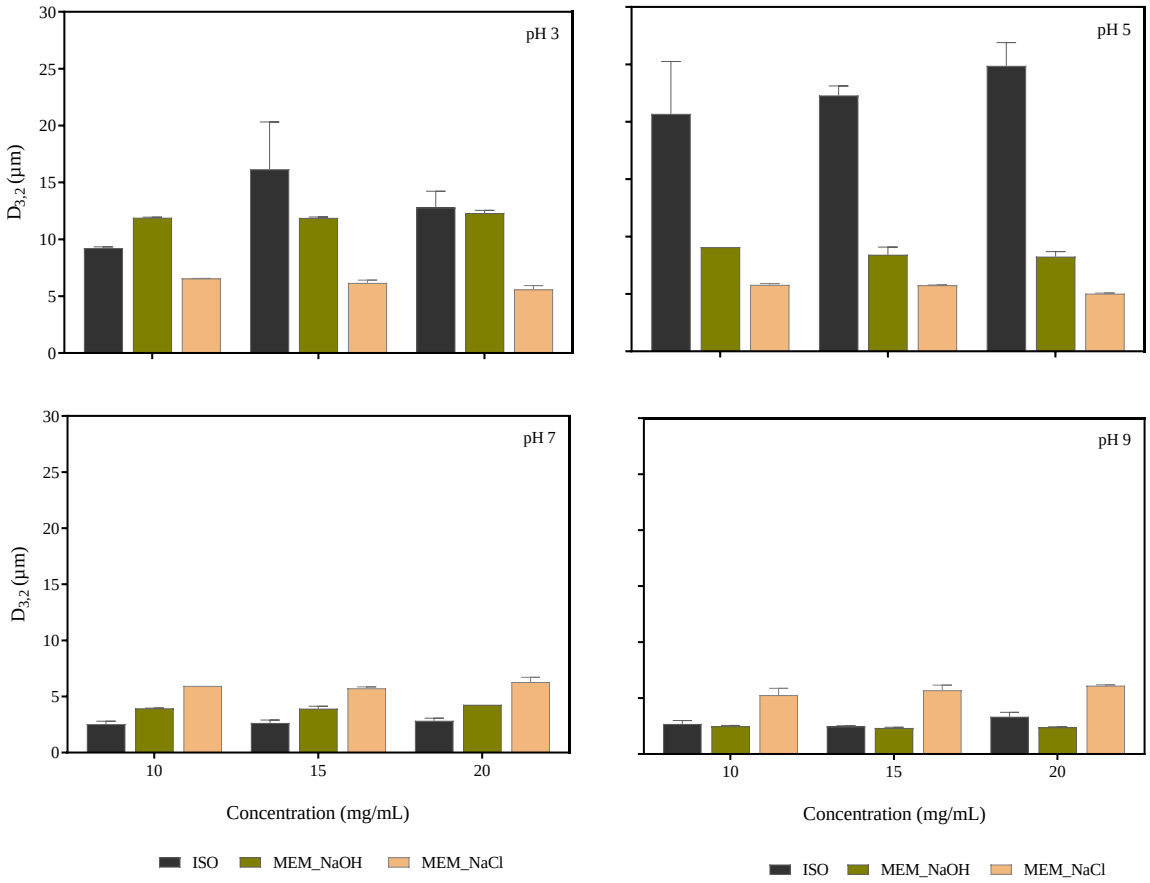

Figure 9. Surface weighted mean ($D_{3,2}$) of emulsions formed by isoelectrically precipitated isolate (ISO) and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at different concentrations and pH values.

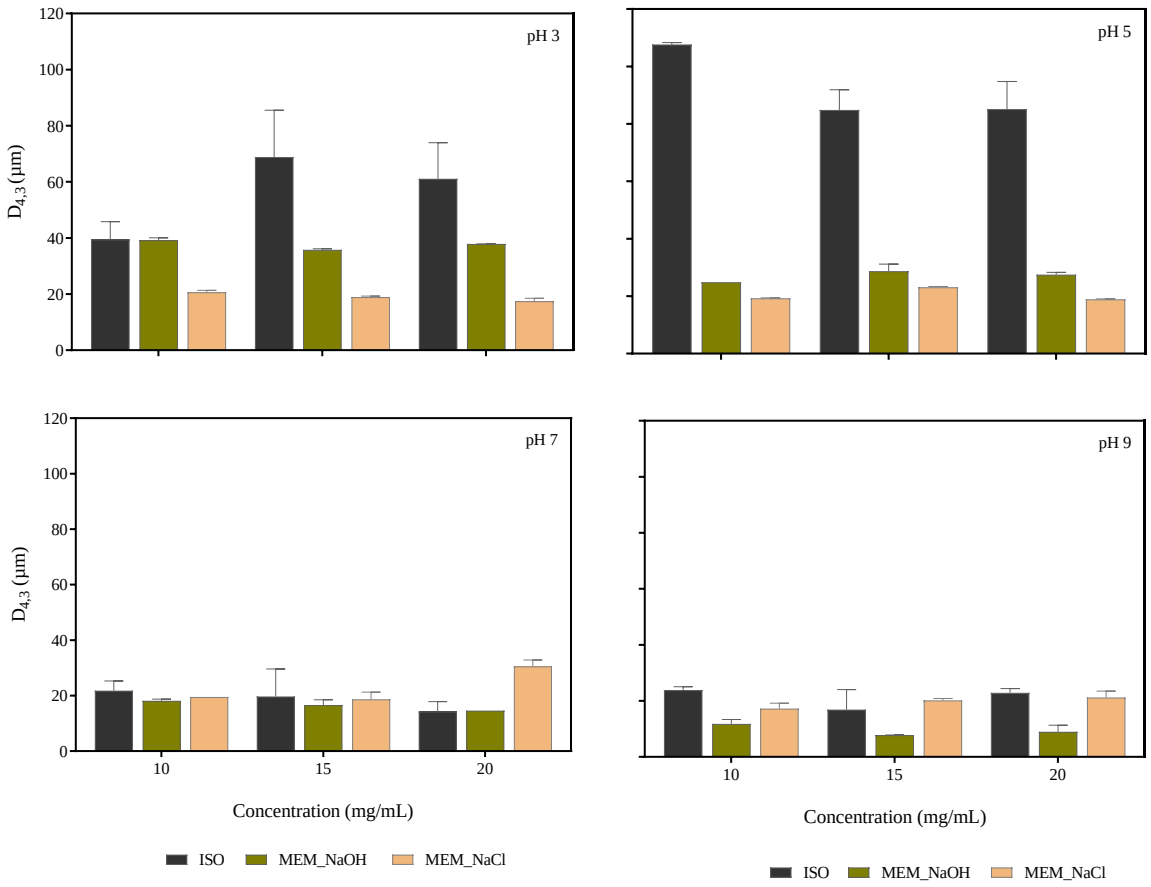

Figure 10. Volume weighted mean ($D_{4,3}$) of emulsions formed by isoelectrically precipitated isolate (ISO) and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at different concentrations and pH values.

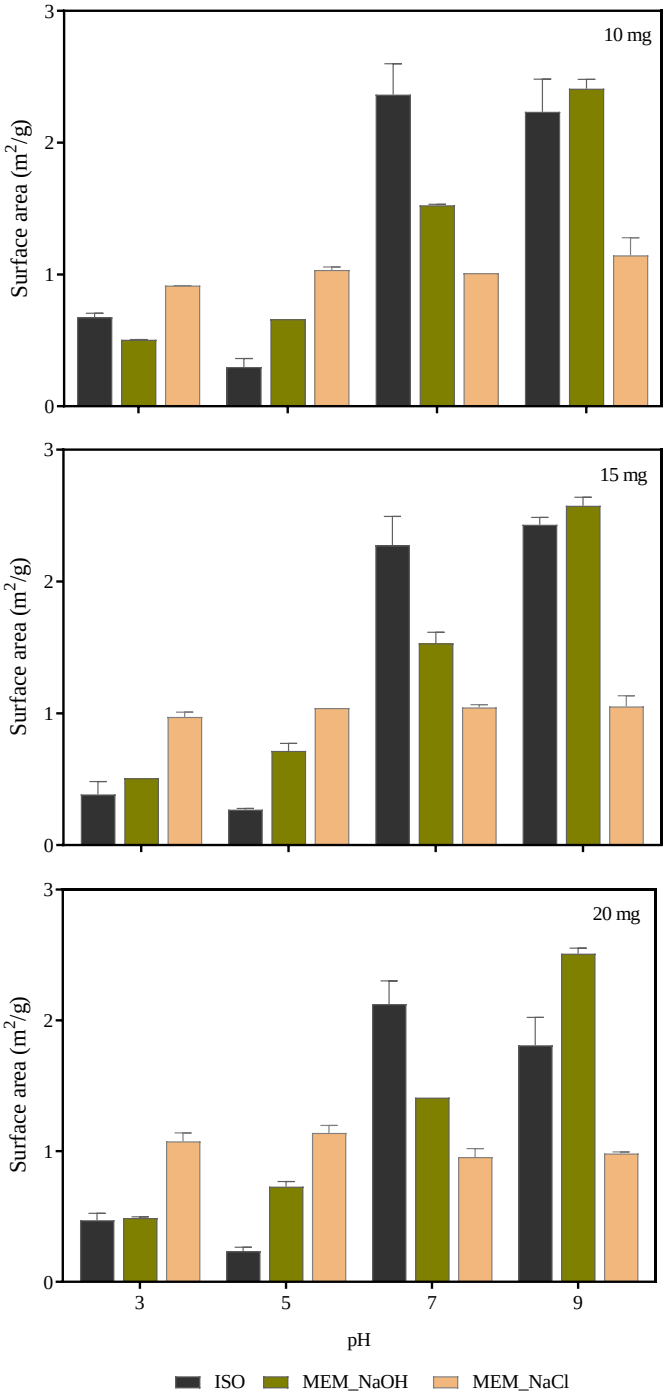

Figure 11. Surface area (m^2/g) of emulsions formed by isoelectrically precipitated isolate (ISO) and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl) at different concentrations and pH values.

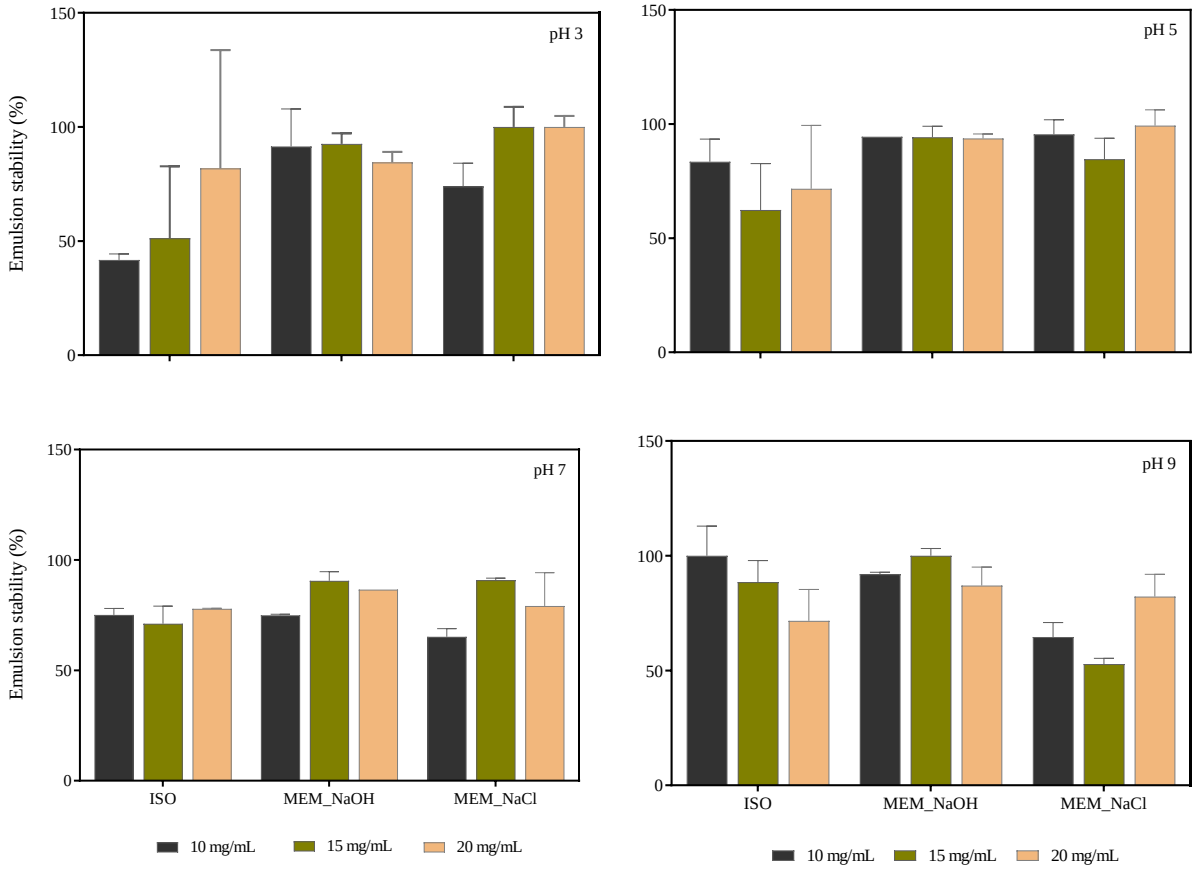

Figure 12. Percentage oil-in-water emulsion stability of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at different concentrations and pH values.

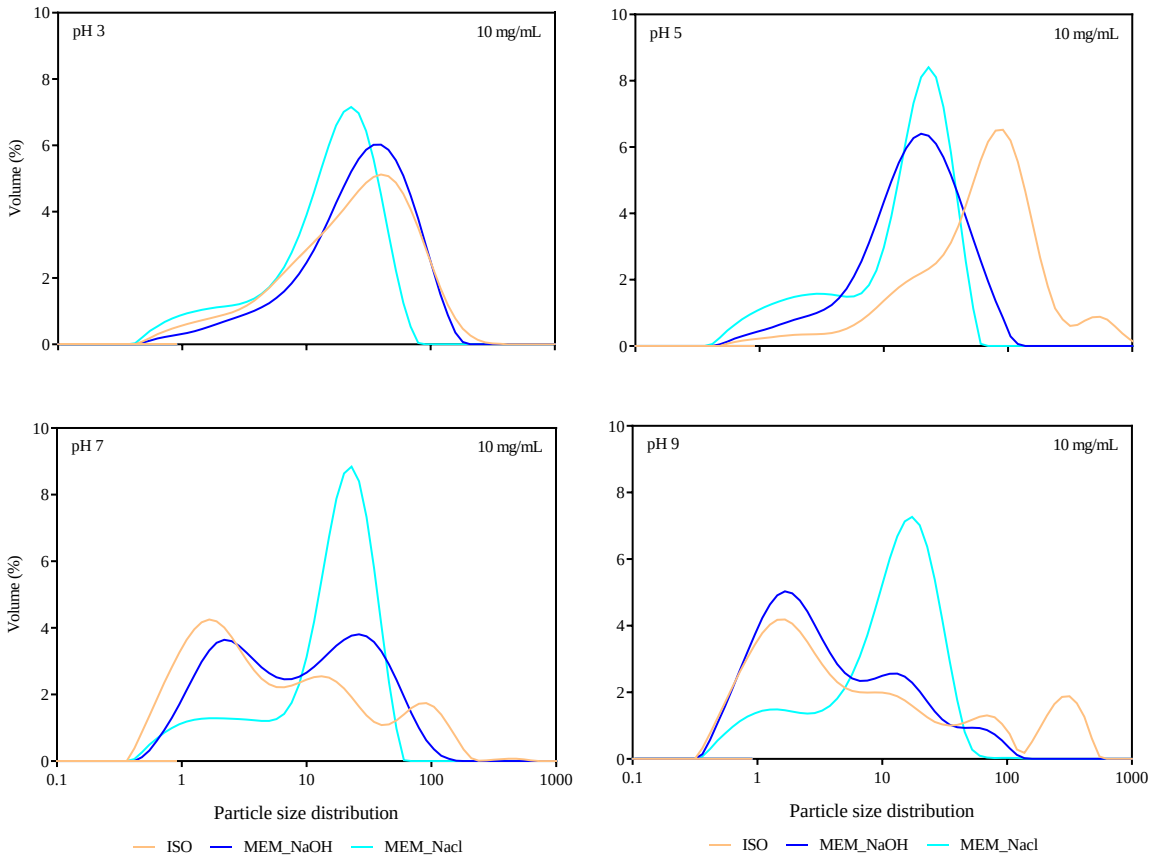

Figure 13. Oil droplet size distribution of emulsions stabilized by isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl) at 10 mg/mL and pH 3, 5, 7 and 9.

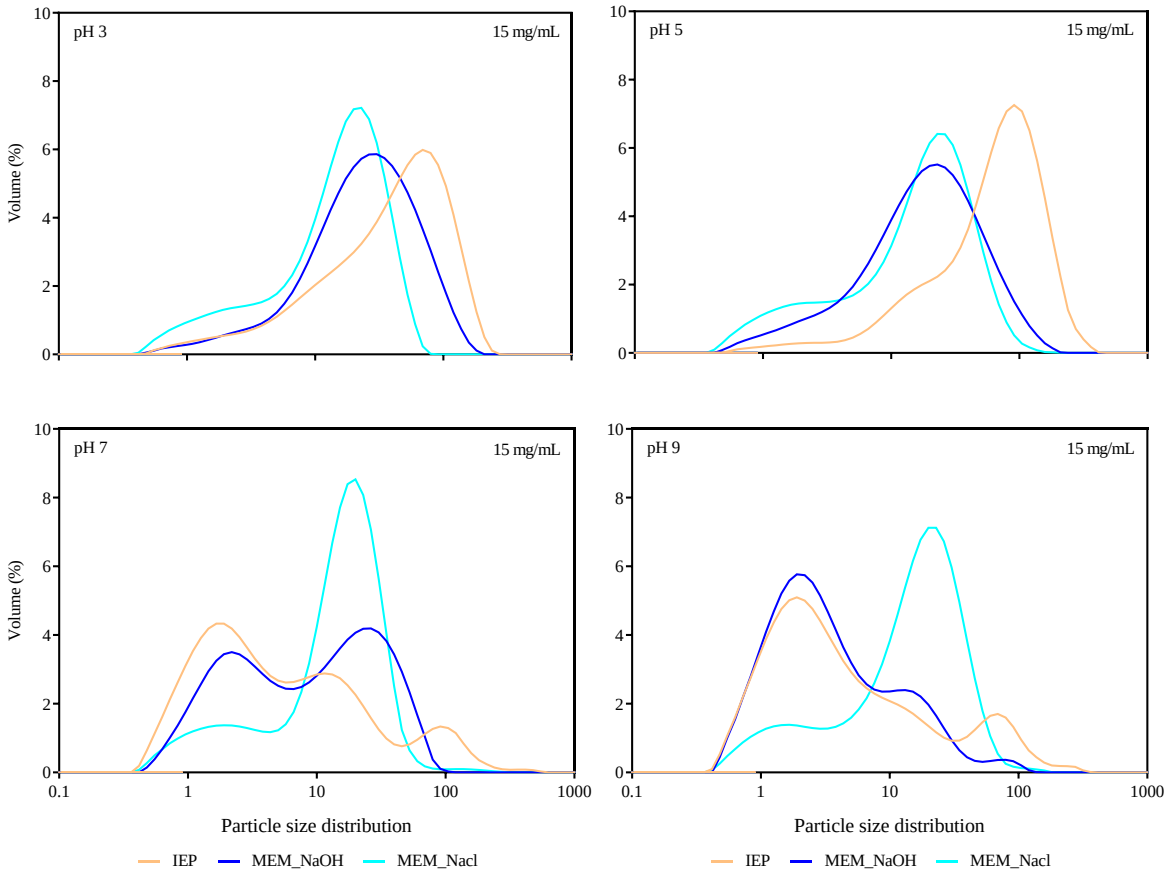

Figure 14. Oil droplet size distribution of emulsions stabilized by isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl) at 15 mg/mL and pH 3, 5, 7 and 9.

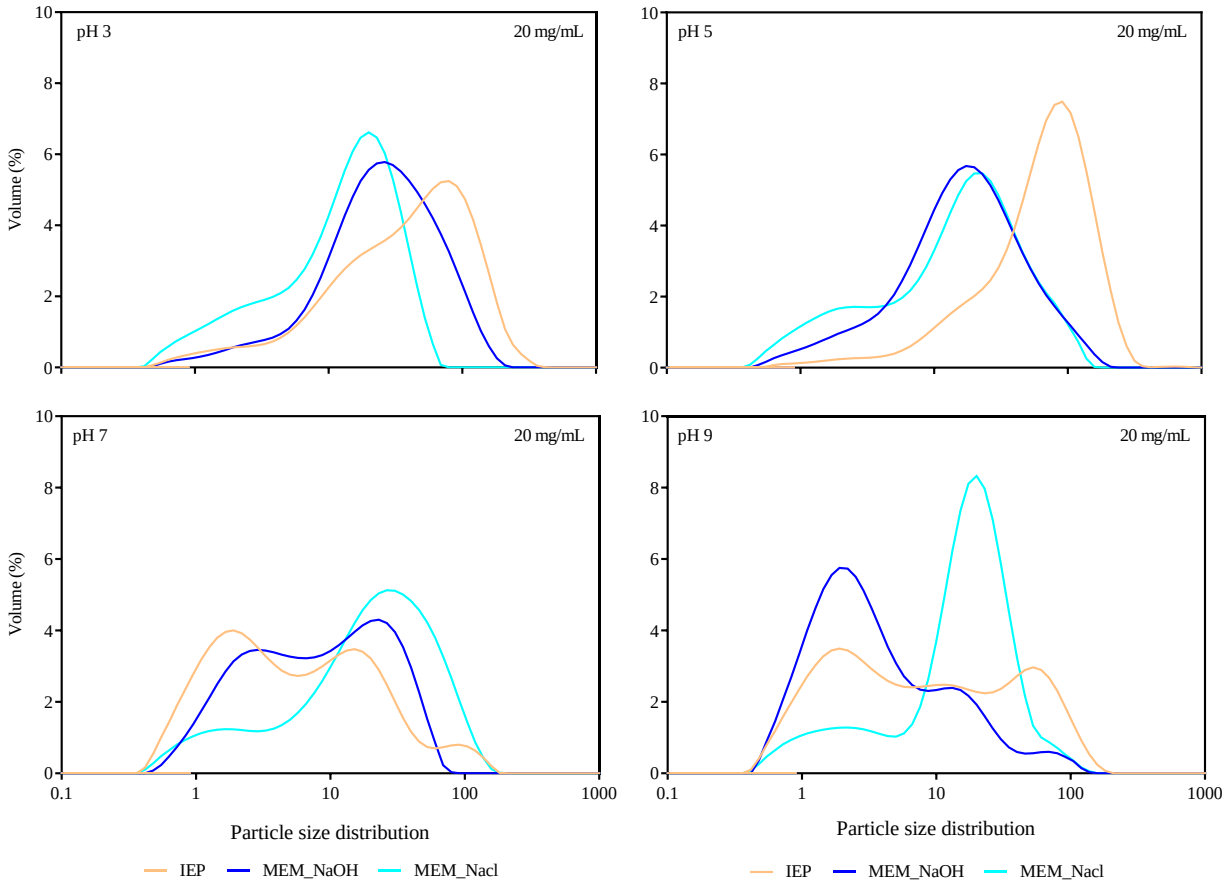

Figure 15. Oil droplet size distribution of emulsions stabilized by isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl) at 20 mg/mL and pH 3, 5, 7 and 9.

3.3.11 Water (WHC) and oil (OHC) holding capacity

The ability of proteins to hold water or oil is an important functional property when formulating certain foods because it affects the texture, flavor retention, mouthfeel, and shelf life of the food product (Singhal et al., 2016). Inability of a protein to bind water could result in stiff and dry food products. Figure 16 shows that the membrane isolates had overall higher WHC. Similar to the WHC values obtained in this work, Boye et al. (2010) found the WHC for lentil proteins obtained by isoelectric precipitation to be insignificantly higher than those obtained through ultrafiltration. Alonso-Miravalles et al. (2019) reported a similar WHC pattern among the lentil protein isolates but noted that the difference arising from the preparation method was significant. The effect of pH on their WHC was clear from the results obtained, which depicted a minimal but consistent reduction in WHC with increasing pH from the acidic to alkaline pH range. WHC is the lowest (<0.1 mg/mL) at pH 9, where all samples have their highest solubility and protein content (>92%), while it is highest at pH 3 (>0.37 mg/mL). This confirms the negative correlation observed by Horstmann et al. (2017) between the protein content and WHC of proteins. In contrast, an increase in protein concentration led to better water retention properties for the lentil proteins (except for ISO at pH 7). The WHC values in the current study are lower on average but within the range of 0.43 – 4.2 mL/g (or g/g, assuming that 1 mL of water is equivalent to 1 g of water) reported for other lentil protein isolates (Alonso-Miravalles et al., 2019; Aydemir & Yemenicioğlu, 2013; Boye et al., 2010; Joshi et al., 2011).

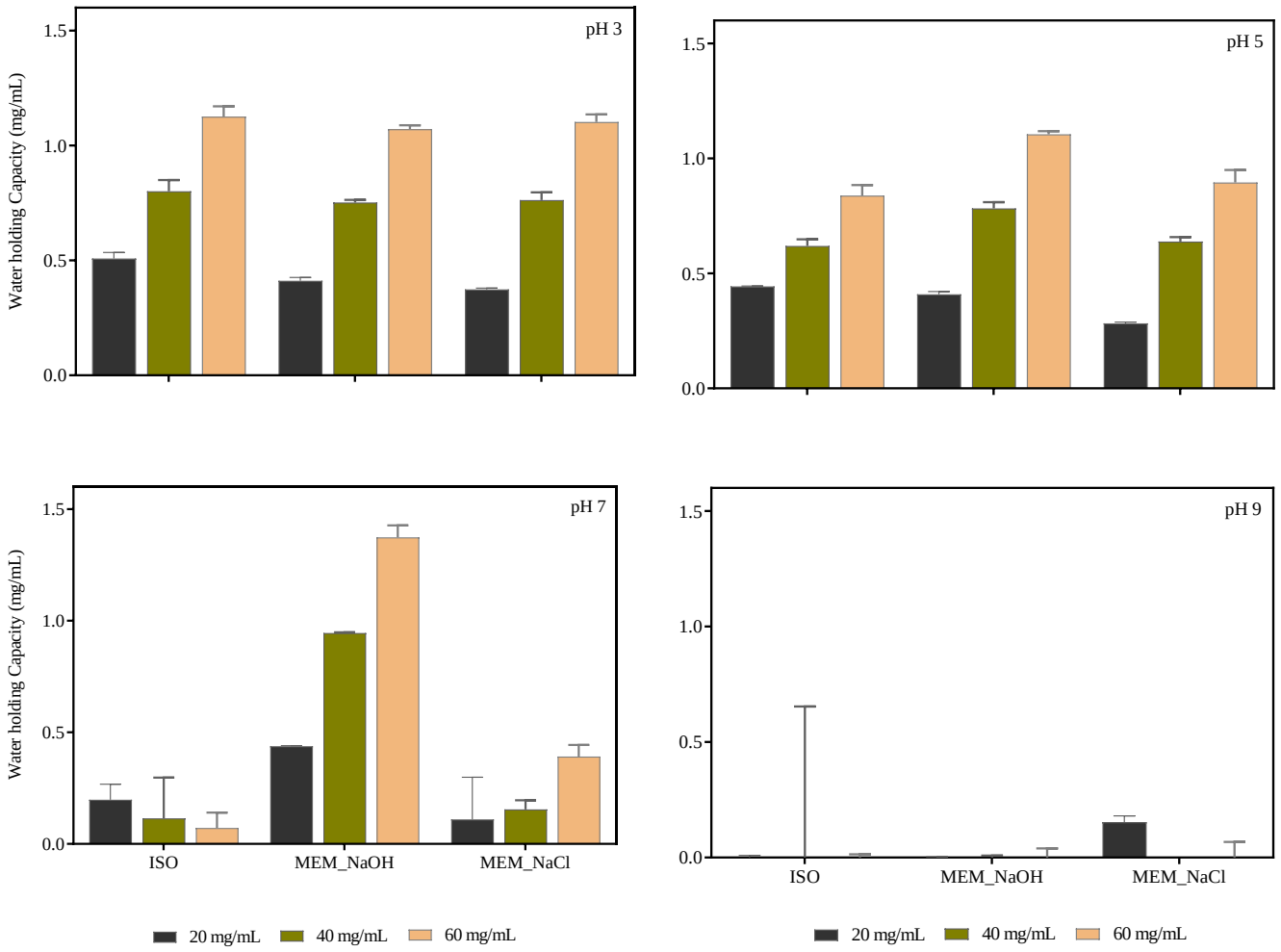

Figure 16. Percentage water-holding capacity of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at different concentrations and pH values.

The OHC of ISO, MEM_NaOH, and MEM_NaCl proteins is reported in Figure 17. Except for ISO, increase in the concentration of protein corresponded to higher OHC. Higher surface hydrophobicity has been reported to be one of the prerequisites for better OHC (Ajibola et al., 2016). However, that was not the case with ISO, which had a lower surface hydrophobicity than MEM_NaOH but exhibited better OHC. The results suggests that other parameters including the protein surface area and charge may have contributed to the OHC of the proteins. The higher OHC obtained for ISO is in contrast to previous reports (Boye et al., 2010; Alonso-Miravalles et al., 2019) indicating that the reverse was the case with other isolated lentil proteins.

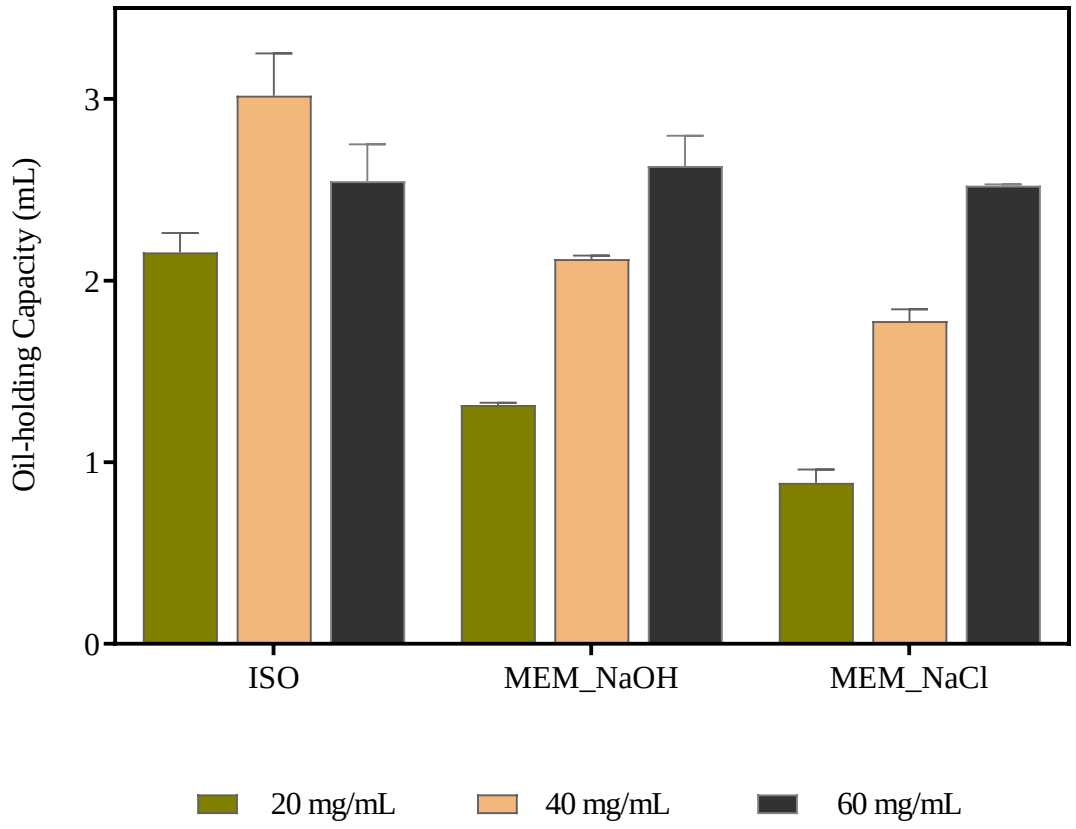

Figure 17. Percentage oil-holding capacity of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at 20, 40 and 60 mg/mL.

3.3.12 Foaming capacity (FC) and foam stability (FS)

FC and FS are generally dependent on the interfacial film formed by proteins, which incorporate air bubbles in suspension and reduces the rate of coalescence (Ma et al., 2011). FC of ISO, MEM_NaOH, and MEM_NaCl at various pH and concentrations are given in Figure 18. The FC of ISO was significantly higher ($p < 0.05$) than those of MEM_NaOH and MEM_NaCl proteins at most pH values and concentrations. In agreement with the present results, Boye et al. (2010) reported better foaming properties for isolates prepared by isoelectric precipitation (79%) when compared to those prepared by ultrafiltration (69%) at pH 7. A similar trend was reported by Arogundade et al. (2016) for yam bean protein isolates but the opposite was observed by Alonso-Miravalles et al. (2019) and Malomo et al. (2014) for lentil protein and hemp seed proteins, respectively. Since, solubility is one of the prerequisites for foam formation, the differences in FC may be attributed to variations in the solubility of the proteins. For example, Alonso-Miravalles et al. (2019) reported that the lentil proteins prepared by ultrafiltration had higher solubility across all pH range when compared with the isoelectric precipitated protein. However, ISO had significantly higher solubility than MEM_NaOH at pH >5.0, which could account for the higher FC of ISO in this study. This higher FC in ISO may result from its enhanced ability to form air bubbles a result of its greater unfolded conformation, which could be inferred from its higher interaction and solubility in water. Nevertheless, ISO only had higher solubility than MEM_NaCl at alkaline pH values, which was not directly reflected in the FC, because ISO had higher FC than the other samples at some concentrations under acidic (pH 3 and pH 5) condition. In fact, the FC for all MEM_NaOH and MEM_NaCl was observed to increase from pH 3 to pH 5 then reduced to attain the least FC at pH 9, which is the point of highest solubility. The results therefore indicate that higher FC of ISO at neutral to alkaline pH values may be due to other structural properties

including the flexibility of the lentil proteins. FS of the lentil protein isolates was dependent on pH as shown in Figure 19. In general, FS for all samples were higher at acidic pH values than the neutral and alkaline environments. FS for ISO and MEM_NaOH were highest at pH 3, irrespective of the concentration used, while MEM_NaCl was highest at pH 5. Higher FS at acidic pH values could result from their ability to form cohesive viscous membranes that could maintain a stable foam by reducing foam drainage (Malomo et al., 2014). Specifically, lower solubility observed at the acidic pH ranges compared with the alkaline pH ranges, increases protein-protein interaction responsible in the formation of the interfacial membrane. Nonetheless, the lowest FS was 73% while the highest FS was 100%, indicating that foams produced by the lentil proteins were generally very stable. FS in this work at pH 7 (75% – 100%) is significantly higher than the 6% - 45 %, and <44% FS values reported by Alonso-Miravalles et al. (2019), and (Boye et al., 2010), respectively, but similar to the 84% average reported by Jarpa-Parra et al. (2014).

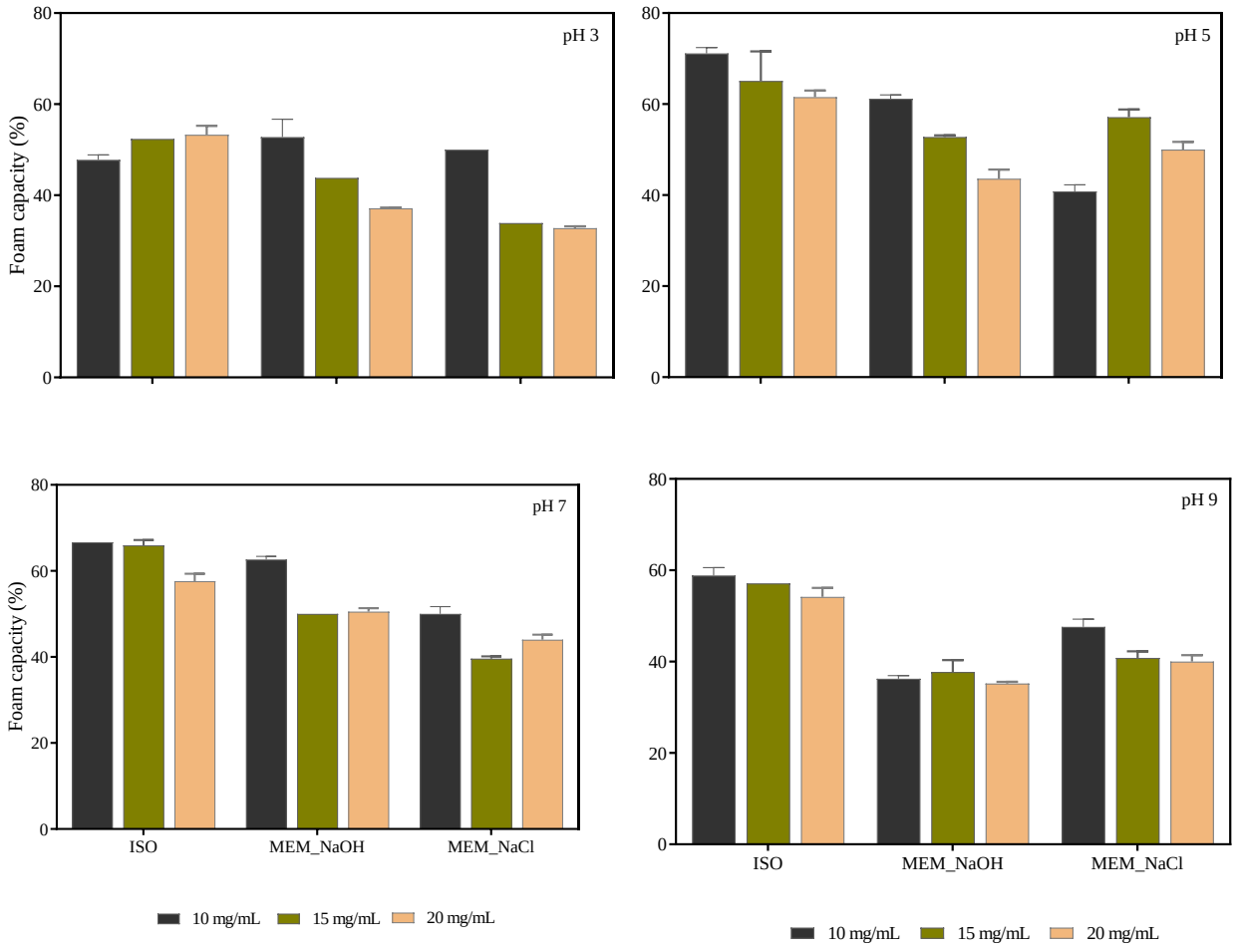

Figure 18. Percentage foaming capacity of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at different concentrations and pH values.

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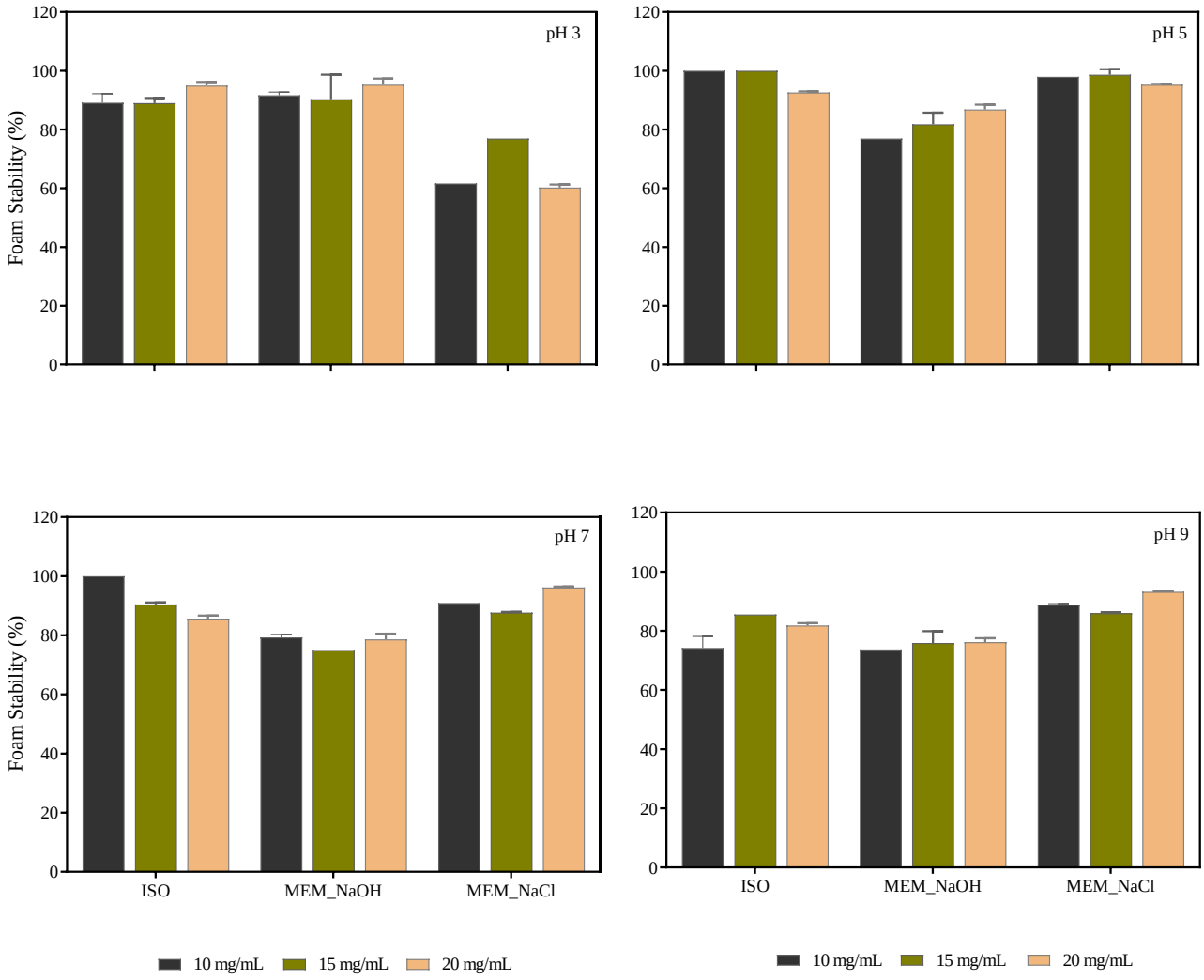

Figure 19. Percentage foaming stability of isoelectrically precipitated isolate (ISO), and and membrane isolates using alkaline (MEM_NaOH) and salt (MEN_NaCl) solution at different concentrations and pH values.

3.3.13 Least gelling concentration (LGC)

The gelling ability of the lentil protein isolates shown in Figure 20 and indicates similar LGC for ISO (11.5%), MEM_NaOH (11%), and MEM_NaCl (10.75%). The LGCs of the lentil proteins were determined as an index for their gel-forming ability and indicates the minimum concentration required for a protein to form a self-supporting gel. The gels are formed by the heat-induced aggregation of proteins to form a three-dimensional network that is resistant to flow under pressure (Jarpa-Parra, 2018). Other methods have been used in the formation of gels, but the commonly used method involves the application of optimal heat not greater than 100 ^{o}C (Joshi et al., 2017). Good gelling properties of food proteins is an important characteristic in the preparation of many viscous food such as gels, baked goods, and meat products. The results obtained by Jarpa-Parra et al. (2014) similarly demonstrates that the method of extraction had no significant effect on the LGC of lentil protein isolates. The LGC values of ISO, MEM_NaOH, and MEM_NaCl in this study is generally comparable with previously reported values of 8 to 16% (Alonso-Miravalles et al., 2019; Aydemir & Yemenicioğlu, 2013; Boye et al., 2010; Joshi et al., 2011)

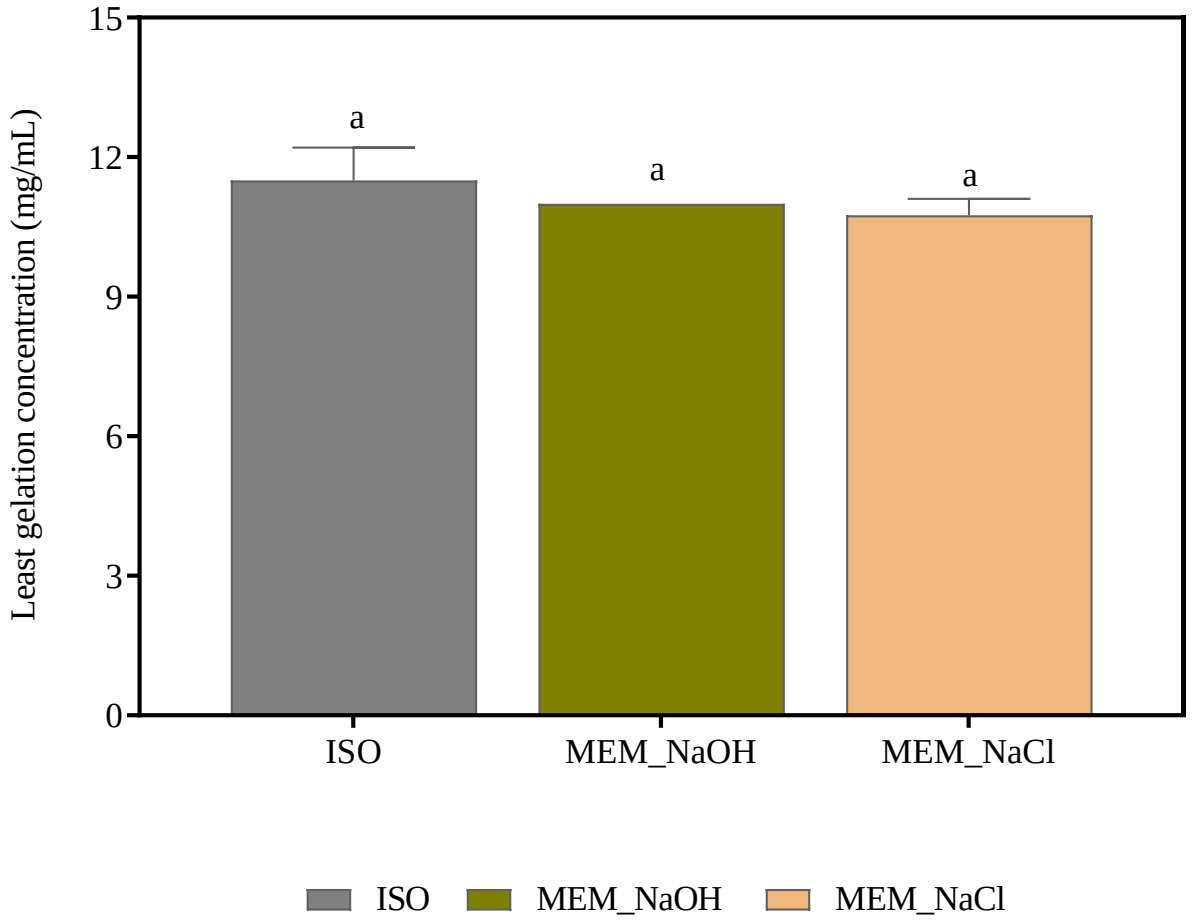

Figure 20. Least gelation concentration (mg/mL) of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl)

3.3.14 Heat coagulability (HC)

Coagulation is defined as a reversible change in the structure of proteins, which is commonly induced my heat, but can also be brought about by acids, mechanical action, or addition of coagulants (Kinsella & Melachouris, 1976). Therefore, HC is an index used to estimate the susceptibility of proteins to heating and was characterised by substantial reduction in solubility resulting from aggregation of unfolded protein molecules (Kinsella & Melachouris, 1976). Percent HC was determined as a loss in protein solubility after heating to about 100 ^{o}C for 15 min and the results are presented in Figure 21. Based on the marginal loss in solubility of ISO, MEM_NaOH, and MEM_NaCl proteins observed at pH 9 (1 – 16 % HC), it can be concluded that the protein isolates were most stable to heat at this pH. Acidic pH values displayed significantly lower solubility and least heat stability for the lentil protein in comparison (71 – 99 % HC). It was stated by Damodaran (2017) that higher protein thermal stability has a positive correlation with the presence of hydrophobic amino acids. This was proven to be true for this study since ISO had a lower presence of hydrophobic amino acids and was also generally the least stable, whereas the more hydrophobic MEM_NaCl was the most thermally stable at all pH values.

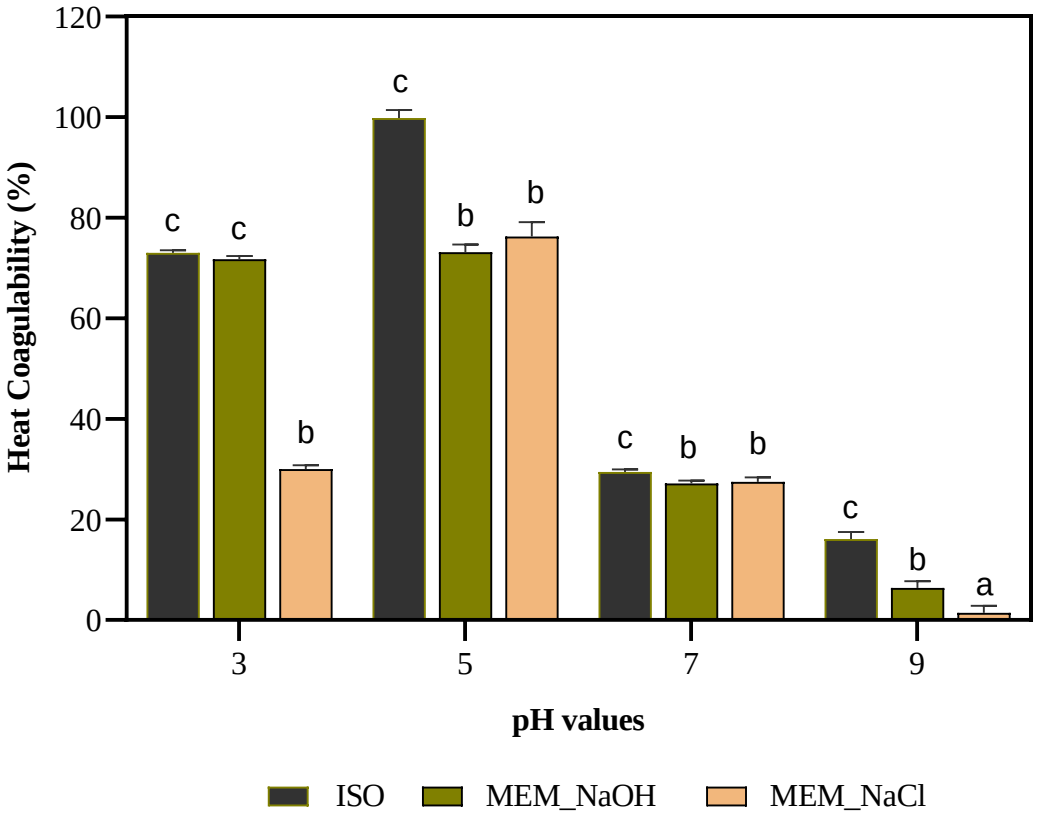

Figure 21. Percentage heat coagulability of isoelectrically precipitated isolate (ISO), and membrane isolates using alkaline and salt solution (MEM_NaOH and MEN_NaCl) from pH 3.0 - 9.0

3.4 Conclusion

The beneficial effects of the protein isolation were apparent in the significantly higher solubility and crude protein content of the isolated lentil proteins compared to that of the defatted flour. Crude protein composition, surface hydrophobicity, and functional properties of ISO, MEM_NaOH, and MEM_NaCl, were significantly different but generally comparable to each other. Where applicable, the effect of the change in pH of the protein environment was apparent on the various physicochemical and functional properties of the lentil proteins, emphasizing the impact of the protein environment on the structural conformation, as well as the functionality of the protein. For example, higher solubility was observed at alkaline pH values, while higher FS and WHC were achieved at acidic pH values. All isolates appeared to have bands consistent with legumin and vicilin-like proteins but MEM_NaOH was primarily made up of legumins. The membrane proteins displayed superior functionality in the WHC and emulsion stability. However, this is a far cry from its expected superior functional properties over that of ISO. Overall, the isolated lentil seed proteins demonstrated excellent functional properties depending on the pH being examined and should be further utilized in the formulation of various food products.

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TRANSITION STATEMENT

Physicochemical and functional properties of other pulse proteins have been reported to improve when separated into their component fractions. However, there is little information on the various properties of the lentil seed protein fractions. The next chapter focuses on the use of traditional dialysis method to separate the lentil seed protein into its albumin, globulin, glutelin, and prolamin fractions. This study is carried out under the assumption that the various fractions have different structural conformations from the lentil proteins isolates and therefore have different functional properties.

CHAPTER FOUR

4.0 PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF LENTIL SEED PROTEIN FRACTIONS

4.1 Introduction

Pulses are edible seeds of leguminous crops (*Leguminosae* or *Fabaceae*) that are among the most significant sources of plant protein (Boye et al., 2010). Unlike animal protein, pulses are inexpensive and low in fat and sodium, which makes it unsurprising that it is often called "poor man's meat" (Shevkani et al., 2019). They are high sources of protein, resistant starch, dietary fibre, vitamins, minerals and other phytochemicals like phenols, carotenoids, tocopherols, saponins, phytic acid, and phytosterols (Zhang et al., 2018). Pulses are second to cereals as essential sources of food, but they contain up to 2 to 3 times more protein than cereals, ranging from 16 to 21% in chickpea and 21% in yellow peas to 23% in green lentils (Ladjal-Ettoumi et al., 2016; Lam et al., 2018; Sharif et al., 2018). Critical efforts have been aimed at the use of these pulse proteins in the development of novel food products. Functional properties such as solubility, foaming, gelation and emulsion, influence the sensory characteristics and consumer acceptability of food products made from plant proteins. Shevkani & Singh (2014) reported the successful use of kidney bean and field pea isolates as a substitute for wheat gluten in muffins. Similarly, lentil seed proteins were used to replace egg or milk in angel food cake in the study conducted by Jarpa-Parra et al (2017). Successful incorporation of kidney bean, field pea, and lentil seed proteins in both studies is the functionality of the pulse proteins and specifically their ability to act as foam stabilisers and emulsifiers.

Some functional properties of isolated lentil seed proteins have been reported in literature (Alonso-Miravalles et al., 2019; Boye et al., 2010; Joshi et al., 2011; Kaur et al., 2007; Ko et al., 2017;

Ladjal-Ettoumi et al., 2016). Although lentil proteins comprise of 16% albumins, 70% globulins, 11% glutelins and 3% prolamins, there is a dearth of information on the characterization of these protein fractions of lentil seeds (Boye et al., 2010). Various studies carried out on other pulse proteins highlight the significant differences between the structural properties of protein isolates or concentrates and the individual protein components of the pulses which majorly include albumin, globin, glutelin and prolamin fractions. For example, Ajibola et al (2016) found the albumin fraction of African yam bean protein to be more soluble and have better foaming, gelling and oil holding properties than that of the corresponding protein concentrate. Properties like molecular size distribution, structural conformation, and amino acid composition among others, predict the functional properties of the protein. Thus, knowledge of the physicochemical properties of protein fractions are equally as important in determining how they would behave during the processing, storage and consumption if they are used as ingredients in food products. Therefore, the aim of this study is to investigate and compare the physicochemical and functional properties of albumin, globulin, glutelin and prolamin fractions of lentil seeds.

4.2 Materials and Methods

4.2.1 Materials

Green lentil seeds were purchased from a grocery store in Winnipeg (MB, Canada) and stored at -20 ^{o}C. The lentil seeds were ground using a coffee grinder (Coffee Grinder PC2770). Dialysis membranes with 6-8 kDa molecular weight cut-off (MWCO) as well as standard protein MW markers were purchased from Fisher Scientific (Oakville, ON, Canada). All other reagents used were of analytical grade and procured from either Fisher Scientific or Sigma Aldrich (St. Louis, MO, USA).

4.2.2 Preparation of defatted lentil seed flour

Milled lentil seed flour was defatted under room temperature using a flour:acetone ratio of 1:10 (w/v) under continuous stirring for 2 h. The solution was decanted, and the defatting process was repeated for another 2 h. The resulting defatted flour was dried in an open pie dish in a fume hood overnight, milled again to obtain a smaller particle size, and subsequently stored using an airtight container at -20^{o}C.

4.2.3 Isolation of lentil protein fractions

Sequential extraction of the lentil protein fractions was carried out using the Osborne's method as described by Adebiyi and Aluko (2011). Briefly, defatted flour was mixed with 2% NaCl solution and continuously stirred for 4 h (10% w/v), then centrifuged for 30 min at 5600 × *g*. The resulting supernatant was dialyzed against distilled water during which the salt-soluble proteins were precipitated. The dialysis bag was then centrifuged to obtain the supernatant, which contained water-soluble proteins (albumin, ALB) while the precipitate consisted of salt-soluble proteins (globulin, GLB). The flour residue obtained from the NaCl extraction was mixed with 70% ethanol for 4 h and centrifuged to obtain a supernatant, which was then dialysed to obtain the ethanol-soluble fraction (prolamin, PRL). The flour residue obtained from the ethanol extraction was mixed with 0.05 M NaOH for30 min, centrifuged and the supernatant dialyzed against water to obtain the glutelin fraction (GLT). The dialysis was performed at 4^{o} C for 4 days with three water changes daily. The volumes of all four fractions were reduced through evaporation using a vacuum rotary evaporator, freeze-dried, and stored at -20^{o}C.

4.2.4 Protein content and solubility of lentil proteins

Analysis for total protein content was carried out using the modified Lowry method (Markwell et al., 1978) after the samples have been dissolved in 0.1 M NaOH solution. Solubility of lentil seed

proteins was determined according to the method described by Malomo, He, and Aluko (2014) with slight modifications. A 10 mg/mL solution was obtained by adding 5 mL of 0.1 M either NaOH, phosphate, acetate, or Tris-HCl buffer solution (pH 3.0 – 9.0) to 50 mg lentil proteins in a centrifuge tube. The solution was thoroughly mixed with a vortex, left to hydrate for an hour, then centrifuged for 30 min at 5600 x *g*. The supernatant was then subjected to protein content determination (Markwell et al., 1978.

The protein solubility was calculated thus:

$$\text{Protein solubility (\%)} = \frac{\text{Protein concentration of supernatant}}{\text{Total protein concentration}} \times 100$$

4.2.5 Heat coagulability

Heat coagulability of the proteins was measured by determining the amount of soluble protein remaining after heating. Preparation of the samples were carried out according to the methods of Malomo et al. (2014) with some modifications. Briefly, each sample was dispersed in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris-HCl (pH 9) buffer solutions, to attain a 10 mg/mL concentration. The mixture was heated in pre-heated water (100 ^{o} C) for 15 min, cooled to room temperature, then centrifuged at 5600 x *g* for 30 min. Percent heat coagulability was obtained by calculating the percentage difference between the initial total protein content of the samples and that of the supernatant.

4.2.6 Emulsion formation & stability

Oil-in-water emulsions were prepared according to the method described by Chao et al. (2018) with slight modifications. Protein slurries of 10, 15, or 20 mg/mL concentrations were separately prepared in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris-HCl (pH 9) buffer solutions, followed by addition of 1 mL of pure canola oil. Emulsions were prepared by homogenization of the oil/water mixture for 1 min at 20,000 rpm using a Polytron PT 10-35 homogenizer (Kinematica

AG, Lucerne, Switzerland) equipped with a 12-mm non-foaming shaft. A Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., Malvern, U.K.) was used to measure the mean oil droplet size ($d_{3,2}$) and volume weighted mean ($d_{4,3}$) of the emulsions while distilled water was used as a dispersant. Under constant shearing, each emulsion samples were added dropwise 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 was attained. The instrument was set to automatically take measurements in triplicates, and emulsions for each sample were prepared in duplicates. After the readings were taken, the emulsions were stored for 30 min without agitation and oil droplet size measurements were repeated to determine the stability of the emulsions. The results obtained, including the oil droplet size of the emulsions were used as indicators of their emulsifying ability, while the emulsion stability was calculated as:

$$\frac{Mean\ oil\ droplet\ size\ at\ 0\ min}{Mean\ oil\ droplet\ size\ at\ 30\ min} \times 100$$

4.2.7 Foam formation and stability

Foams were formed as previously described by Chao, Jung, and Aluko (2018). Slurries were prepared by adding 10, 15, or 20 mg/mL (protein weight basis) sample dispersions in graduated centrifuge tubes containing 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris-HCl (pH 9) buffer solutions. The mixtures were homogenized at 20,000 rpm for 1 min using the polytron PT 3100 homogenizer equipped with a 20 mm foaming shaft (Kinematica AG, Lucerne, Switzerland). The foam volume was recorded as the foam capacity (FC), while foam stability was expressed as volume of foam remaining after 30 min.

$$\text{Foam Capacity } = \frac{\text{volume after whipping} - \text{volume before whipping}}{\text{volume before}} \times 100$$

4.2.8 Water and oil holding capacity

Analyses of water holding capacity (WHC) and oil holding capacity (OHC) of the lentil seed protein fractions were determined following slight modifications of the method described by Malomo et al. (2014). Samples were dispersed in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris-HCl (pH 9) buffer solutions to attain final concentrations of 20, 40, or 60 mg/mL. Aqueous as well as similarly prepared oil mixtures were vortexed, left to stand for 30 min, then centrifuged at 5600 x *g* for 30 min. The centrifuge tubes were inverted to drain the excess water or oil from each sample and reweighed. WHC/OHC was expressed as grams of water or oil retained per gram of sample.

4.2.9 Gel formation capacity

The capacity of the proteins to form gels was determined by finding the least gelling concentration (LGC) according to the method previously described by Malomo et al. (2014). Appropriate amounts of the protein samples were suspended in 0.1 M phosphate buffer (pH 7) at various concentrations (2% to 20%, w/v, protein weight basis). The samples were vortexed thoroughly for 5 min and placed in a water bath (heated to 95 ^{o} C). The gels were cooled under tap water, left in the refrigerator for 14 h (4 °C) and then inverted to determine the least sample concentration at which the gel did not slip.

4.2.10 In vitro protein digestibility

To determine the *in vitro* protein digestibility of the protein fractions, a total of 187 mg of each fraction was suspended in 30 mL of DDW, which was adjusted to pH 8 using 0.1 M NaOH solution and maintained at a temperature of 37 ^{o} C. This analysis was carried by modifying the method used by Hsu et al. (1977). An enzymatic solution containing 1.6 mg trypsin, 3.1 mg chymotrypsin and 1.3 mg peptidase per mL of solution was prepared and kept in an ice water bath. The enzymatic

solution was added to each sample at a 1:10 v/v ratio and the pH of the mixture was recorded every half minute for a period of 10 min using a pH meter. The analysis was carried out in duplicates for each sample, and the in vitro protein digestibility was calculated using the regression equation provided by Hsu et al. (1977) as follows:

% Protein digestibility (Y) = 210.46 – 18.10Xf

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

4.2.11 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 & Chemists, 2010), while their crude fiber and fat contents were analyzed according to the methods of the American Oil Chemists' Society (Mehlenbacher et al., 2010).

The amino acid composition of the lentil fractions was determined by hydrolyzing the samples for 24 h with 6 M HCl and then using the HPLC Pico-Tag system (Bidlingmeyer et al., 1984). Cysteine and methionine contents were determined separately (Gehrke et al., 1985), following a performic acid oxidation, while the tryptophan content was determined after hydrolysis with NaOH (Landry & Delhaye, 1992).

4.2.12 Sodium dodecyl sulphate (SDS)- and native- polyacrylamide gel electrophoresis (PAGE)

The freeze-dried lentil seed proteins were subjected to native- and SDS-PAGE according to the method of Aluko & McIntosh (2004), with sight modifications. Dispersions of 10 mg/mL was made for each sample using Tris-HCl buffer solution (pH 8.0), containing 2.5 % of SDS and 0.01% bromophenol blue with or without the addition of β-mercaptoethanol, resulting in a reducing or non-reducing SDS solution. The native buffer on the other hand similarly uses Tris-HCl (pH 8.8) but includes L-Alanine instead of SDS. The respective mixtures were heated at 100 ^{o}C for 10

min, cooled to room temperature, then centrifuged for 10 min at 1000 x *g*. After centrifugation, an aliquot of the supernatant for each sample was then separately loaded onto PhastGel® 8–25% gradient gels before beginning the separation of the proteins using the PhastSystem Separation and Development unit (GE Healthcare, Montreal, Canada). Protein standards of 10 – 200 kDa stained with Coomassie brilliant blue were used as a molecular weight marker, and the gels were scanned using GE Health labscan 5, while the bands were analysed using the ImageQuant TL software.

4.2.13 Surface morphology (SEM)

Surface morphology of the protein gels was studied by viewing under a scanning electron microscope (FEI Quanta E-SEM). Gels formed using the least gelling concentration as obtained through the method of Malomo et al. (2014), were frozen, then directly deposited on an aluminium specimen holder using double-sided adhesive carbon tape. They were sputtered with a thin layer of gold before SEM observation at an accelerating voltage of 10 kV.

4.2.14 Fluorescence Intensity

The fluorescence intensity (FI) spectra of each protein fraction were determined at 25 $^{\circ}$C using the Jasco FP-6300 spectrofluorometer (Jasco, Tokyo, Japan) equipped with a 1 cm path length cuvette. This was done according to the method outlined by Ajibola et al (2016). Protein samples weighing 10 mg (based on protein weight) were dispersed separately in 1 mL of 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and tris (pH 9) buffer solutions. The stock solutions were centrifuged, and the supernatants were further diluted with the appropriate buffers to a concentration of 0.002% (v/v), in preparation for the fluorescence measurements. The fluorescence spectra were recorded at excitation wavelengths of 280 nm (tyrosine and tryptophan) with emissions recorded from 290 to 500 nm. The final fluorescence spectra of each obtain were then obtained by subtracting the emissions of the buffer blanks from those of the respective samples.

4.2.15 Surface hydrophobicity

Surface hydrophobicity of the LPF was determined according to a modification of the method outlined by Karaca et al (2011). The protein samples were dispersed in 0.1 M potassium phosphate buffers (pH 7.0), vortexed thoroughly, then left to hydrate for 1 h. The mixtures were centrifuged at 10,000 x *g* for 10 min and the supernatants were collected separately then serially diluted to obtain protein concentrations ranging from 50 to 250 µg/mL. A 20 µL aliquot of the aromatic fluorescent probe, 8-Anilino-1-naphthalenesulfonic acid (ANS), which was prepared to a final concentration of 0.8 M in 0.1 M sodium phosphate buffer phosphate buffer (pH 7.0) was added to every 4.0 ml of diluted protein solution. The solutions were incubated in dark for 15 min before fluorescence intensity was measured using a spectrofluorometer with excitation and emission wavelengths of 390 and 470 nm, respectively. Fluorescence intensity values for the mixtures without ANS were subtracted from the fluorescence intensity of the protein solutions containing ANS. The coefficient of linear regression analysis of the FI against protein concentration (mg/ml) was used as an index of protein surface hydrophobicity (S_0).

4.2.16 Circular dichroism

CD measurements of the ALB, GLB, GLT and PRL fractions, were carried out at 25 °C in a J-810 spectropolarimeter (Jasco, Tokyo, Japan). The Far-UV spectral range of 190-240 nm, and the near-UV spectral range of 250-320 nm, were used in the determination of the secondary and tertiary structures of each fraction, according to the method illustrated by Agboola and Aluko (2009). Protein stock solutions were prepared by dispersing each sample in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and tris (pH 9) buffer solutions, to attain a concentration of 2mg/mL for far-UV determination, and 4 mg/mL for near-UV determination. The mixtures were centrifuged at 10,000 x *g* for 10 min, and the supernatants were collected to be analysed in cuvettes with path lengths of

0.05 cm and 0.1 cm, for far- and near- UV, respectively. All the results were obtained as the average of three consecutive scans with automatic subtraction of the buffer spectra.

4.2.17 Thermal properties

Thermal characteristics of the various protein samples were examined using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE). Lentil fractions were first dispersed in 0.1 M phosphate buffer (pH 7) to a final concentration of 200% w/v, then each sample was weighed in an aluminium pan which was later hermetically sealed. Calorimetric measurements were carried out by heating sealed pans containing the samples, as well as sealed empty pans from 40 °C to 140 °C at a rate of 10 ^{o} C/min. The empty pan was used as a reference and all experiments were conducted in duplicates. Onset temperature (T_o), denaturation temperature (T_d), and enthalpy of denaturation (ΔH) was computed using a computer software (Universal Analysis 2000, Version 4.5).

4.2.18 Statistical analysis

Except where indicated, triplicate replications were used to obtain mean values and standard deviations. The IBM SPSS statistics software (version 26) was used to perform one-way ANOVA and values were tested for statistical significance ($p < 0.05$) using Duncan's multiple range test.

4.3 Results and discussions

4.3.1 Chemical composition and protein yield

The protein yields of ALB, GLB, GLT, and PRL fractions were 8.49%, 17.07%, 22.94%, and 0.17% (Table 1b). The results showed that GLT was the major protein fraction in lentil seeds, followed by the GLB fraction. Similar to other reports of the prolamin fraction obtained from lentil seeds (Boye et al., 2010a; Sulieman et al., 2008), PRL in this work was also found to have a very low yield. Moisture contents in the lentil protein fractions were significantly different ($p < 0.05$)

ranged from 3.93% in GLB and 5.18% in GLT to 10.16% in ALB (Table 1a). There was no significant difference between the crude fibre and fat content in all fractions. Low amounts of fat (0.37 – 0.62%) and ash content (1.28 – 3.84%) found in this work were similarly reported for red and green lentil flours containing <1.8 % fat and <2.8 % ash content (Barbana & Boye, 2013; Health Canada, 2015; Nosworthy et al., 2018). Reduced fat content in the lentil fractions resulting from the defatting procedure reduces their interaction with proteins which could potentially restrict isolation through the inhibiting the protein dissolution. As a result, lower fat content is occasionally consistent with a higher crude protein content in legumes (Ajibola et al., 2016; Johnston et al., 2015). In addition to the fat content, Ajibola et al (2016) noted that other components like carbohydrates and ash co-extracted with proteins could potentially limit the crude protein content of the legumes. This was confirmed in the work of Johnston et al (2015) and Mundi & Aluko (2012) however, that was not the case in this study. For example, GLB had a significantly higher non-fibre carbohydrate content of 89.68% compared to that of ALB and GLT with 2.89% and 13.12%, respectively. However, GLB also had a significantly higher crude protein content (94.18%) compared to ALB (79.38%) or GLT (79.87%). This could be attributed to the amount of lectin present in the protein. Lectins are known to covalently bind carbohydrates to form complexes which could limit protein isolation (Mundi & Aluko, 2012). Historically higher amount of lectins in legumes like red kidney beans could account for the inverse correlation between the carbohydrate and protein content. Deviation from this pattern as obtained in this work could therefore be evidence of a reduced amount lectin in the lentil protein fractions.

The amino acid composition of ALB, GLB, GLT, and PRL introduces the nutritional value and quality of the fractions as presented in Table 2. To the best of our knowledge, this is the first report of the amino acid composition of lentil seed glutelin and prolamin, though the values for albumin

and globulin have been previously documented. Depending on their side chain, amino acids can be referred to as sulphur-containing, hydrophobic, hydrophilic, branch-chained, acidic, basic or aromatic (Nelson et al., 2008). Apart from protein synthesis, amino acids play various other significant roles in the body system like muscle metabolism, the synthesis of hormones, haemoglobin, and antibodies. The body is able to synthesize 11 of these amino acids (non-essential amino acids), while the other nine amino acids must be supplied through the diet because optimal concentrations of these amino acids cannot be synthesized *de novo* by humans (Bischoff & Schlüter, 2012; Meyers et al., 2006). As a result, the nutritive value of proteins increases with increasing number of essential amino acids in adequate amounts. This can be assessed through their amino acid score, which is a comparison with essential amino acid (EAA) pattern recommended by WHO and FAO (2007). In comparison with the amino acid requirements for adults, PRL surpassed the cysteine requirement 16 times with an amino acid score of 1673.0%. PRL generally had a substantially higher amount (12.5%) of sulphur-containing amino acids (SCAA) compared to ALB (2.9%), GLB (1.1%) and GLT (2.0%). SCAAs have been shown to have anti-inflammatory and antioxidant effects in the human body. Cysteine has also been reported to reduce the symptoms of irritable bowel syndrome, while methionine has been reported to relief pain and the incidence of arthritis (Meletis & Barker, 2005; Uneyama et al., 2017). PRL also had notably higher hydrophobic amino acids (46%) whereas GLB which had the lowest (37%).

All the lentil seed protein fractions had high amounts of most of the essential amino acids. Few exceptions were the low amounts of methionine in ALB, GLB, and GLT, and the relatively low tryptophan, and lysine contents in the GLB and PRL fractions, respectively. After methionine, cysteine was the second least amino acid in GLB. Amino acid requirements can, therefore, be met by simply combining either ALB, GLB, or GLT with the PRL fraction. Glutamic and aspartic

acids, which includes glutamine and asparagine, respectively, were the major nonessential amino acids present in the lentil fractions. Glutamic and aspartic acid are neurotransmitters that also protect the body against oxidative damage, while glutamine is highly important for metabolism, nitrogen exchange, and pH homeostasis (Curi et al., 2016; Wu, 2013). GLB, especially, had significantly higher amount of negatively charge amino acids (NCAAs), which is similar with other globular seed proteins.

Table 1: a) Proximate composition of defatted green lentil Osborne fractions: albumin (ALB), globulin (GLB), and glutelin (GLT) fractions; b) protein yield of defatted green lentil Osborne fractions: albumin (ALB), globulin (GLB), glutelin (GLT), and prolamin (PRL) fractions.

(a)

	ALB	GLB	GLT
Moisture content	10.16 ± 0.62^{a}	3.93 ± 0.62^{b}	5.18 ± 0.74^{b}
Crude fibre	0.04 ± 0.01^{a}	0.08 ± 0.06^{a}	0.06 ± 0.04^{a}
Fat	0.42 ± 0.20^{a}	0.62 ± 0.70^{a}	0.37 ± 0.08^{a}
Ash	3.84 ± 0.06^{b}	1.28 ± 0.04^{c}	4.5 ± 0.06^{a}
Non-Fibre Carbohydrates	2.89 ± 0.95^{c}	-	13.12 ± 0.76^{b}
Protein content	79.38 ± 0.53^{b}	94.18 ± 0.94^{a}	79.87 ± 0.53^{b}

(b)

	ALB	GLB	GLT	PRL
Protein yield	8.49 ± 0.30^{c}	17.07 ± 0.45^{b}	22.94 ± 0.68^{a}	0.17 ± 0.01^{d}

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

Table 2: Percent amino acid composition of Lentil protein fractions

AA	ALB	GLB	GLT	PRL
Asx	12.16 ± 0.04	12.81 ± 0.14	12.05 ± 0.22	8.86 ± 0.25
Thr[1]	4.82 ± 0.00	3.11 ± 0.08	4.22 ± 0.00	2.19 ± 0.13
Ser	4.96 ± 0.04	5.70 ± 0.19	5.51 ± 0.07	7.11 ± 1.27
Glx	16.86 ± 0.14	18.97 ± 0.00	17.44 ± 0.35	11.18 ± 0.32
Pro	4.34 ± 0.06	4.18 ± 0.15	4.48 ± 0.11	8.13 ± 0.19
Gly	4.00 ± 0.13	3.06 ± 0.0#	4.14 ± 0.12	5.43 ± 0.07
Ala	4.71 ± 0.06	3.16 ± 0.11	4.39 ± 0.16	4.91 ± 0.10
Cys[1]	1.46 ± 0.02	0.33 ± 0.08	0.67 ± 0.11	10.44 ± 0.56
Val[1]	5.79 ± 0.08	4.88 ± 0.04	5.56 ± 0.15	5.62 ± 0.06
Met[1]	1.43 ± 0.02	0.57 ± 0.17	1.24 ± 0.01	2.49 ± 0.03
Ile[1]	4.50 ± 0.42	4.53 ± 0.51	4.66 ± 0.36	2.58 ± 0.08
Leu[1]	7.57 ± 0.04	8.74 ± 0.07	8.52 ± 0.12	3.81 ± 0.05
Tyr	4.20 ± 0.48	3.36 ± 0.02	3.79 ± 0.12	5.83 ± 0.65
Phe	5.02 ± 0.01	6.06 ± 0.08	6.04 ± 0.08	3.52 ± 0.00
His[1]	2.22 ± 0.36	2.03 ± 0.27	2.60 ± 0.11	3.70 ± 0.13
Lys[1]	8.89 ± 0.18	6.63 ± 0.32	5.21 ± 0.12	4.03 ± 0.16
Arg	5.31 ± 0.02	9.09 ± 0.43	8.59 ± 0.09	7.65 ± 0.20
Trp[1]	1.35 ± 0.01	0.50 ± 0.01	1.02 ± 0.01	0.58 ± 0.13
AAA	10.56 ± 0.46	9.91 ± 0.05	10.84 ± 0.03	9.93 ± 0.52
BCAA	17.86 ± 0.29	18.14 ± 0.54	18.75 ± 0.09	12.01 ± 0.04
HAA	40.36 ± 0.17	36.29 ± 1.09	40.38 ± 0.04	47.91 ± 1.39
PCAA	16.43 ± 0.52	17.76 ± 1.03	16.40 ± 0.08	15.38 ± 0.09
NCAA	29.02 ± 0.18	31.78 ± 0.14	29.49 ± 0.57	20.04 ± 0.07
SCAA	2.89 ± 0.00	0.90 ± 0.25	1.91 ± 0.10	12.93 ± 0.54

Albumin (ALB); Globulin (GLB); Glutelin (GLT); Prolamin (PRL); 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; [1]Essential amino acids.

4.3.2 In vitro protein digestibility

Digestibility describes the susceptibility of a protein to undergo proteolysis and provide amino acids for absorption. This is used to evaluate the nutritional quality of proteins, as highly digestible proteins are more desirable and are considered to be of better nutritional value than those with a lower digestibility. Antinutritional compounds like tannins, and protease inhibitors such as chymotrypsin and trypsin, could adversely affect digestibility if not inactivated during processing (Liener, 2012; Martín-Cabrejas et al., 2009). In addition, beta structures abundantly present in pulse proteins have also been reported to contribute to lower digestibility by inhibiting the proteolytic activity of digestive enzymes (Yu, 2005). The use of a combination of proteases was done to simulate gastrointestinal digestion of the lentil seed protein isolates and the results are presented in Fig. 1b. From the presented data, the GLB fraction underwent a sharp drop from pH 8.0 to pH 7.0 in 30 seconds, and then slowly declined to pH 6.7 by the 10-min mark. In the case of ALB, there was only a slight decline from pH 8.0 to pH 7.8 within the first 30 seconds, and the pH remained relatively stable at pH 7.8 until the time elapsed. Similar to GLB, GLT also had a sharp reduction form pH 8.0 to pH 7.3 in 30 seconds, then gradually declined to 6.9 after 10 min. The pH drop recorded in these results is as a result of the H+ produced during the hydrolysis of peptide bonds that occur when the proteins are being digested. Peptide bond hydrolysis yields a carboxylic (COOH) group, which ionizes to release protons, hence continuous protein hydrolysis generally leads to decreases in pH of the reaction mixture. The sharp pH decline in the GLB fraction, therefore, indicates that it has the highest digestibility with readily available and susceptible peptide bonds. Digestibility values of the GLB and GLT lentil fractions (Fig. 1b) were generally comparable to other documented results obtained which ranged from 79.3.% to 88.0% for lentil protein isolates (Aryee & Boye, 2016; Carbonaro et al., 2012; Nosworthy et al., 2018).

ALB on the other hand had a significantly lower digestibility (69.1%), which was more comparable with that of flaxseed protein (68.0%) (Marambe et al., 2013). The lower digestibility of ALB may be due to the presence of protease inhibitors in this protein fraction, which usually contains non-storage proteins.

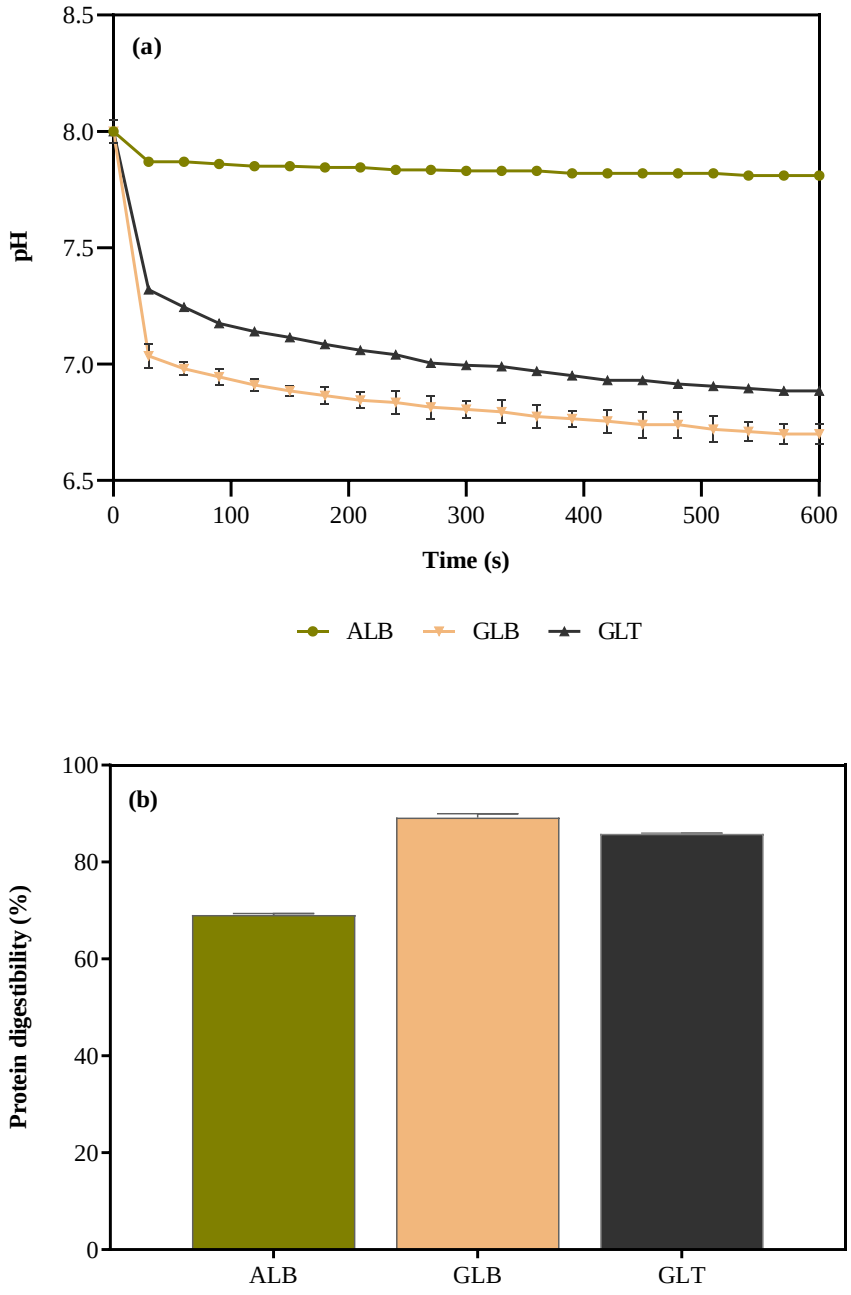

Figure 1. Protein digestion progress measured as time-dependent decreases in pH (a) and protein digestibility values (b) of green lentil seed protein fractions: albumin (ALB), globulin (GLB), and glutelin (GLT).

4.3.3 Intrinsic fluorescence

Fluorescence spectra in Fig 2. provide a sensitive means of revealing the conformational changes occurring in the lentil seed protein fractions at different pH values. This information is important in characterizing the proteins and also determining the location of the aromatic amino acid residues within the protein configuration (Schmid, 1990). These aromatic amino acids (tryptophan, tyrosine, and phenylalanine) become excited when exposed on the protein surface to UV light, and increased exposure leads to an increased fluorescence intensity (FI) (Eftink, 2000). Monitoring the FI of the samples as a function of pH is important because as potential food ingredients, the protein isolates will be subjected to pH changes during food processing. The results showed that the intrinsic fluorescence of the proteins was dependent on the type of protein as well as the pH of the environment. However, ALB appeared to have a relatively stable maximum FI wavelength (λmax) at across pH 3 (350 nm), pH 5 (349-350 nm), pH 7 (347 nm) and pH 9 (348 nm). The red shift in λmax obtained for ALB indicates that the tryptophan (Trp) residues present in ALB were in a hydrophilic environment (Eftink, 2000). There was also a gradual decline in the maximum FI of ALB across pH 3, 5, 7 and 9, implying a higher degree of protein-water interactions and a more unfolded conformation with increasing pH. At pH 3 and pH 5, the results obtained for GLB and GLT were very similar. No fluorescence peaks were observed in both samples at pH 3, which indicates complete unfolding of protein structure and total exposure of the aromatic groups to the hydrolphilic environment. The λmax values for GLT (337 – 344 nm) at pH 5, 9, and 7 showed that the Trp residues were in a hydrophobic environment (Eftink, 2000). Results obtained from for GLB and GLT are similar to the 336 nm and 338 nm λmax reported for the red kidney bean albumin and globulin fractions, and Bambara proteins, respectively (Ajibola et al., 2016; Arise et al., 2017b). PRL results varied considerably with changes in pH indicating that Trp residue in pH

7 were fully exposed to the solvent (λmax of 351), while those in pH 3 (337 nm), 5 (343 nm), and 9 (339 nm) were not completely in contact with the solvent.

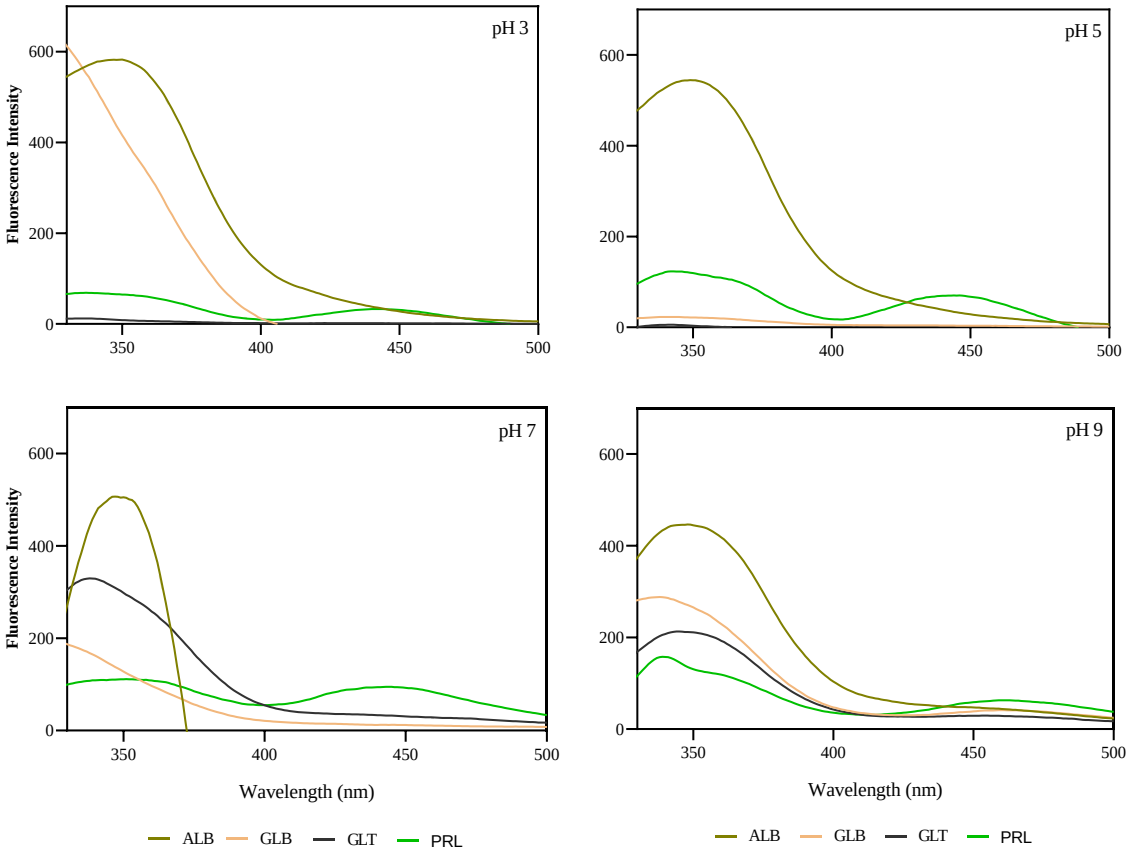

Figure 2. Intrinsic fluorescence intensity of green lentil seed protein fractions: albumin (ALB), globulin (GLB), glutelin (GLT) and prolamin (PRL) at different pH values

4.3.4 Surface hydrophobicity

Surface hydrophobicity (S_o) of the lentil seed protein fractions at pH 7 are presented in Fig 3. The increase in S_o from ALB, GLT and GLB to PRL, shows a progress in the unfolding of the proteins and exposure of hydrophobic patches, with the implication of PRL having the least folded protein structure. It could also be an indication of the number of hydrophobic amino acids on the surface of the folded proteins (Jahaniaval et al., 2000). S_o is important in predicting the functional properties of proteins such as emulsifying and foaming activity, because it represents the capacity of the proteins to interact at the molecular level (Arogundade et al., 2016; Kato & Nakai, 1980). Higher hydrophobicity and improved protein-protein interaction in PRL, for example, is key to the viscoelasticity of gluten during breadmaking (Cauvain, 2003). The significant difference in the S_o of lentil fractions ($p < 0.05$) implies that the solvent used in extraction, extraction method, and/or amino acid composition of the proteins have a substantial impact on molecular interactions with their polar environment. Significantly higher S_o value for PRL is consistent with the level of hydrophobic amino acids present, which is at least 6% greater than those of the other protein fractions. However, ALB had higher contents of hydrophobic amino acids but lower S_o when compared to GLB, which suggest that those in GLB are either mostly present on the surface of the protein or that its protein structure is more unfolded to expose them. The reverse could also be the case for ALB, implying that at pH 7, the hydrophobic amino acids present are buried in the core of a less denatured or unfolded protein structure. Similar to what was obtained in this work, the S_o of albumin proteins has consistently been reported to have the lowest when compared to those of the globulin, glutelin and prolamin fractions (Chen et al., 2018; Idowu et al., 2021; Nwachukwu & Aluko, 2018; Stone et al., 2015).

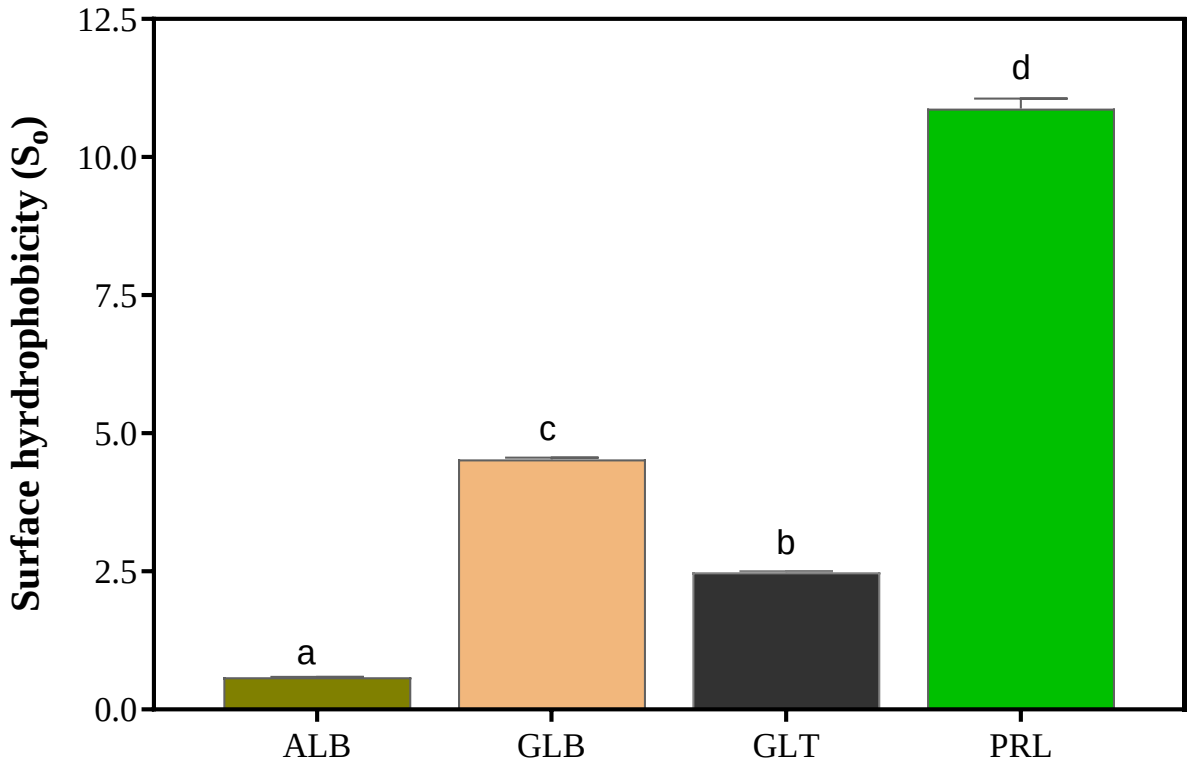

Figure 3. Surface hydrophobicity of green lentil seed protein fractions: albumin (ALB), globulin (GLB), glutelin (GLT) and prolamin (PRL).

4.3.5 Circular dichroism (CD)

CD spectroscopy is a powerful tool used to provide a deeper understanding of the secondary and tertiary structures of proteins. Figures 4 and 5 show the CD spectra for far- and near-UV measurements, respectively. The far-UV spectra show that the secondary structures are dominated by β-sheets and random coils, with lower amounts of α-helix. This higher level of β-sheets suggests that the proteins have an open secondary structure conformation. At pH 3, all samples had a negative ellipticity but otherwise showed different ellipticity patterns. GLT spectrum had the lowest and almost zero ellipticity at pH 3, which indicates absence of regular secondary structures. Also at pH 3, the ALB had a slight positive band at around 195 nm and a weak double minimum between 210 – 212 nm and at 222 – 224 nm, which characterizes the presence of α-helix and β-sheet structures. Less intense positive bands between 191 and 200 nm for all samples at pH 3 indicate presence of β-sheet structure. PRL had a negative peak similar but more intense than GLB at around 208 nm denoting a higher presence of random coils or α-helix. All samples at pH 5 also had almost zero CD values, except PRL and GLT with characteristic weak positive bands occurring between 190 – 200 nm, which indicate presence of β-sheet. Since the secondary structures are usually buried in the interior site of the proteins, the general increase in ellipticity indicates more unfolding of polypeptide chains with increasing pH (Xue et al., 2013). Likewise, the positive maximum at pH 5, 7, and 9 indicates increased β-sheet structures (compared to pH 3). However, similar to the discrepancy reported by Agboola & Aluko (2009), the observed spectra did not particularly translate into a significant increases in the calculated percentage of β-sheet present in PRL at pH 3-9 (Table 2). With the exception of GLB, very similar ellipticity patterns can be seen for the other samples at pH 7 and 9, showing the typical positive bands at 200 – 205 nm, suggesting the presence of both β-sheets and random coils in the protein structures. In contrast,

GLB had well defined α-helix secondary structure at pH 7 and 9, which were evident from the double intensity at about 206 and 223 nm. Results obtained are consistent with previous reports that confirmed processing conditions such as pH alter protein secondary structures (Arise et al., 2017b; Malomo & Aluko, 2015a; Mundi & Aluko, 2013). The secondary structure composition of ALB for all pH analysed remained between 5.2 to 9.37%, 29.2 to 34.4%, 16.7 to 19.05%, and 41.3 to 43.5 % for α-helix, β-sheet, β-turn, and random coils, respectively (Table 3). The upper and lower limits were generally comparable to the secondary structure conformation of albumin and glutelin from cumin fractions reported by Chen et al (2018). Significant differences were however seen in GLB, which had α-helix, β-sheet, β-turn, and random coils ranging from 5.1 to 15.2%, 19.3 to 34.9%, 13.4 to 19.2% and 40.8 to 53.0%, respectively.

The near-UV spectra in Fig. 5 shows different ellipticity patterns across pH 3, 5, 7 and 9, that demonstrate the pH dependence of tertiary structure conformations. For example, Phe and Trp peaks present at 254 and 285 nm in the pH 3 spectra of PRL underwent a redshift to 270 and 288 nm, respectively. Maximum ellipticity of GLB remained at around 267 nm for both pH 7 and 9, but pH 9 had a higher mean ellipticity and a more intense peak, depicting a more organised structure for GLB at pH 9. Very weak Phe transition occurred between 265 – 268 nm for GLT and ALB at pH 5 and 9, respectively. Except for the aforementioned spectra for GLB and GLB, their other spectra lacked any distinct peaks, indicating that there were only minor tertiary structures present.

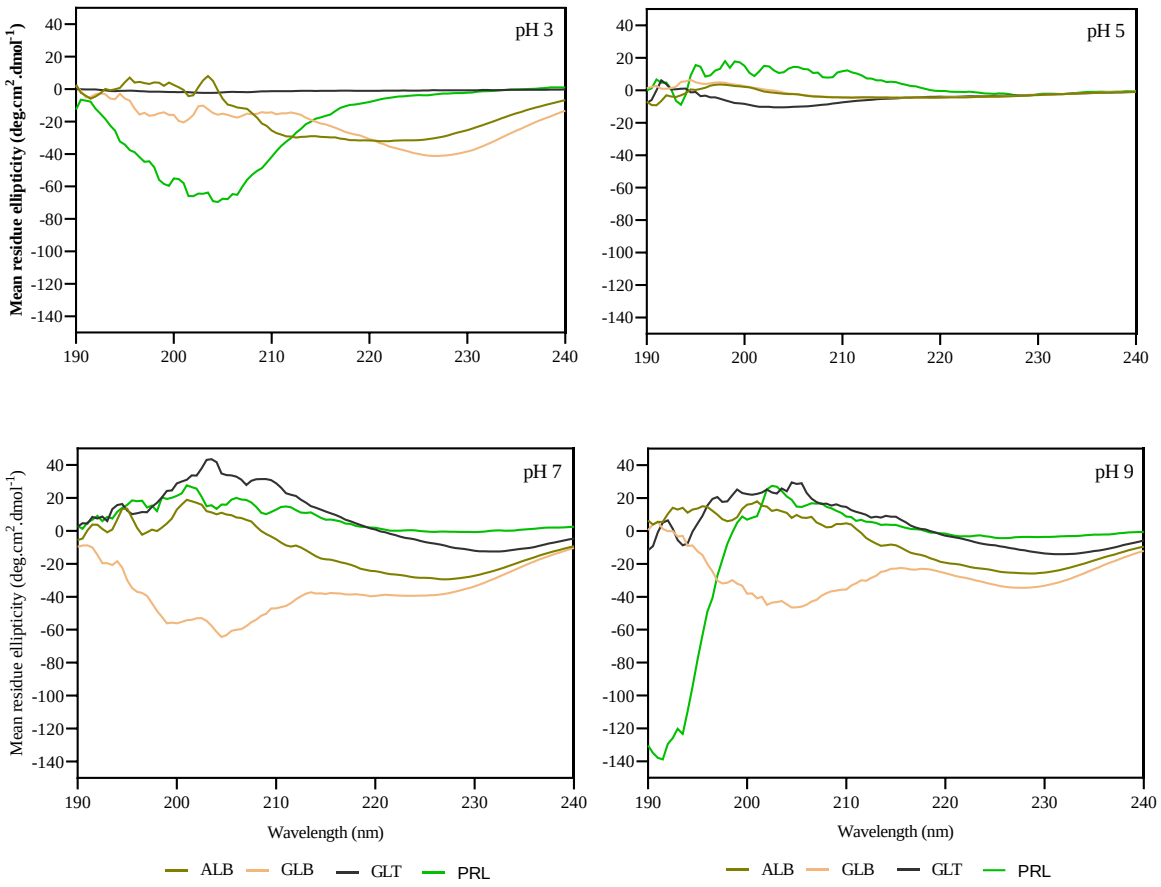

Figure 4. Far-UV circular dichroism spectra of green lentil seed protein fractions: albumin (ALB), globulin (GLB), glutelin (GLT) and prolamin (PRL) at different pH values at different pH values

Table 3. Secondary structures obtained from deconvolution of far-UV circular dichroism spectra

pH	Samples	α-helix (%)	β-sheets (%)	β-turns	Unordered
3	ALB	4.77 ± 0.17	14.57 ± 0.46	18.35 ± 0.20	43.05 ± 1.00
	GLB	6.12 ± 0.17	11.42 ± 0.46	13.60 ± 0.10	51.30 ± 1.10
	GLT	2.42 ± 0.03	17.60 ± 0.00	18.60 ± 0.00	41.40 ± 0.10
	PRL	4.93 ± 0.46	12.97 ± 0.32	16.55 ± 0.90	47.60 ± 0.70
5	ALB	2.60 ± 0.14	17.20 ± 0.14	19.05 ± 0.30	41.3 ± 0.80
	GLB	2.55 ± 0.07	17.45 ± 0.14	19.20 ± 0.10	40.80 ± 0.00
	GLT	2.82 ± 0.11	16.95 ± 0.07	19.00 ± 0.20	41.45 ± 0.60
	PRL	2.25 ± 0.14	18.17 ± 0.53	17.70 ± 1.20	41.45 ± 2.6
7	ALB	4.50 ± 0.35	15.30 ± 0.35	17.55 ± 0.60	42.95 ± 0.70
	GLB	7.10 ± 0.49	9.50 ± 0.35	13.85 ± 0.00	53.00 ± 0.20
	GLT	2.52 ± 0.18	18.50 ± 0.28	17.50 ± 0.90	40.50 ± 1.80
	PRL	2.37 ± 0.11	18.67 ± 0.67	18.35 ± 0.90	39.55 ± 0.60
9	ALB	4.47 ± 0.67	15.42 ± 0.46	16.70 ± 0.80	43.45 ± 0.30
	GLB	7.62 ± 0.18	9.67 ± 0.03	13.40 ± 0.10	52.00 ± 0.40
	GLT	3.05 ± 0.56	17.67 ± 1.45	17.35 ± 1.20	41.25 ± 2.90
	PRL	3.07 ± 0.39	14.82 ± 0.67	14.45 ± 1.70	49.75 ± 2.60

- Albumin (ALB); Globulin (GLB); Glutelin (GLT); Prolamin (PRL)

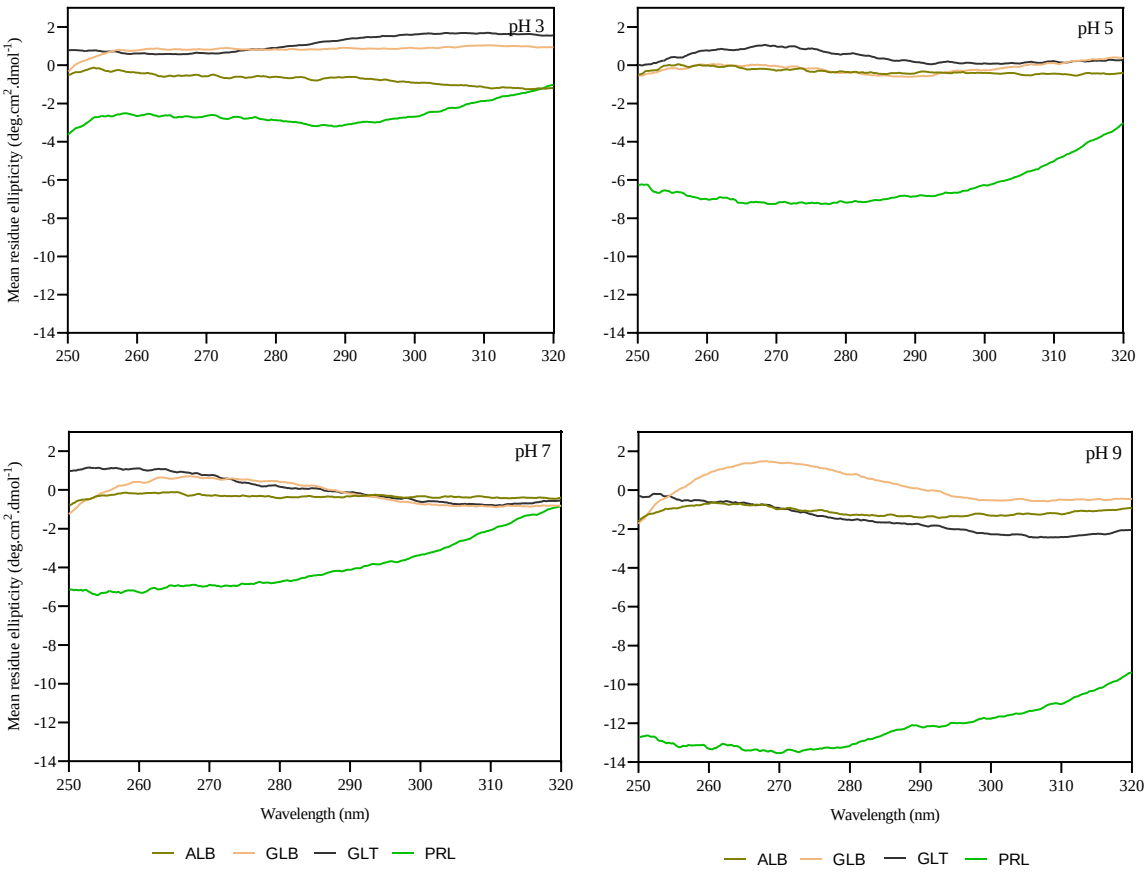

Figure 5. Near-UV circular dichroism spectra of green lentil seed protein fractions: albumin (ALB), globulin (GLB), glutelin (GLT) and prolamin (PRL) at different pH values at different pH values

4.3.6 Thermal properties

Information illustrating the thermal properties of the protein fractions obtained from a differential scanning calorimeter (DSC) is shown in Table 4. Peak denaturation temperatures (T_p) for dry ALB GLB, GLT, and PRL samples were similar and ranged between 90.57 to 91.02 ^{o}C. This indicates the thermal stability of the lentil proteins and potentially reflects the nature of their tertiary or quaternary structures (Tang & Sun, 2011). For proteins with higher T_p for example, it could indicate they have more intact hydrogen bonds and can be concluded that their tertiary structures are also more compact. Contrary to the T_p obtained for the dry samples, the results were significantly different and lower for the wet samples, with ALB, GLB, GLT, and PRL, having maximum T_p of 88.05, 82.52, 90.64, and 79.08 ^{o}C, respectively. This is especially true for PRL and GLB, which had a 11.94 and 8.26 ^{o}C, respectively, decreases in their T_p when a pH 7 buffer was added. This decrease puts emphasis on the contribution of their polar groups to the stability of their structures. In addition, wet GLB and PRL proteins had a significantly higher change in enthalpy when compared with that of their corresponding dry samples, while the opposite was the case for wet ALB and GLT proteins. This indicates that a higher amount of energy (J/g) was needed to unfold the helical structures in the wet GLB and PRL samples during denaturation, which indicate the presence of stronger protein-water interactions than in the ALB and GLT protein solutions.

Table 4: Thermal properties obtained using differential scanning calorimetry

Dry Samples			
Sample	Onset T_o (°C)	Maximum Tp (°C)	Area ΔH (J/g of sample)
ALB	85.69 ± 0.54	90.83 ± 0.04	0.46 ± 0.03
GLB	86.37 ± 0.13	90.78 ± 0.08	0.05 ± 0.01
GLT	86.03 ± 0.06	90.57 ± 0.00	0.28 ± 0.04
PRL	85.82 ± 0.30	91.02 ± 0.32	0.42 ± 0.22
Wet Samples[1]			
Sample	Onset T_o (°C)	Maximum Tp (°C)	Area ΔH (J/g of sample)
ALB	80.78 ± 0.37	88.05 ± 0.21	0.27 ± 0.33
GLB	75.89 ± 0.17	82.52 ± 0.25	1.46 ± 0.13
GLT	85.87 ± 0.22	90.64 ± 0.13	0.09 ± 0.01
PRL	77.13 ± 1.36	79.08 ± 0.47	1.19 ± 0.11

[1]samples were prepared in a 200 mg/mL concentration using a phosphate buffer.

4.3.7 SDS-PAGE

Polypeptide composition of the lentil seed protein fractions is represented in the gel electrophoresis (SDS-PAGE) patterns as shown in Figure 6. The non-reduced SDS-PAGE showed that ALB had seven polypeptides, while GLB and GLT had four polypeptides each. Calculations of the estimated molar weight and relative contents of PRL could not be obtained due the low solubility of PRL in the solvent used. The estimated molecular weights of ALB ranged from 20 – 97 kDa in both reducing and non-reducing conditions. After the addition of mercaptoethanol, eight polypeptide bands in ALB were reduced to six but the highest band intensity at 20 kDa was maintained. GLB had six bands (22, 25, 30, 32, 36, and 51 kDa) with the polypeptide at 51 kDa having the largest size under non-reducing conditions. In the reducing condition, there was a small shift in the polypeptide bands but the band at 51 kDa remained the highest proportion.

Like the wide range of polypeptides observed in ALB, bands present in GLB indicate that they majorly made up of legumins but also have vicilins and convicilins present. GLT displayed the presence of inter-molecular disulphide bonds by the significant changes to the bands at 21, 22, 24, 27, and 47 kDa in non-reducing conditions to 24, 47, and 92 kDa Similar reports have also noted that only subtle differences were noted in the polypeptide compositions of lentil, Bambara, and African yam bean proteins in their non-reducing or reducing condition (Alonso-Miravalles et al., 2019; Arise et al., 2017b; Joshi et al., 2011).

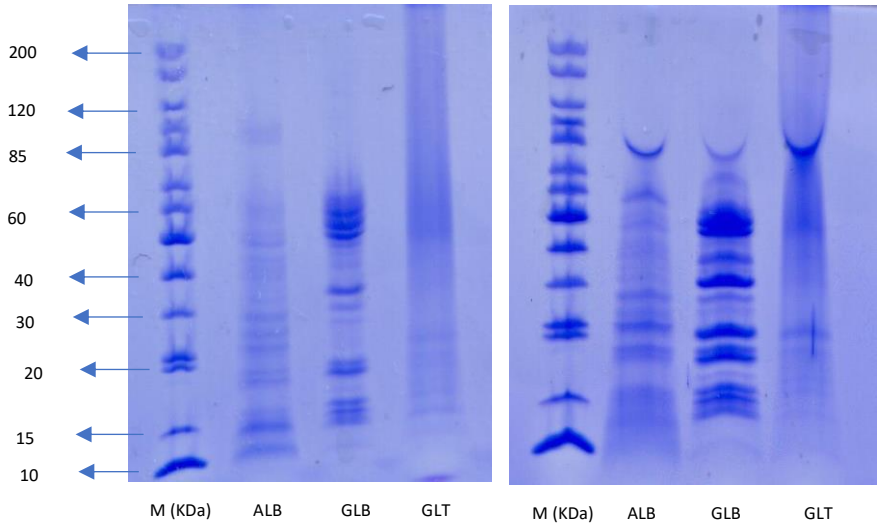

Figure 6. SDS-PAGE of green lentil seed protein fractions: albumin (ALB), globulin (GLB), and glutelin (GLT) under non-reducing (A) and reducing (B) conditions.

Table 5: Molecular weight (MW) and relative content of the major subunits of defatted green lentil Osborne fractions: albumin (ALB), globulin (GLB) and glutelin (GLT), under non-reduced condition

	ALB		GLB		GLT	
	MW (KDa)	RC (%)	MW (KDa)	RC (%)	MW (KDa)	RC (%)
1	20.00	12.64	22.00	21.72	21.00	11.07
2	21.00	18.85	25.00	15.87	22.00	9.51
3	24.00	14.07	30.00	5.18	24.00	15.55
4	27.00	8.80	32.00	8.81	27.00	13.58
5	29.00	8.70	36.00	9.70	47.00	50.29
6	31.00	8.42	51.00	38.72		
7	45.00	10.99				
8	97.00	17.51				

Table 6: Molecular weight (MW) and relative content of the major subunits of defatted green lentil Osborne fractions: albumin (ALB), globulin (GLB) and glutelin (GLT), under reduced condition.

	ALB		GLB		GLT	
	MW (kDa)	RC (%)	MW (kDa)	RC (%)	MW (kDa)	RC (%)
1	20.00	41.38	20.00	22.42	24.00	26.49
2	24.00	13.48	23.00	12.13	47.00	40.53
3	27.00	14.07	25.00	13.43	92.00	32.97
4	32.00	6.59	34.00	17.42		
5	67.00	12.26	40.00	7.41		
6	95.00	12.22	52.00	25.38		

4.3.8 Scanning electron microscopy (SEM)

Micrographs of lentil seed protein fractions at 100 µm are shown in Figure 7. The SEM revealed a difference in structure and composition of GLT fraction compared to ALB and GLB. ALB and GLB fractions were found to have a more compact and dense structure with very little pores. GLT on the other hand, had an almost flaky, plate-like structures which appeared less dense, and more porous. ALB and GLB were also observed to have more globular granules, while GLT had a more flat and irregular particles. All fractions however, appeared to have smooth surfaces and a homogenous mixture.

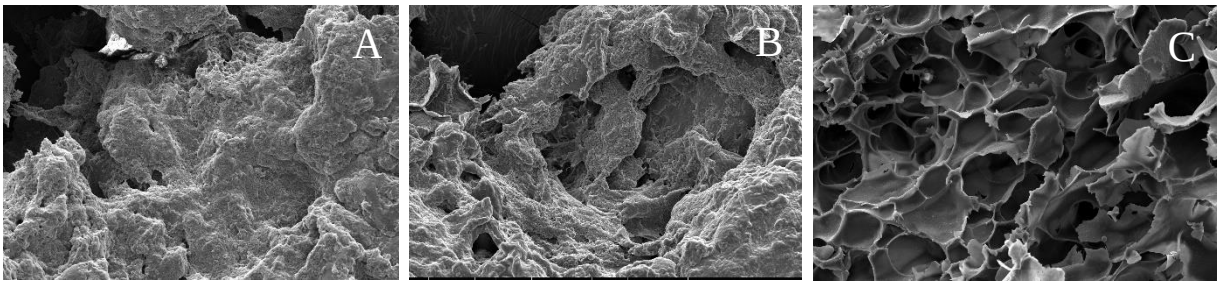

Figure 7. Scanning electron micrographs at 100 µm for green lentil seed protein fractions: albumin (ALB), globulin (GLB), and glutelin (GLT), as A, B, and C, respectively.

4.3.9 Protein Solubility

Solubility is among the most important properties of a protein and is usually a prerequisite for other functional properties of the protein such as emulsification, gelation, and foaming. The solubility of lentil seed protein fractions is associated with the equilibrium between the proteins and the thermodynamics of interactions with the solvent. Figure 8 shows the solubility of ALB, GLB, GLT, and PRL at different pH values ranging from 3 to 9. The results indicate that solubility of the protein fractions is heavily dependent on pH. Based on the points of minimum solubility of each sample, it can be concluded that the isoelectric points of each fraction were at pH 5 for ALB and GLB but at pH 4 for GLT, and PRL. This corresponds with the commonly recorded isoelectric points for pulse proteins in literature (Ajibola et al., 2016; Ladjal-Ettoumi et al., 2016). The isoelectric point is the point at which a protein is least soluble due to the lack of surface charge and reduction in the electrostatic repulsive forces (Singhal et al., 2016). Outside of the isoelectric point, protein solubility increased considerably with increasing pH value until the maximum solubility of 98%, 100%, and 100% was obtained at pH 9 for ALB, GLB, and PRL, respectively ($P > 0.05$). Surface hydrophobicity has been reported to play a crucial role in the solubility of a protein (Karaca et al., 2011; Malomo & Aluko, 2015), but other physicochemical and structural properties should also be considered. For instance, ALB had a significantly lower surface hydrophobicity ($p < 0.05$) which translated into an overall higher solubility trend at most of the pH values when compared to other protein fractions. However, this does not seem to be the case for PRL, which had the highest surface hydrophobicity but higher solubility than GLT at acidic and alkaline pH values.

Irrespective of the characteristic increase in solubility of all fractions after the minimum solubility had been attained, GLB appeared to be the only fraction in this study to exhibit the typical reported

U-shaped curve of pulse proteins. Wide variations in solubility among the protein fractions were observed at pH 3, while pH 7 presented almost the same solubility values for all fractions except GLT. At pH 3, GLT was insoluble at 0%, while PRL, ALB, and GLB, had solubility values of 28%, 57%, and 91%, respectively. On the other hand, the solubility of ALB, GLB, and PRL at pH 7 were 71%, 73%, and 74%, while that of GLT was 88%. The significantly higher solubility of GLB under acidic conditions could make it a promising ingredient for use as an ingredient to formulate acidic food products.

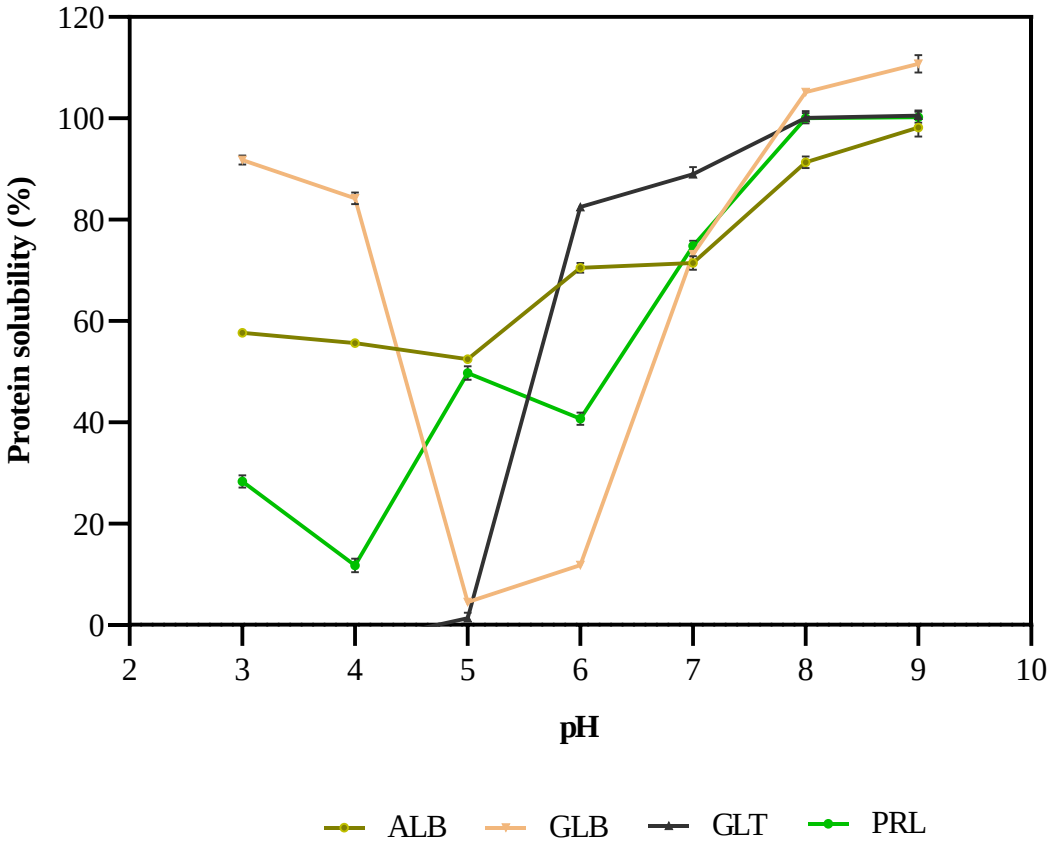

Figure 8. Percentage solubility of green lentil seed fractions: albumin (ALB), globulin (GLB), glutelin (GLT) and prolamin (PRL) from pH 3.0 - 9.0.

4.3.10 Emulsion formation & stability

The effects of protein concentration and pH on oil droplet size ($d_{3,2}$) of emulsions stabilised by ALB, GLB, and GLT are shown in Figure 9 - 11. Across all pH and concentration ranges, ALB and GLB demonstrated similarly small oil droplets, while that of GLT was consistently larger except at pH 9. The better emulsion forming property of GLB as indicated by smaller oil droplets, could be attributed to a more unfolded protein, which is reflected in the higher surface hydrophobicity when compared to ALB and GLT. A good emulsifier is often characterised by its ability to quickly unfold and form a protective layer around the oil droplets in order to encapsulate them. Although GLT displayed a higher hydrophobicity than ALB, smaller oil droplets in ALB can be possibly due to its higher number of hydrophobic amino acids compared with GLT. The oil droplet size of GLT was at least triple that of the other protein fractions at pH 3 and pH 5. This is to be expected as a result of the insolubility of GLT at pH 3, 4 and 5. In a similar vein, GLB exhibited the highest solubility and therefore, had the best emulsifying properties at pH 3. The oil droplet size of ALB experienced a consistent but minimal decrease across all pH range, which is also consistent with its solubility pattern at acidic, neutral, and alkaline pH values. In addition, this is also in agreement with the stable, but gradually declining maximum FI recorded, which indicates more flexible ALB protein conformations across pH 3, 5, 7 and 9 (Fig. 2). Distribution of the emulsion oil droplet sizes is shown Figure 12 - 14. Across all protein concentration, the lentil seed protein fractions showed a monomodal particle size distribution at pH 5, in addition to ALB and GLT at pH 3 and pH 7. This demonstrates that the protein fractions were more efficient in producing uniform oil droplets at that acidic pH values. Particularly, emulsions stabilised by ALB fraction had the least range of oil droplet distributions, confirming its previously suggested superior emulsifying properties. The results indicate that pH, had greater effect than protein

content, with respect to ability of the protein factions to interact with oil droplets and produce better emulsions.

Emulsifying activity as a function of short-term stability was carried out over a 30-min interval and recorded as emulsion stability as shown in Figure 15. The emulsion stabilising effect of proteins result from the formation of a membrane matrix that surrounds oil droplets to prevent coalescence. ALB had an overall higher emulsion stability particularly at acidic and alkaline pH values as expected, which is consistent with the smaller oil droplets. GLT, which demonstrated relatively poor emulsion forming activity (large oil droplet sizes) especially at acidic pH produced the most stable emulsions at pH 3. This differs from other reported works in literature, which presume that the emulsion stabilizing ability of proteins is usually minimal at low protein solubility (Joshi et al., 2012; Rahmati et al., 2018; Zang et al., 2019). High stability of the GLT emulsion at pH 3 could be due to formation of strong interfacial membranes around the oil droplets. However, the same highly stable emulsion was not produced for GLT at pH 5, which similarly demonstrated larger oil droplets. The low stability of GLT-stabilized emulsions at pH 5 and pH 7 suggests formation of weak interfacial membranes, which is less resistant to oil droplet coalescence or flocculation.

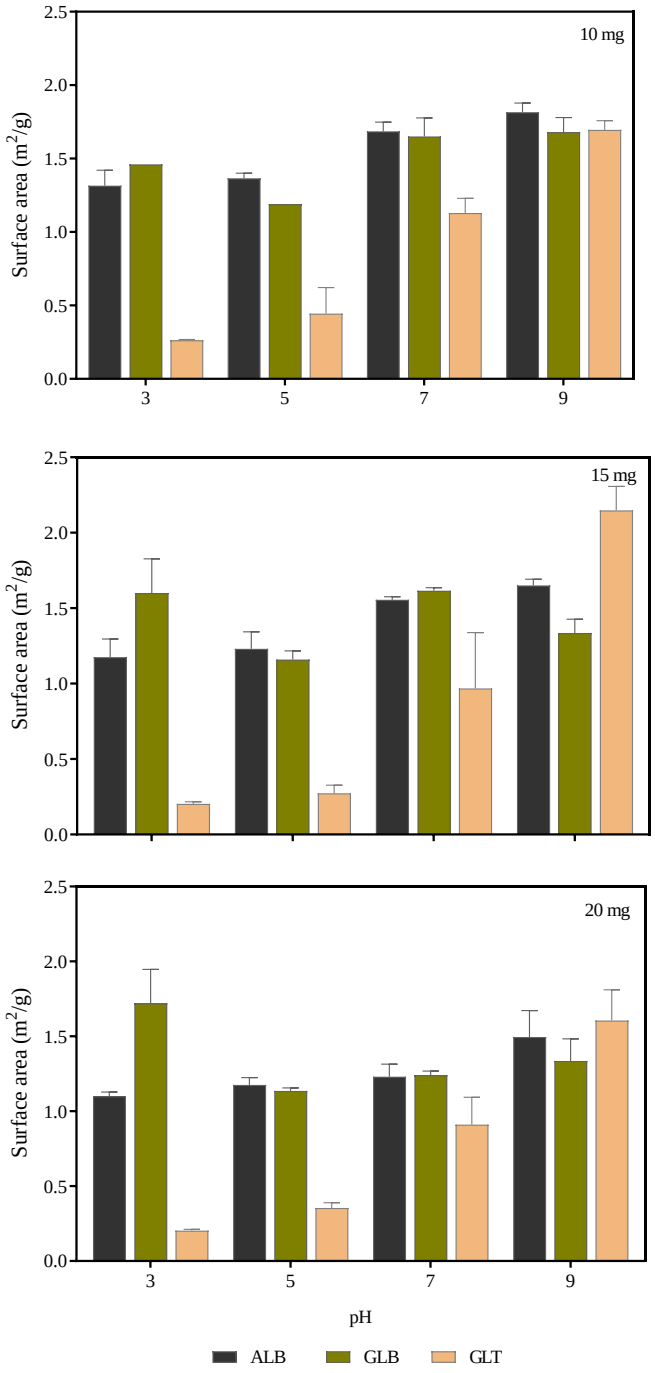

Figure 9. Surface area (m^2/g) of emulsions formed by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at different concentrations and pH values.

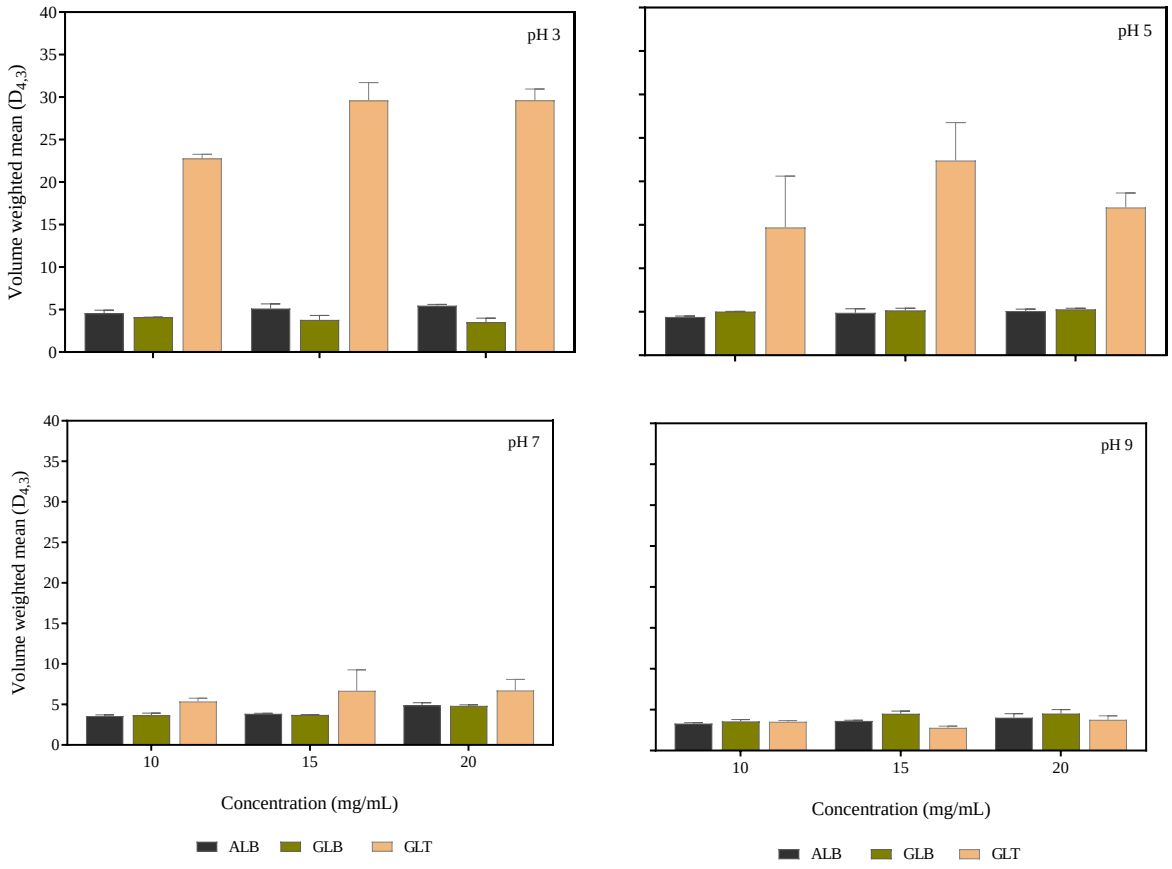

Figure 10. Surface weighted mean ($D_{3,2}$) of emulsions formed by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at different concentrations and pH values.

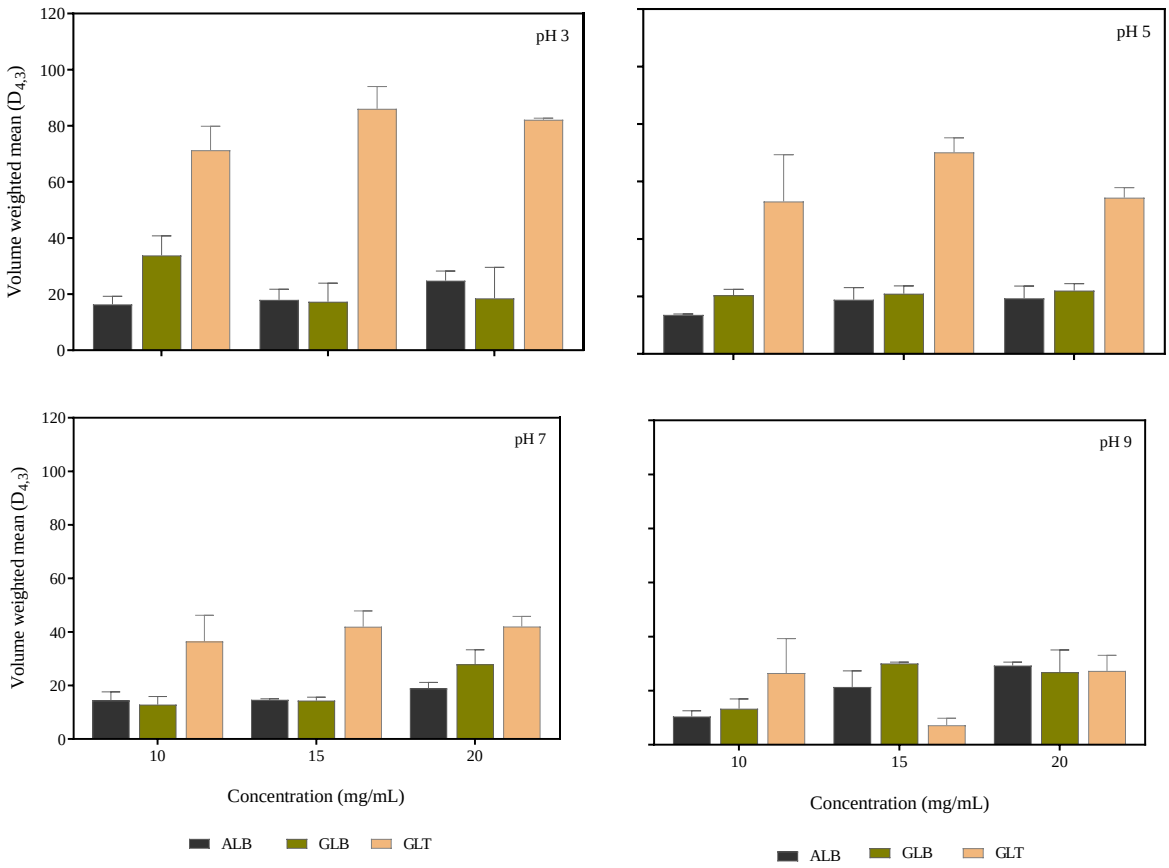

Figure 11. Volume weighted mean ($D_{4,3}$) of emulsions formed by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at different concentrations and pH values.

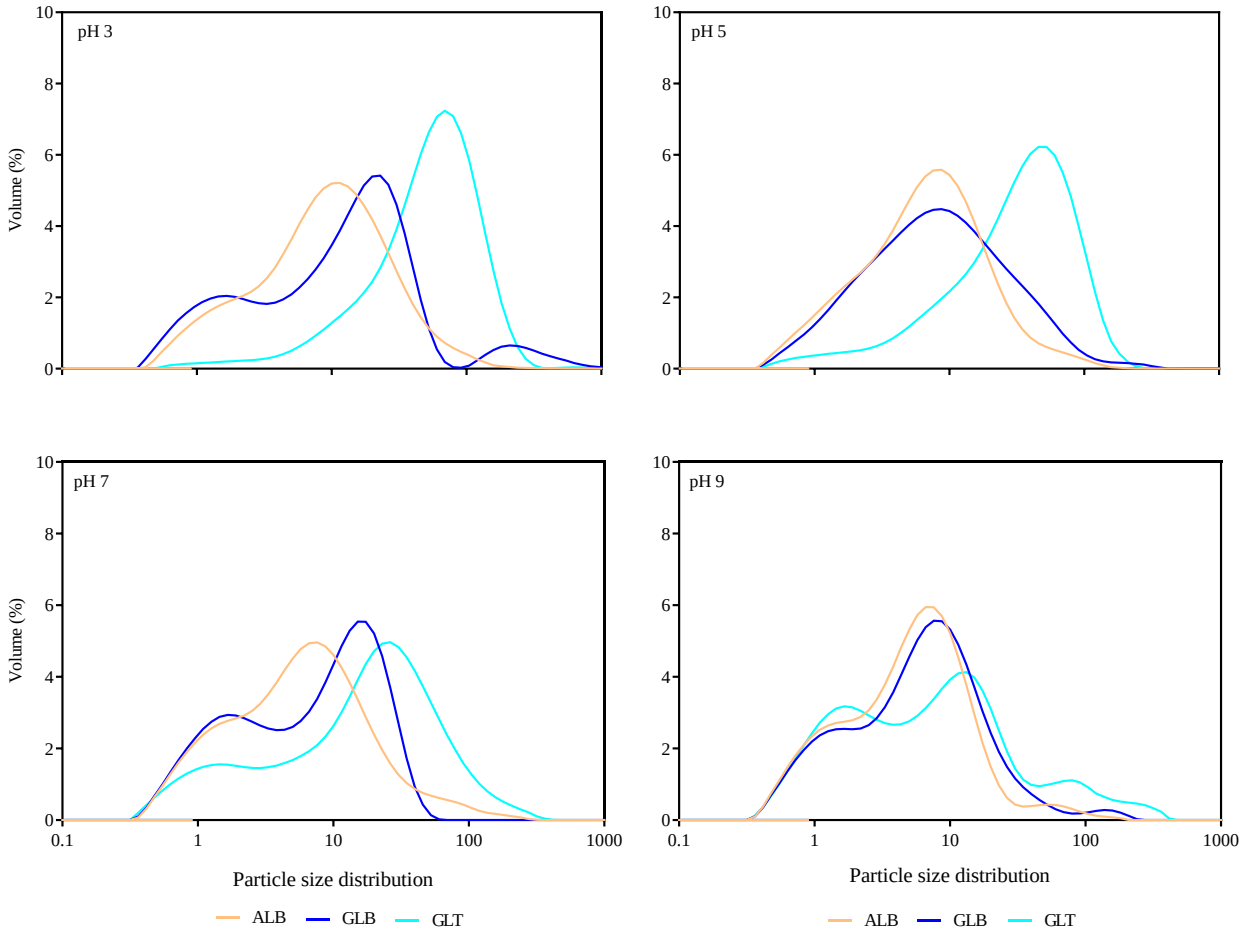

Figure 12. Oil droplet size distribution of emulsions stabilized by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at 10 mg/mL and pH 3, 5, 7 and 9.

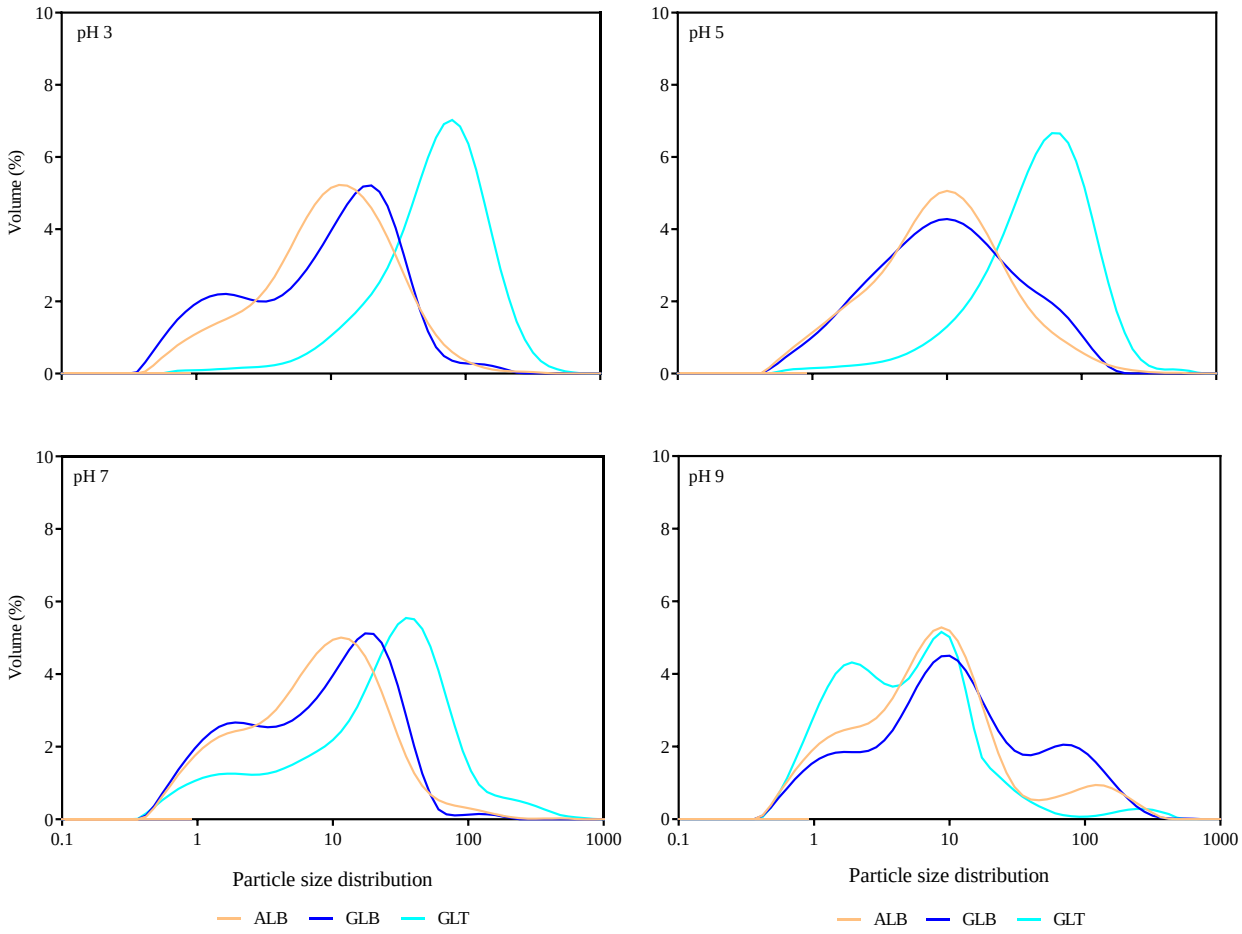

Figure 13. Oil droplet size distribution of emulsions stabilized by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at 15 mg/mL and pH 3, 5, 7 and 9.

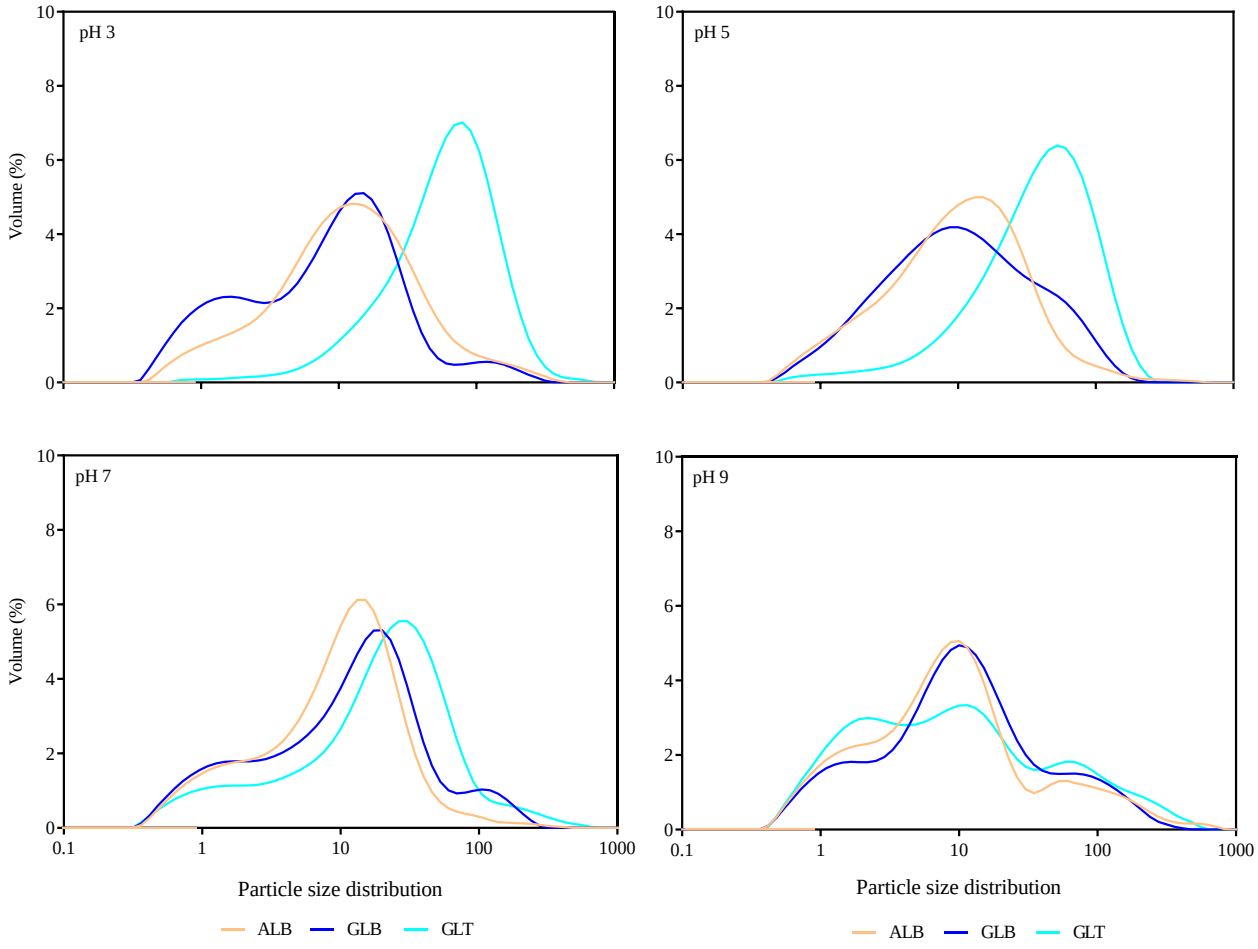

Figure 14. Oil droplet size distribution of emulsions stabilized by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at 20 mg/mL and pH 3, 5, 7 and 9.

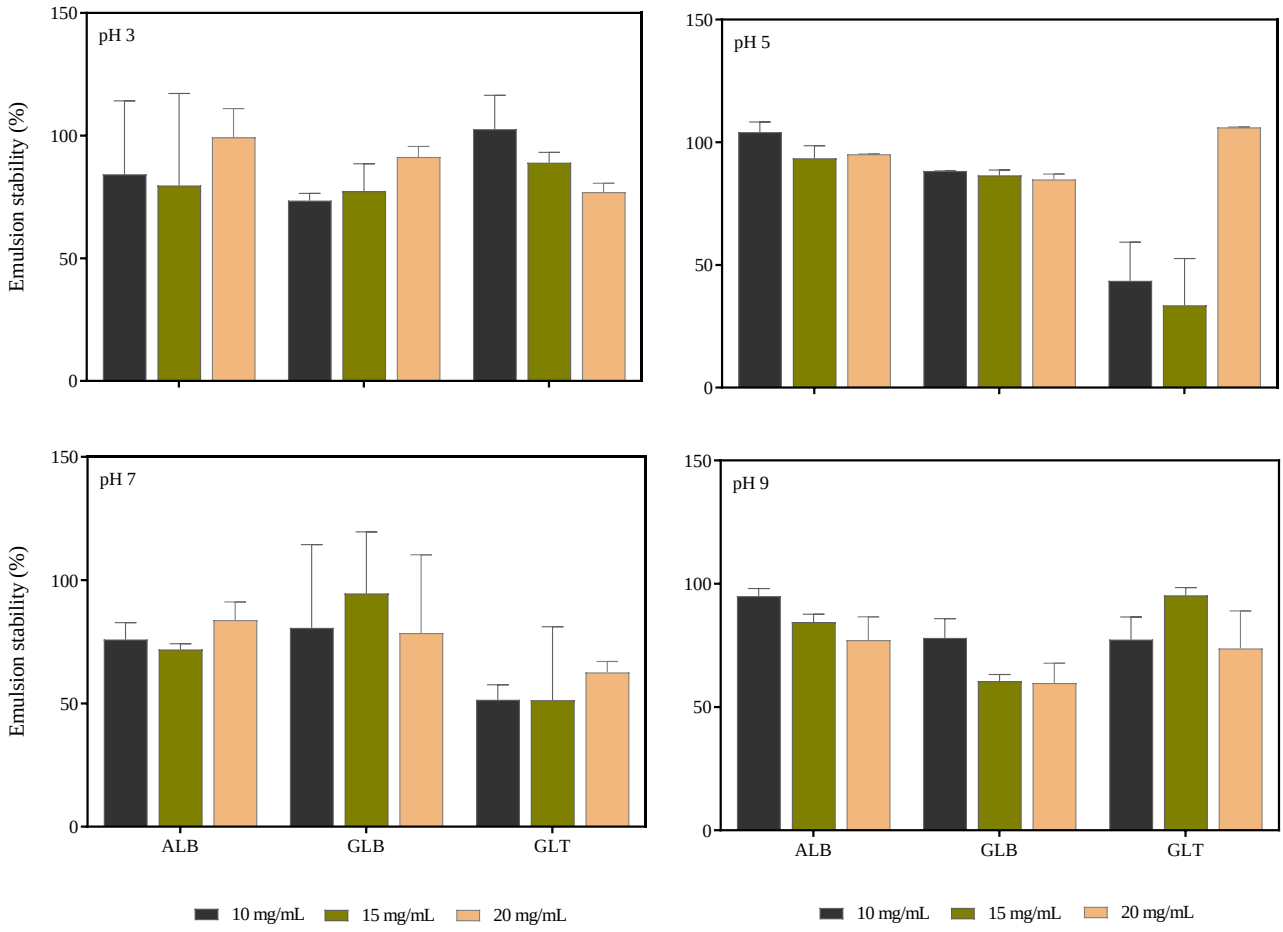

Figure 15. Stability of oil-in-water emulsions formed by green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at different pH values at different pH values.

4.3.11 Water and oil holding capacity

Water or oil absorption capacity are terms used to describe the amount of water or oil that a gram of protein can hold. The ability of proteins to retain water or oil can be important in food products such as baked goods and meat formulations, as they influence texture and mouthfeel. The water holding capacity (WHC) of proteins, which indicates the ability of proteins to retain water without dissolution, is equally as important in food formulations. As shown in Figure 16, GLT had the highest WHC at pH 3, 5, and 7, while that of ALB was higher at pH 9. Although ALB had a WHC at pH 9, WHC for all samples including ALB were particularly low at that pH. This is due to the negative correlation of the increased solubilities (>98%) of all the lentil seed protein fractions at pH 9 with their WHC. Similar to its poor OHC, GLB also demonstrated an inability to retain water at all pH and concentrations investigated. Overall, the combination of good OHC and WHC for GLT suggests that it would be better suited than ALB, and GLB, in the formulation of processed foods, which require water retention to maintain quality. Results obtained in this study (0 – 1.5 mg/ml) are lower than what has been previously reported in literature. For example, Alonso-Miravalles et al. (2019), Matina Joshi et al. (2011), Mundi and Aluko (2012), Shevkani et al. (2015), and Shevkani, Singh, et al. (2015), reported WHC of 1.4 – 4.9 mg/ml for lentil, kidney bean, field pea, and cowpea proteins .

Oil holding capacity (OHC) as shown in Figure 17 are 1.6, 2.3, and 2.9 mg/ml for ALB; 0.6, 1.1, and 1.0 mg/ml for GLB; and 1.7, 3.2 and 4.3 mg/ml for GLT at 20, 40 and 60 mg/ml, respectively. The lower OHC of GLB when compared to the ALB is similar to the results reported for kidney beans (Mundi & Aluko, 2012).. This could result from a more folded or globular structure of GLB, which reduces interaction with the lipid phase. Although surface hydrophobicity of GLB is the highest in comparison, this did not translate to a higher OHC, emphasizing that other

properties of the protein such as its structure, played a more crucial role. Particularly, the lower content of hydrophobic amino acids in GLB could also account for its lower OHC.

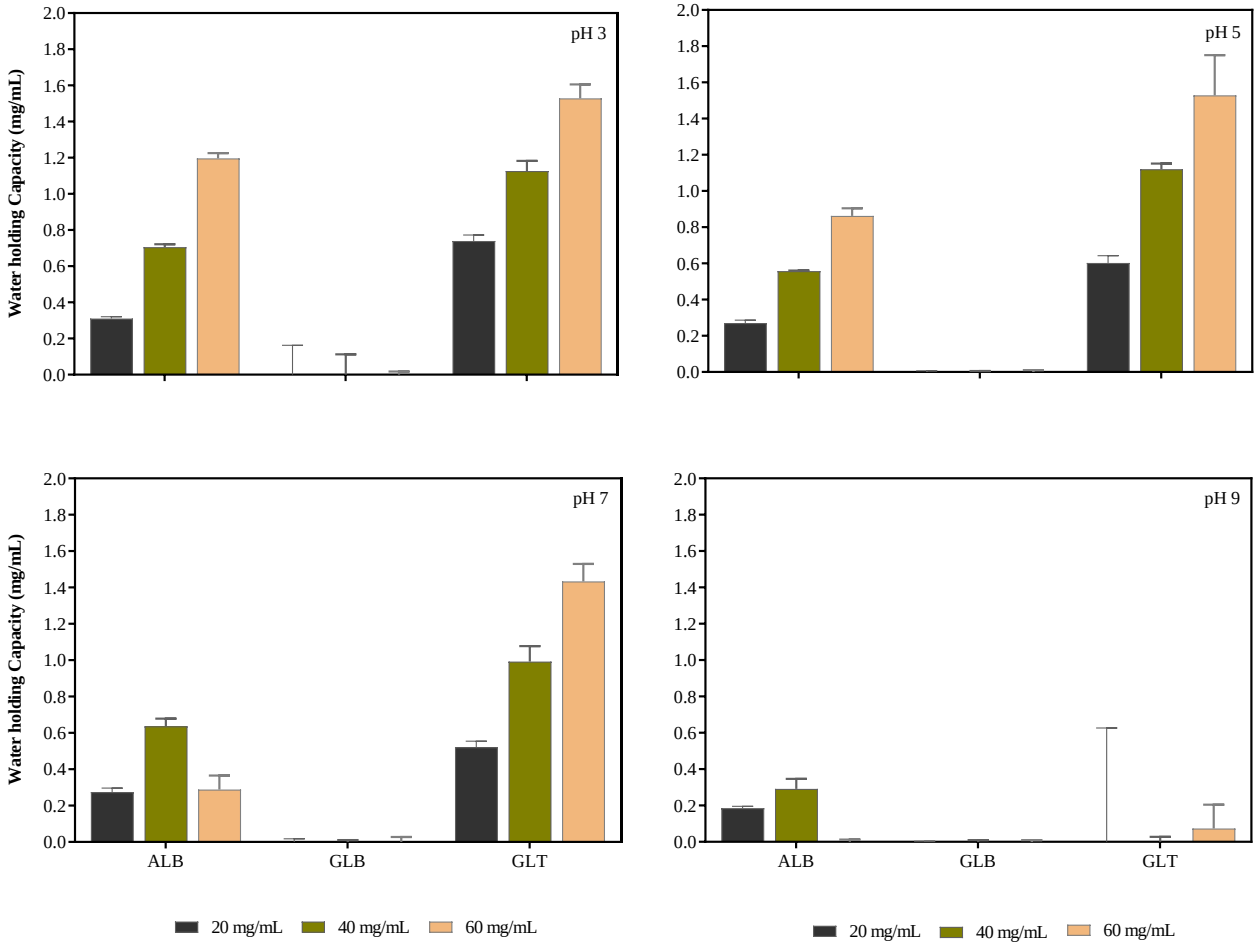

Figure 16. Water-holding capacity of green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) and at different pH values

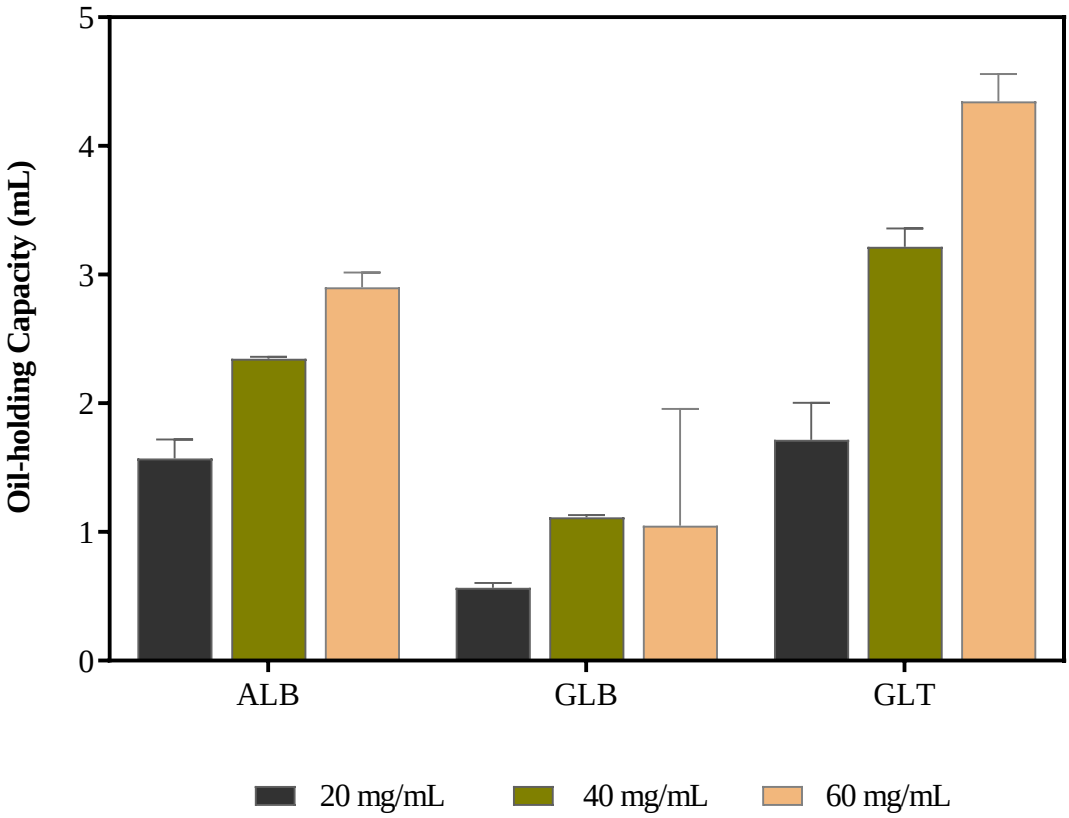

Figure 17. Oil-holding capacity of green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at 20, 40 and 60 mg/mL.

4.3.12 Foam formation and stability

Foams are formed as proteins disperse and adhere to an air-water interface that decreases surface tension when partly or wholly unfolded. This creates an interfacial layer around the air bubbles that prevents the foam from fracturing directly after formation and often during storage (Boye et al., 2010). Foaming properties of proteins are considered an important factor in food products where aeration and overrun are essential (Shevkani, Singh, et al., 2015). Examples of such food products include cakes, ice-cream mixes, whipped toppings, and fudges. Foaming capacity (FC) represents the percentage increase in volume after air incorporation, while foam stability (FS) measures ability of the protein to retain the air in the form of bubbles (Jarpa-Parra, 2018). FC (Fig. 18) and FS (Fig. 19) of proteins fractions were determined at different pH values (3, 5, 7 and 9) and protein concentrations (10, 15, and 20 mg/ml). Changes in the pH and concentration of the proteins were observed to have some effects on FC and FS. For example, the highest FC was recorded for ALB (32 – 38 %), GLB (58 – 65%), and GLT (43 – 52 %), at pH 9, while lower FC was observed at pH 3 and pH 5. Shevkani et al. (2015), reported greater foaming properties corresponding with higher solubility of proteins, and this can account for the higher FC of the lentil seed protein fractions at pH 9, since they were most soluble at this pH. However, the reverse is the case for FS. Neutral to alkaline pH range with higher solubility values were observed to have significantly lower FS compared to more acidic pH values with lower solubility. Foams created by ALB were most stable at pH 3 and 5 (100%) and least stable at pH 9 (57 – 78 %). Similarly, GLB and GLT had higher FS at pH 3 (86 – 94% and 90 – 95 %, respectively) but produced the less stable foams at pH 9 (85 – 92 % and 59 – 70%, respectively). The lower FS at pH 9 could be due to high net charge, which limited protein-protein interactions to form weak interfacial membranes. Overall, foams formed by GLB were very stable across all measured pH (> 81 %);

however, its FC was relatively lower (< 66 %). Ghumman et al. (2016) also reported higher FC and FS for lentil seed albumins compared to globulins. Lentil seed albumin was reported to have FC and FS of 77% and 67%, respectively, while globulin had 16% and 7%, respectively. Shevkani et al. (2019) attributed the lower foaming properties of GLB to be because of their folded structure, which could limit the protein-protein interactions necessary to form thick interfacial membranes that promotes foam stability.

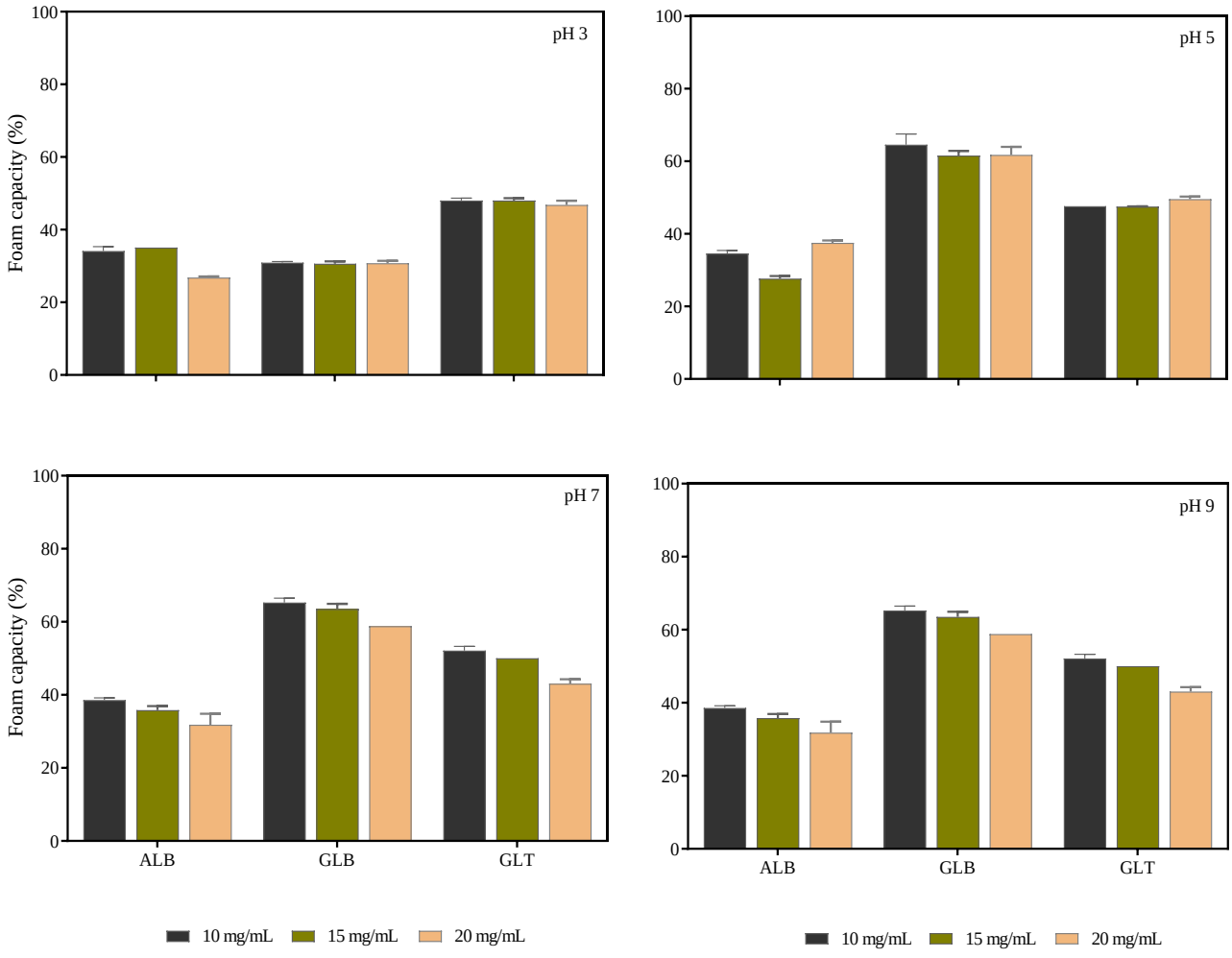

Figure 18. Percentage foaming capacity of green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at different pH values

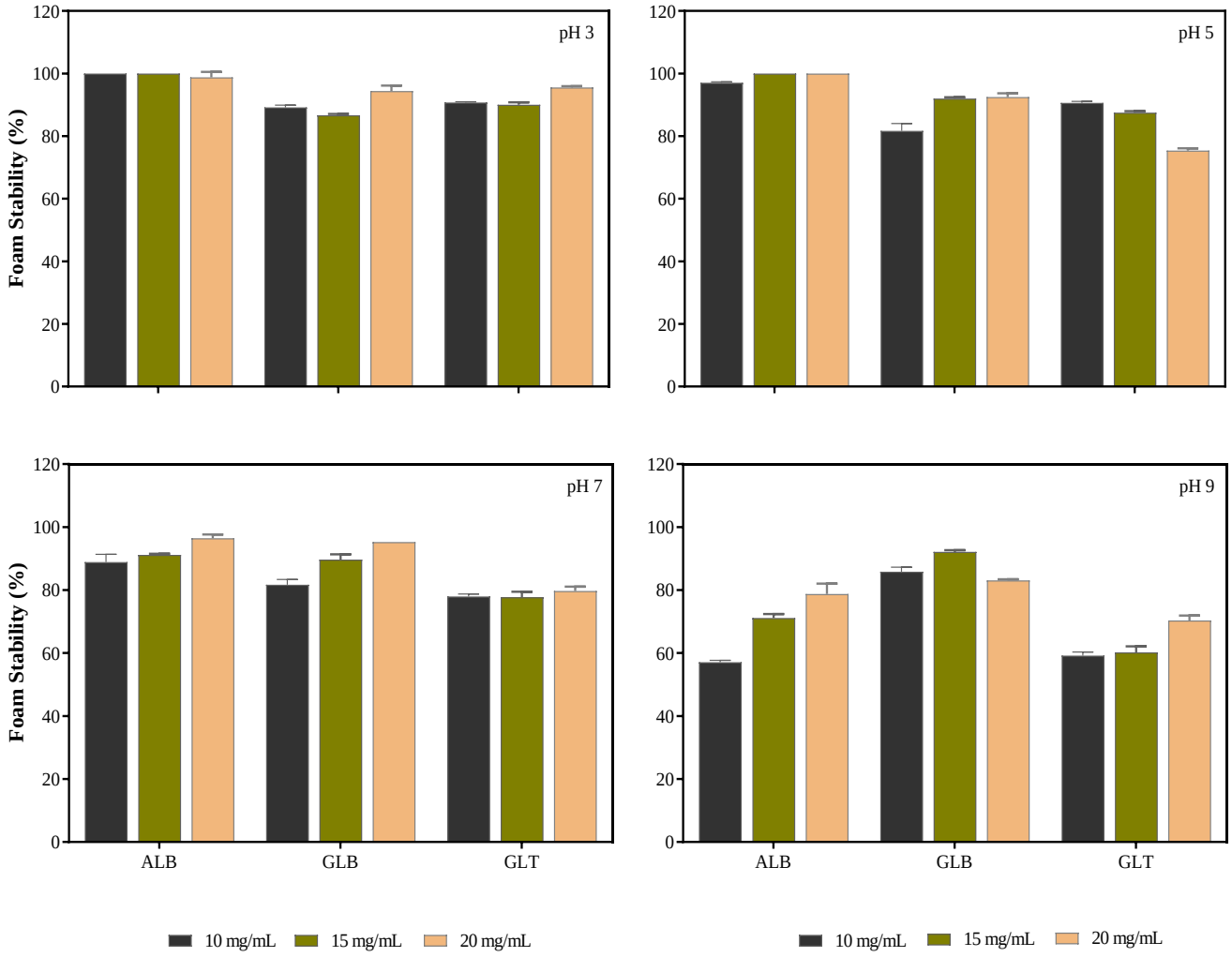

Figure 19. Percentage foam stability of green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) at different pH values

4.3.13 Least gelation concentration (LGC)

Gel formation by proteins involves a complex process including denaturation, aggregation, and network formation, which is usually induced by heating. The capacity to form gels can be estimated by determining the LGC, which may be defined as the lowest concentration of protein required to form a self-supporting gel that would not slide due to gravity when inverted (Boye et al., 2010). Therefore, proteins with lower LGC values are considered to have a more superior gelling capacity. As shown in Fig. 20, GLB had a significantly higher ($p < 0.05$) LGC (11.5%) than ALB (8.5%) and GLT (10.5%). Gelation capacity is largely dependent on modes of interaction involving electrostatic, hydrophobic, hydrogen, and disulphide bonds (Ma et al., 2011). The higher content of hydrophobic amino acids in ALB would promote strong intermolecular interactions and can account for its better gel-forming ability. In contrast, reduced hydrophobic amino acid content of GLB could justify its higher LGC. The LGC of GLT obtained in this work is similar to the 10% obtained for the alkali-soluble fraction of yellow field pea protein reported by Adebiyi and Aluko (2011). The LGC of ALB was lower while that of GLB was higher than the LGC of albumin (16%) and globulin (6%) reported by Mundi & Aluko (2012).

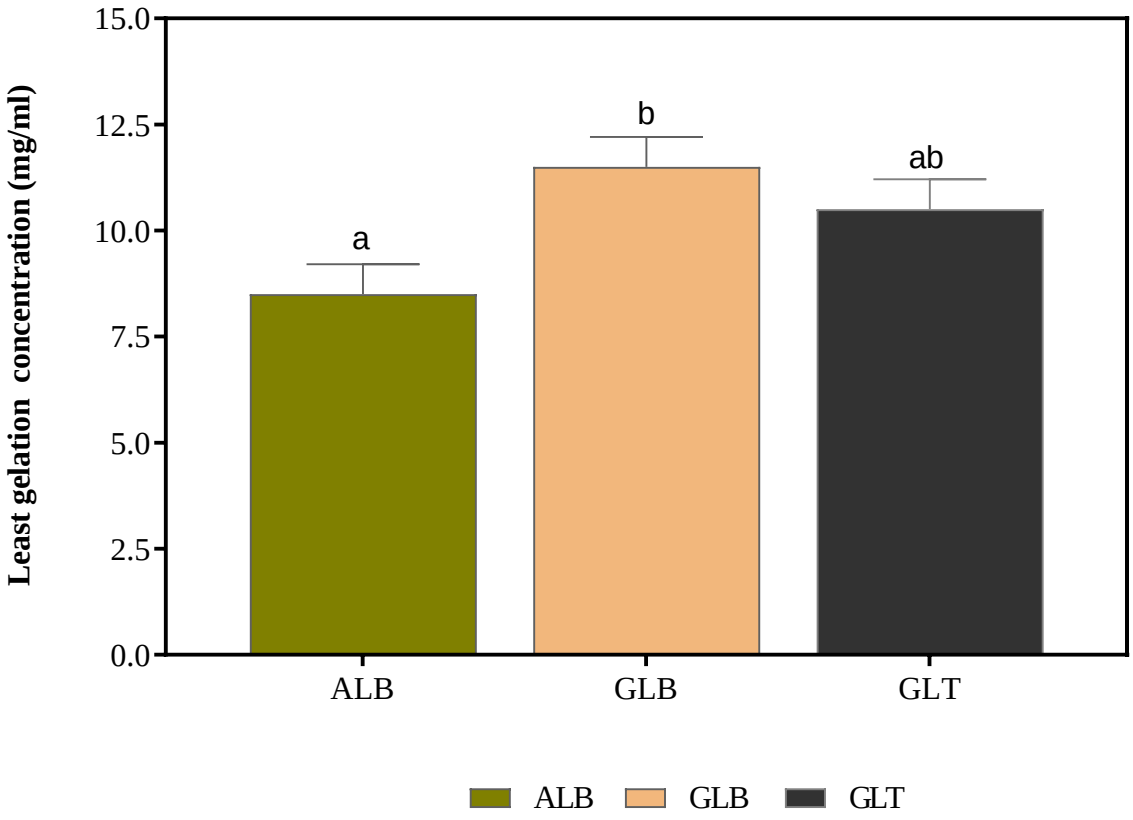

Figure 20. Least gelation concentration (mg/ml) of defatted green lentil Osborne fractions: albumin (ALB), globulin (GLB) and glutelin (GLT)

4.3.14 Heat coagulability (HC)

Proteins are liable to the damaging effects of heat (especially wet heat) during various stages of processing (Damodaran, 2017). The extent to which a protein can withstand denaturation resulting from the application of heat is an important property to be considered in the production of foods such as desserts, and baked goods (Aryee et al., 2018; Östbring et al., 2020). Alternatively, the ability of proteins to easily coagulate with heat treatment could also be considered as favorable properties in curd-like products and other breakfast foods. Susceptibility of a protein to structural changes during a heat treatment can be influenced by the nature of the protein, concentration, ionic strength, and hydration activity of the protein (Damodaran, 2017). HC measures the typically substantial and irreversible reduction in solubility caused by the aggregation of unfolded protein molecules. HC results shown in Figure 21 demonstrates that the lentil seed protein fractions were most resistant to heat treatments at pH 9 (3 – 20 % HC), with the highest HC occurring around their isoelectric point (pH 5) for ALB, GLB and GLT (72 – 98% HC). Heating to 100 ^{o} C was also extremely damaging to GLT at pH 3 with 100% loss of solubility, indicating no thermal stability (100% HC) and suggests high susceptibility to heat-induced protein-protein hydrophobic interactions that produced insoluble protein aggregates. These results correspond with the DSC thermal properties of ALB, GLB, and GLT, obtained in this study with GLT demonstrating a higher denaturation temperature at pH 7 and GLB displaying the lowest. The HC of the lentil seed protein fractions in this work are comparable to those obtained from previous works on canola protein isolates, mustard seed protein, soybean isolate, coriander protein, pea isolate, and whey protein isolates (Aluko et al., 2005; Aluko & McIntosh, 2001; Hojilla-Evangelista & Evangelista, 2017; Voutsinas et al., 1983).

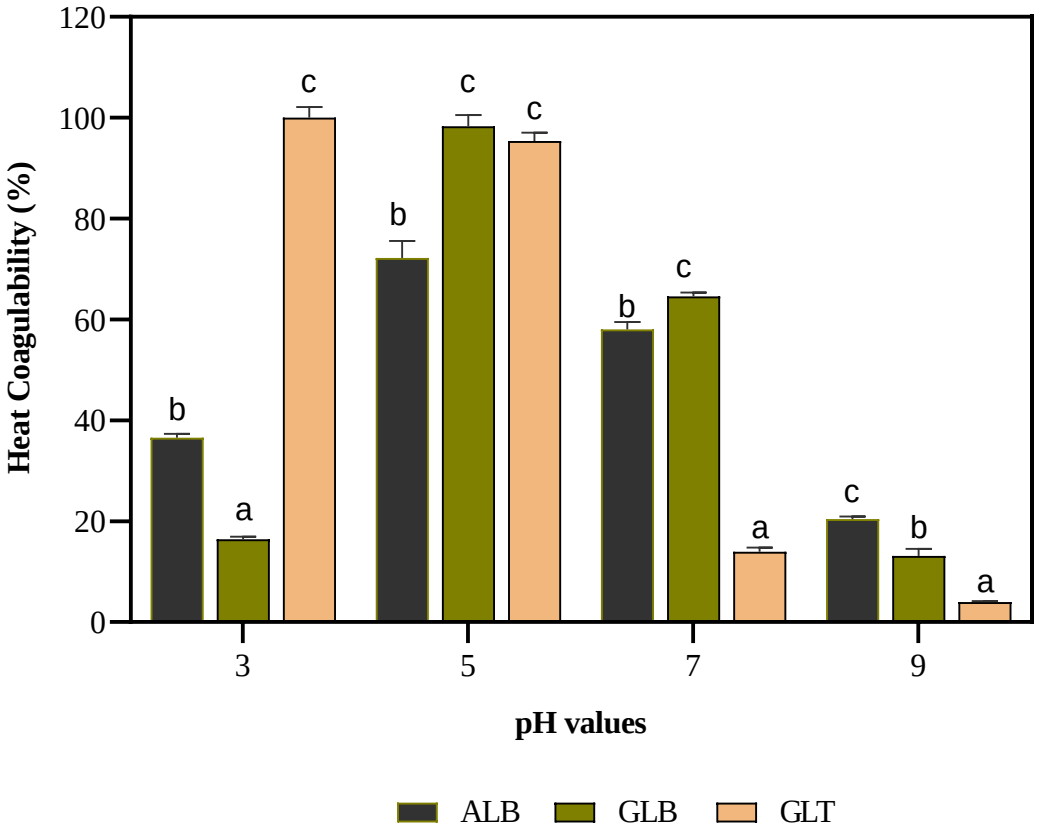

Figure 21. Percentage heat coagulability of green lentil seed protein fractions: albumin (ALB), globulin (GLB) and glutelin (GLT) from pH 3.0 - 9.0

4.4 Conclusion

SCAAs which have been shown to have anti-inflammatory and antioxidant effects in the human body was very high in PRL. Therefore, PRL can be used in the fortification of foods which require these SCAAs. Besides its nutritional role, PRL had a surface hydrophobicity that was at least 6% greater than those of the other lentil protein fractions, which is also a reflection of the significantly higher amount of HAA on the surface of the protein molecule. Higher hydrophobicity can also be predicted to have an impact on the foaming activity and emulsifying properties of PRL. Therefore, further studies should be carried out on how the low yield of the PRL fraction can be increased in order to further investigate its functional properties. In general, the functional properties obtained from the ALB, GLB, GLT, and PRL lentil fractions are favorably comparable to those obtained from other legume protein sources. As a result, findings from this study shows that there is a potential use for the individual lentil protein fractions as ingredients in the food industry.

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CHAPTER 5

GENERAL SUMMARY AND CONCLUSIONS

As the lentil seed becomes increasingly popular due to its good nutritional quality, this work was designed to examine potential uses of the proteins as functional ingredients in food formulation by evaluating their structural and functional properties. Results showed that extraction conditions or changes in the protein environment in terms of pH, or/and concentration had significant effects on some functional properties but not others. The highest solubility for all isolated lentil seed proteins and fractions in this work was attained at pH 8 – 10. As expected, isoelectric points of all lentil seed proteins as estimated using point of least solubility appeared between pH 3 – 5. However, except for GLB which had a >80% solubility at acidic pH values (pH 3 and pH 4), the others did not display the characteristic high solubility of pulse proteins in acidic environments. High solubility of GLB in both acidic and alkaline pH environments makes it the ideal protein for use in acidic drinks, desserts, non-acidic beverages, and other liquid beverages. Due to the very low yield of prolamin, some of its functional properties were not investigated in this study. Prolamin also had a significantly lower protein content, which could be as a result of its limited solubility in the aqueous solution used for protein measurement when compared to the other protein fractions. However, prolamin had a substantially higher amount of SCAA, as well as a better solubility at pH 5. This could make it ideal for use in acidic beverages, especially those that need to be fortified with generous amounts amino acids like methionine and cysteine. An inverse correlation between solubility and WHC was observed, with a consistent reduction in WHC as pH or solubility increased from the acidic to alkaline pH range. As a result, the highly soluble GLB protein demonstrated an inability to hold water at all pH values. Increase in protein concentration had a positive impact on all OHC except that of ISO but did not seem to have much of an impact on the

foaming capacity and stability. Similar to the pattern observed in their WHC, higher FS was observed at acidic pH values. Although FC of all isolates and fractions were <80% at all pH and concentrations evaluated, they had exceptional FS at acidic pH values. Extraction conditions appeared to have no significant effect on the gelling ability of the lentil seed protein isolates. Among the fractions, the superior gelling ability of ALB when compared to that of GLB and GLT is one of the important characteristics needed for successful incorporation into several foods such as jellies, puddings, and meat products. Highest HC was generally observed at pH 9 while the lowest was observed at pH 5. Consistent with their thermal properties obtained at pH 7, GLT also had a lower HC than other fractions, making it suitable for use in some desserts and baked products. Alternatively, higher HC of the proteins at pH 5 and 7 also make them good appropriate for use in curd-like products and other foods that may require some degree of coagulation or heat-induced changes in rheological properties and texture. The isolates, especially ISO were highly digestible when compared to the protein fractions. As a result, higher digestibility of ISO when compared with MEM_NaOH or MEM_NaCl may be due to the degree of denaturation and resultant deviation from the native folded conformation that may have occurred during acid-induced isoelectric pH precipitation. This can be further confirmed by the almost zero ellipticity observed for ISO at pH 3 and 5 of the far-UV CD spectra, for example. The significant impact resulting from changes in pH can also be observed in the CD and intrinsic fluorescence spectra for all isolated lentil seed proteins and fractions. Moreover, analytical methods like fluorescence spectroscopy can also be used to assess food quality by monitoring the degree of microbial contamination.

Overall, this study also provides new information about the functional properties of lentil seed proteins and fractions, in relation to their physicochemical properties and their potential

application as ingredients in food formulation. In addition, this work investigated and compared the impact of various extraction conditions and protein environment on their functional properties in order to provide information on the most functional lentil seed protein isolates or fractions at various conditions examined. This information could enhance the utilization of lentil seed proteins in the food industry as ingredients that can be used to formulate novel food products.

Therefore, future work should be geared towards their application in actual food formulations such as fortified beverages and baked goods, or as emulsion stabilizers in sausages, salad dressings and other food products. However, further work is required on improving the yield of prolamin and investigation of its functional properties. The use of an alternative industry-wide accepted defatting solvent like ethanol, should also be investigated for its potential to increase protein yield and defatting efficiency, while retaining the native structure of the protein.