STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF FERRITIN (IRON-BINDING PROTEINS) ISOLATED FROM MANITOBA LEGUME SEEDS

BY

FOLASHADE GESINDE

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Department of Human Nutritional Sciences

University of Manitoba

Winnipeg

ABSTRACT

Fourteen Manitoba legume seeds and a pea protein isolate were evaluated for ferritin production.

Optimized ferritin concentrate yields from 5 selected isolates ranged from 19.07% (Chick Peas) to 9.69% (Moon Dal Washed). Iron concentrations were between 0.45g (Green Lentils Whole) and 0.30g (Chick Pea)/100g. SDS-PAGE revealed presence of the major ferritin polypeptides in the concentrates. The levels of iron in ferritin appear to be directly related to the amount of negatively charged amino acids. Intrinsic fluorescence and circular dichroic spectra showed that the ferritin concentrates had denatured protein conformations at pH 2 and 7, which is critical to their digestibility and bioavailability. Gel-permeation chromatography revealed differences in elution volumes between pre- and post-digestion ferritin concentrates, and kinetics studies confirmed susceptibility to proteolysis and a high potential for iron release.

Results demonstrated the feasibility of phytoferritin production from Manitoba pulses, which could serve as a better iron supplement than inorganic iron during IDA treatment.

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"Unto the King eternal, immortal, invisible, the only wise God, be honour and glory for ever and ever. Amen". I thank God from the depth of my heart, who, indeed does ALL things well. To Him alone be all the glory for helping me to complete this project as with each one I have set my mind on.

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DEDICATION

I dedicate this thesis to my sons, Timothy and David, and my daughter, Zoe.

You are my why and I bless God for you.

CHAPTER ONE

1.0 INTRODUCTION

Iron Deficiency Anaemia (IDA) is the most common nutrient deficiency in the world (WHO, 2005; WHO, 2008). IDA is characterized by a reduction in the circulating haemoglobin concentration and directly infringes on oxygen transport within blood cells. Its global prevalence is currently estimated at between 25 and 33% of the world's population and occurs in both developed and under-developed countries. IDA mostly affects school aged children and women of child-bearing ages and its overall economic burden seem to have gotten worse over the last two decades. (Murray-Kolb et al., 2003; De Benoist et al., 2008).

Many vital interactions occur between metals and organic compounds in all living organisms and are precursors for many biochemical processes occurring within the cells. They are responsible, not only for metabolism, but also for some regulatory mechanisms of gene expression (Andrews et al., 2003; Briat et al., 2006; Arosio et al., 2009). Iron is one of such essential micronutrients and its importance in the proper functioning of the human body cannot be over emphasised.

IDA has severe negative impacts, the effects of which are more pronounced in critical life stages such as: active growth, aging, pregnancy and lactation and the eventual health of the child (Walker et al., 2007). Reduction in work efficiency (or physical work capacity) by anemic sufferers results in a large negative economic impact (Beard et al., 1996); poor pregnancy outcome and compromised health of the child such as a decreased rate of normal cognitive development in iron-deficient children, with negative social and economic effects (Pollitt, 1993 and Walker, 2007).

IDA can occur as a result of genetic inheritance, but there are two major diet–related conditions that have been reported as being directly responsible for the wide prevalence of the deficiency: 1) Inadequate intake of dietary iron or consumption of poorly available forms of iron. This is especially true for underdeveloped economies and 2) poor or diminished bioavailability of iron in foods, due to high levels of dietary inhibitors. This has been implicated particularly in the developed countries, including the United States of America (USA) and Canada.

There is scientific evidence that most plant-based foods contain significantly high levels of substances which act as dietary inhibitors and impact on the bioavailability of iron in foods (Sandberg, 2002). Such inhibitors, including vegetable components (phytate and polyphenols) and calcium, are major players in the problem of iron deficiency. They interfere with iron absorption and have been associated with the fact that only 10-15% of iron is actually absorbed during consumption of non-vegetarian diets (Zielinska-Dawidziak & Siger, 2012); the numbers are even much lower for vegetarian diets (Hunt 2003).

It is, therefore, understandable that efforts are being geared by international agencies to drastically reduce IDA. Pharmaceutical treatment is through iron supplementation in the form of ferrous salts (Perez-Exposito et al., 2005). However, the use of inorganic iron salts as nutritional supplements to alleviate iron deficiency has been associated with negative side effects, including diarrhea, constipation and decreased growth (Hyder et al., 2002 and Dewey et al., 2002). Due to these serious adverse effects of inorganic iron supplementation, some of which causes inconsistency in consumption of the pills, the WHO has recommended the use of more readily bioavailable forms of iron (Stoltzfus & Dreyfuss, 1998).

The organic form of iron is called ferritin. Ferritins are ubiquitous and spherical iron storage and detoxification proteins, and are widely distributed in animals, plants and microorganisms (Yang, 2014). The storage molecule essentially consists of a protein shell and an iron core; it is estimated that up to 4500 iron atoms in the form of hydrated ferric oxide can be enclosed within the core (Theil, 2004). Since the protein shell is susceptible to enzyme digestion in the gastrointestinal tract, dietary ferritin iron can be easily released and made available for absorption as already demonstrated by several studies. Another advantage of ferritin is that the iron is sequestered within a protein cage, which reduces the negative effects (low bioavailability) caused by metal-complexing food components such as phytates, calcium, ascorbic acid, oxalates and polyphenols.

There are two sources of dietary ferritin, animals and plants (phytoferritin); however, the proposed work focuses on phytoferritins due to low cost and widespread availability of plant raw materials. Phytoferritins have been shown to be highly bioavailable (Beard et al., 1996 and Murray-Kolb et al., 2003), hence can be used to boost plasma iron during deficiency. For example, soybean meal ferritin has been shown to be as efficient as ferrous sulfate in restoring haemoglobin levels (Beard et al., 1996) while in vitro tests confirmed full release of iron following complete dissolution of kidney bean ferritin at gastric pH of 2.0 and 37^{o}C (Hoopler et al., 2008). And even when complete dissolution did not occur, evidence suggests that digestion-resistant ferritin can be absorbed intact from the gastrointestinal tract through the mucosal cells (Lönnerdal et al., 2006). In vitro work with Caco-2 intestinal epithelial cells also confirmed that soybean ferritin can pass through the apical cell membrane in intact form by means of endocytosis (San Martin et al., 2008). Due to the abundance of plant resources, plant ferritin (phytoferritin) could serve as a source of bioavailable iron for ameliorating iron-

deficiency symptoms in humans. Another advantage of phytoferritins is their larger shell cavity, which can store 1.2-1.4 times as much iron as mammalian ferritins (Crichton et al., 1978). Phytoferritins are oligomers with molecular weights of up to 500 kDa while individual polypeptide subunit weights can be >20 kDa (Crichton et al., 1978). For example, soybean and pea seed ferritins are each composed of two subunits of 26.5 and 28.0 kDa, which are referred to as H-1 and H-2, respectively (Laulhere et al., 1989 and Masuda et al., 2001). Phytoferritins are usually assembled from 12 or 24 polypeptides that are folded into four helix bundles (Theil, 2004). In general legumes have higher levels of iron and ferritin than cereal grains (May et al., 1980, Ambe et al., 1987 and Ragland & Theil, 1993), hence the focus of the proposed work will be Manitoba-grown legume seeds. The high ferritin levels in legumes have been attributed in part to the large iron requirement during nitrogen fixation by nodules, from where ferritin is recycled to the seed during nodule senescence (Theil, 2004). Ferritin concentrations can vary between 8-80 µg/g of seed, with pea, mungbean and soybean reportedly among the highest (Lukac et al., 2009). While consumption of ferritin-enriched plant products have been suggested, the presence of iron-binding compounds such as phytates may reduce bioavailability during oral consumption.

Thus ferritin nutraceuticals can be consumed along with regular foods but without the reduction in bioavailability associated with various food components. Therefore, application of protein extraction and fractionation methods that eliminates phytates and concentrates phytoferritin within a protein-enriched product could provide a novel approach to the use of ferritin as a nutraceutical agent against iron-deficiency.

1.1 OBJECTIVES OF STUDY

1.1.1 STUDY RATIONALE

Currently, information on extractability (including yield), structural (including resistance to gastrointestinal proteases) and functional (iron release) potential of ferritin from Manitoba-grown legumes is not available. Due to the low ferritin content in seeds, production of a purified product is not deemed commercially feasible. Therefore, the isolation protocol was focused on production of a crude extract with >50% ferritin content; this approach is to enhance technology transfer and eventual commercialization.

Plant ferritins (Phytoferritins) have been demonstrated to be highly bioavailable (Murray-Kolb et al., 2003) and can be effectively used to boost plasma iron during deficiency in humans. Even though animal sources contain higher ferritin levels, the proposed work will focus on plant ferritins (phytoferritin) because it is less expensive and plant raw materials are readily available. For example, Alberta Agriculture and Rural Development reported two consecutive yearly increases in the Canadian certified seed production of grass and legume seed species. Manitoba and Alberta are known to be the heartland of certified grass and legume seed production, with both provinces growing over 50,000 acres in 2013 (Wong, 2013).

The overall goal is to produce a ferritin concentrate that can be used as an ingredient to formulate nutraceutical products (pills or tablets) for IDA treatment. Based on the reported purified ferritin contents (up to 90 µg/g seed) of legumes, crude ferritin concentrate yields ranging from 0.5 to 5 mg/g of seed could be achieved in the proposed work.

Therefore, the specific objectives of the proposed work are as follows:

- Determine the yield and purity of ferritin concentrates isolated from legume seeds such as red lentils, yellow split pea, green lentils, mungbean, soybean, chickpea, lima beans and black beans
- Determine susceptibility of the protein cage-iron complex structure in ferritin concentrate to gastrointestinal proteases through simulated stomach and intestinal digestion treatments.
- Determine time-based kinetics of iron release from isolated ferritin concentrates during simulated gastrointestinal digestion.

1.2 HYPOTHESES

- Aqueous-based extraction and fractionation protocols can be used to produce ferritin concentrates from various legume seeds.
- The protein cage will be susceptible to proteolysis, which will lead to a release of its iron load when ferritin concentrate is treated with gastrointestinal proteases.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 IRON

Iron is an essential element, required for plant productivity, human and bacterial nutritional and metabolic functions. It is required in all body tissues critical for cellular respiration, oxidation and reduction enzyme systems as well as muscular functions and the brain and red blood cells. Several reports have also indicated that iron is involved in cellular processes of higher plants, most notably, for photosynthesis and respiration, DNA synthesis and metabolism and is, therefore, a limiting factor for plant productivity and biomass production (Blain et al., 2007; Cassar et al., 2007; Ravet et al., 2009). In legumes, iron is of particular interest since it is a required element in most cellular redox reactions and electron transfer chains. It also plays an important role in nitrogen fixation within the nodules, where iron-proteins such as leghaemoglobin and nitrogenase are essential.

2.1.1 Sources of Iron

Iron exists in two forms: the heme and the non heme. Heme iron is the form found in animal tissue food sources and consists primarily of haemoglobin and myoglobin. Even though heme iron represents only about 10-15% of dietary iron, it is highly bioavailable and easily absorbed (Benito and Miller 1998). The non-heme iron is the form of iron found in plants and all non-meat based foods and (vegetables, grains, iron-fortified breakfast cereal, lentils and beans) so is the type found in all plant ferritins. According to Ambe (1994), legume seed iron occurs mainly as ferritin, with the hull containing a significant amount. For centuries now, the non-heme forms of iron have accounted for 85–90% of the iron content in a typical Western diet (Hallberg, 1981).

However, this form of iron has a very poor bioavailability, with a gastrointestinal tract absorption rate typically between 2-20%, unless supplemented or boosted (Roughead et al., 2002; Baech et al., 2003).

Table 1: Select Dietary Iron Sources and Average Content (mg)

Food	Serving size	Iron (mg)
Liver, cooked	75 g (2 ½ oz)	6.9-13.4
Peas (chickpeas/garbanzo, black-eyed, split), cooked	175 mL (¾ cup)	1.9-3.5
Soybeans, mature, cooked	175 mL (¾ cup)	6.5
Lima beans, cooked	125 mL (½ cup)	2.2
Kale, cooked	125 mL (½ cup)	1.3
Green peas, cooked	125 mL (½ cup)	1.3
Oatmeal, instant , cooked	175 mL (¾ cup)	4.5-6.6
Oat bran cereal, cooked	175 mL (¾ cup)	2.0
Cereal, dry, all types	30 g	4.0-4.3
Tempeh/fermented soy product, cooked	150 g (3/4 cup)	3.2
Lentils, cooked	175 mL (¾ cup)	4.1-4.9
Beans, cooked (white, kidney, navy, pinto, black, roman/cranberry)	175 mL (¾ cup)	2.6-4.9

Pumpkin or squash seeds, roasted	60 mL (¼ cup)	1.4-4.7

Culled from "Canadian Nutrient File 2010"

www.hc-sc.gc.ca/fn-an/nutrition/fiche-nutri-data/index-eng.php

2.1.3 Iron Requirement

Table 2: Standard Daily Iron Requirement in Human Populations by Age and Gender

Age (Years)	Iron (mg)	
	Male	Female
0.5 - 1	11	11
1-3	7	7
4-8	10	10
9-13	8	8
14-18	11	15/27*
19-30	8	18/27*
31-50	8	18/27*
51-70	8	8
70+	8	8

* Requirement for pregnant women

Adapted from Food and Nutrition Board, Institute of Medicine, 2001

The human body generally conserves iron and retains the required level within the system through iron recirculation after the breakdown of red blood cells and by the absence of an iron-excretion process other than through blood loss. The body also limits the level of absorption to below 2 mg daily to avoid reaching toxicity levels (Hentze et al., 2010).

2.1.4 Iron Deficiency

In plants, iron is required in the biosynthesis of chlorophyll, therefore a deficiency will result in chlorosis. Nutritional constraints of iron will therefore, affect plant growth and also modify the accumulation and the partitioning of iron and potassium between young and old leaves (Mahmoudi et al., 2005 and Nikolic and Kastori, 2000).

In humans, certain life stages, such as: active growth, menstruation, pregnancy and lactation, aging and high activity levels, will generally increase iron requirement while chronic illnesses and diet can significantly affect absorption. Approx. 65 to 75 percent of the body's iron is in the blood in the form of haemoglobin and if iron is lacking in the diet, the iron reserves in the body, are used (Anderson and Fitzgerald, 2010). Once this supply (iron reserves) is depleted, the formation of haemoglobin is affected. This condition is termed anemia, which according to the World Health Organization (2007), is the most common and widespread nutritional nutrient deficiency in the world, affecting more than 2 billion people (Theil, 2004).

Camaschella (2015) described the common causes of iron deficiency with some examples as shown in the table below:

Table 1. Causes of Iron Deficiency.

Cause	Example
Physiologic	
Increased demand	Infancy, rapid growth (adolescence), menstrual blood loss, pregnancy (second and third trimesters), blood donation
Environmental	Insufficient intake, resulting from poverty, malnutrition, diet (e.g., vegetarian, vegan, iron-poor)
Pathologic	
Decreased absorption	Gastrectomy, duodenal bypass, bariatric surgery, *Helicobacter pylori* infection, celiac sprue, atrophic gastritis, inflammatory bowel diseases (e.g., ulcerative colitis, Crohn's disease)*
Chronic blood loss	Gastrointestinal tract, including esophagitis, erosive gastritis, peptic ulcer, diverticulitis, benign tumors, intestinal cancer, inflammatory bowel diseases, angiodysplasia, hemorrhoids, hookworm infestation, obscure source Genitourinary system, including heavy menses, menorrhagia, intravascular hemolysis (e.g., paroxysmal nocturnal hemoglobinuria, autoimmune hemolytic anemia with cold antibodies, march hemoglobinuria, damaged heart valves, microangiopathic hemolysis) Systemic bleeding, including hemorrhagic telangiectasia, chronic schistosomiasis, Munchausen's syndrome (e.g, self-induced hemorrhages)
Drug-related	Glucocorticoids, salicylates, NSAIDs, proton-pump inhibitors
Genetic	Iron-refractory iron-deficiency anemia
Iron-restricted erythropoietic	Treatment with erythropoiesis-stimulating agents, anemia of chronic disease, chronic kidney disease*

* Inflammatory conditions may be associated with iron deficiency. NSAIDs denotes nonsteroidal antiinflammatory drugs.

Culled from Camaschella (2015)

2.1.5 Iron toxicity

Excess iron accumulation in plant and animal cells will result in iron toxicity. In plants it causes oxidative stress, which is characterised by instant brown spotting of leaves and a significant effect on metabolism (Kampfenkel et al., 1995). Connolly & Guerinot (2002) reported that iron can act catalytically through the Fenton reaction to generate hydroxyl radicals, which can damage lipids, proteins and DNA. Plants, therefore respond to iron stress in terms of both iron deficiency and iron overload.

In humans, excessive iron deposition into body tissues will cause an iron overload resulting in iron toxicity. Patients with the recessive genetic disorder hemochromatosis are at a higher risk for increased iron absorption and type 2 diabetes (Anderson and Fitzgerald, 2010). Hereditary hemochromatosis is a condition where an individual accumulates an excessive amount of iron, resulting in an overload. It results from a mutation in the human hemochromatosis protein, HFE, which encodes for the HFE protein. It has been associated with liver disease, and is the predominant genetic iron overload disorder diagnosed in Caucasians (Vujic, 2014; Gozzelino and Corradini, 2015**).** If left untreated, the excess iron will build up in tissues and organs, causing damage and may increase the risk for certain cancers, heart diseases and cirrhosis, eventually leading to death. Once iron is absorbed it can only be excreted through blood loss.

2.1.2 Factor Influencing Iron Absorption

Certain dietary factors have been identified as being either inhibitory or enhancing the availability and subsequent usability of iron in diet. Hurrell and Egli (2010) reported that other factors relating to the condition of the individual can positively or negatively influence the effects of these inhibitors and enhancers on iron absorption. These factors include malnutrition or balanced diet, prevailing iron levels (lowered iron levels will facilitate higher absorption), healthy or disease state, as well as obesity.

Inhibitors: The most important inhibitor of iron bioavailability is phytate, which is present in most plant-based diets, including legumes, and is reported to be active even in very small quantities but can be denatured by heat treatment and processing (Hurrell and Egli, 2010). Polyphenols are also found in vegetables, fruits and legumes and are inhibitory to dietary iron absorption. Petry et al. (2013) demonstrated that polyphenol inhibitory action is further enhanced

when acting in synergy with phytates. Calcium, milk and egg proteins, as well as albumin have also been implicated as having inhibitory effects on the absorption of iron in varying degrees within the digestive system (Hurrell et al., 1988; Hurrell et al., 1989; Hurrell et al., 2003; Brazaca and da Silva, 2003). *Mechanism of action of the inhibitory substances are as follows*: During digestion, the polyphenols and phytates in the food or beverage are released into the intestinal lumen and combine with the iron, making it unavailable for absorption, and significantly decreasing the basolateral iron export in cells. The higher the concentration of the polyphenols, the higher the level of iron absorption inhibition reported (Mascitelli and Goldstein, 2011).

Enhancers: Vitamin C (ascorbic acid) has been identified as being definitely beneficial in increasing the bioavailability and absorption of iron from diets (Brazaca and da Silva, 2003). Findings have reported that the inclusion of vitamin C in meal actually reversed or reduced the inhibitory effects of phytate, polyphenols and egg and milk proteins had on iron absorption, especially where the inhibitory substances were present in smaller quantities (Hallberg and Rossander, 1982; Ballot et al., 1987; Siegenberg et al., 1991; Hurrell and Egli, 2010).

Mechanism of action of iron absorption enhancer: Vitamin C, in the stomach mixes with the non-heme iron in the food or beverage and enhances iron absorbance in a two-fold process. First, it prevents the formation of insoluble and non-absorbable iron compounds. Secondly, it reduces the non-heme ferric to ferrous iron. This is said to facilitate the transportation from the stomach to the small intestine where the ferrous iron is easily taken up and absorbed into the mucosal cells (Hallberg et al., 1989).

2.2 IRON PROTEIN (FERRITIN)

Theil (2004) described ferritin as “a novel, alternative dietary iron source” and emphasized the key role it will most likely play in the control and treatment of IDA worldwide. Ferritin is a store for iron protein and a mineralization vehicle (Chasteen and Harrison, 1999). Iron is retained inside the protein cage in a reversible hydrated iron oxide form (Harrison and Arosio, 1996) in animals, plants, and prokaryotes from where the iron release and control is maintained by ferritins (which are the storage proteins).

The dangerous effect of an imbalance in iron levels in both plants and animals previously described, suggests that cellular iron concentrations must be finely regulated to avoid cellular damage since maintaining correct labile iron levels is a critical to preserving iron homeostasis. It also emphasizes the fact that a delicate iron-balance needs to be maintained and highlights the vital regulatory and buffering roles of iron storage.

Ferritins are important iron storage and detoxification proteins that are widely distributed in the living kingdoms. To further establish the necessity for ferritins, Ravet et al. (2009) grew a ferritin-less mutant under elevated iron conditions and recorded major developmental defects associated with iron homeostasis perturbations (disruptions) and oxidative stress. The research findings showed that the absence/loss of ferritins in vegetative and reproductive organs resulted in oversensitivity to excess iron where the ferritin-less plants showed significantly reduced growth as well as defective flower development. They also reported that, the absence of ferritin led to an over-accumulation of iron within the reproductive organs leading to decreased fertility. They concluded that ferritin plays a significant role in the defence machinery against oxidative stress in plants as one of the primary functions although it is not an essential iron source for plant development.

2.2.1 Functional Properties of Ferritin

Ferritin, a ferric iron (Fe)-containing protein, primarily serves the physiological function of storing iron (Harrison, 1986) and it can store up to 4500 Fe atoms as an inorganic complex in a non-toxic and biologically available form**.** This enables ferritin to express two essential roles: a) to be a reserve of the essential elemental iron, from where it can be drawn when required for molecular synthesis of haemoglobin, cytochromes and iron-sulphur compounds and b) to essentially be a regulator responsible for maintaining the delicate balance of iron concentration within cells, thereby protecting them from iron toxicity. This brings to fore, the most important function of ferritin, which is that, by retaining this fine balances at the cellular and systemic levels, it maintains iron concentration in a narrow range. This is vital, especially because, while other dietary metals can be regulated by excretion in the feces and urine, humans do not possess the capacity to remove excess iron from the body (Anderson and McLaren, 2012).

Also, iron stored within ferritin is relatively inert, which protects from oxidative stress. Free iron is potentially toxic, largely due to its ability to participate in redox reactions that catalyse the activation of dioxygen leading to the generation of more reactive oxy radicals, reactive oxygen species (ROS), creating a disease condition known as oxidative stress (Galaris and Pantopolus, 2008; Galatro et al., 2012). New experimental evidence reported by Ravet et al. (2009) stated that the role of ferritin in the defense machinery against oxidative stress included acting as a transient buffer for Fe and not just as an inert storage molecule.

Furthermore, Galaris and Pantopolus (2008) described that loosely bound redox-active iron and hydrogen peroxide may interact with heme-iron in the active site of heme-containing proteins. This will lead to the predomination of two-electron oxidation of heme, causing the formation of

ferryl-heme forms and an unstable free radical localized in different positions depending on the structure of the particular protein.

2.2.2 Bioavailability of Ferritin

Ferritin protein has been described as being relatively resistant to proteolytic digestion (Crichton, 1969), suggesting that plant ferritin is protected from digestion in the stomach and arrives in the intestine intact. It is also highly stable to temperature, heat, and protein denaturants in solution (Theil 1987; Harrison & Arosio, 1996; Lönnerdal et al, 2006). This is further supported by the location of ferritin iron in a solid mineral, which is within the stable protein inside of seed cell plastids and is concentrated in seed hulls (Ambe, 1994). Ferritin stores up about 4500 iron atoms as a hydrate of ferric oxide in the form of minute crystals (Macara and Harrison, 1972). To be absorbed, the microcrystalline particles have to be eventually converted to ferric ions at low pH values and made available during digestion for absorption and use.

The process of absorption begins with the apoferritin (protein complex only), which takes up iron based on its level of saturation. The uptake results in the formation of the microcrystals which forms within the protein cage. Macara and Harrison (1972) described the process in two steps. The first stage is the slow "nucleation stage" which has little or no ferric ion. This is followed by the "rapid growth" stage, characterised by an increase in ferric ion and oxidative activities.

Isotope studies on the basis of data generated from ferritin iron uptake have indicated iron bioavailability of below 20% in mixed diets. Bioavailability is much lower in all vegetarian diets, being between 5 and 15% (Hurrell and Egli, 2010).

2.3 STRUCTURE OF FERRITIN

Theil and Briat (2004) described the ferritin structure as a unique one among proteins and attributed the slow release of iron in ferritin to its being in the form of a solid mineral containing thousands of iron atoms concentrated inside a single ferritin iron-protein complex. The structure of ferritin itself is thought to control the flow of iron from the mineral (Liu et al., 2002).

Theil (1987) reported that although all ferritins share structural properties, cell-specific variations in structure, function, and amount indicate the presence of cell specific features of genetic regulation. Ferritin occurs as a highly conserved structure, composed of 24 subunits assembled into a spherical shell (Galatro et al., 2012). Certain reports have suggested that only one type of polypeptide chain was present in the multimer (Lescure et al., 1991), but Masuda et al. (2001), reported the presence of two subunits (with cooperative functional roles), having molecular weights of 26.5 and 28 kDa and designated as H-1 and H-2, respectively.

The occurrence of a unique N-terminal sequence in plant ferritin, which contains an extension peptide (EP) located on the outer surface of the protein has also been reported to play a part in imparting special oxidative activities to the protein (Ragland et al. 1990; Lobr´eaux et al., 1992; Zhao, 2010).

Furthermore, Galaris and Pantopolus (2008) described that loosely bound redox-active iron and hydrogen peroxide may interact with heme-iron in the active site of heme-containing proteins. This will lead to the predomination of two-electron oxidation of heme, causing the formation of ferryl-heme forms and an unstable free radical localized in different positions depending on the structure of the particular protein.

The functional roles and mechanism of iron storage in ferritin have both been associated with its crystal structure. This three-dimensional structure of human H-type ferritin (Figures 1 and 2 below) was first described by Lawson et al. (1991). Since then, however, various structures of ferritin from different species have been investigated and their iron storage mechanisms have been proposed.

More recently, Masuda et al. (2010) detailed the three-dimensional structural model and novel metal binding site of plant ferritin from soybean. Their research procedure crystallized a recombinant plant ferritin subunit from soybean (SFER4). The high resolution x-ray crystallographic analysis of SFER4 revealed a novel metal binding site between the 3-fold symmetry channel and ferroxidase center. When they deleted this site, there was a delay of the iron oxidation/nucleation process, which led them to conclude that this site functions as a transit point for metal translocation in ferritin. The results obtained considered along with the extremely high conservation of this site, suggest that it is a part of the metal ion pathway widely distributed in ferritin from various species.

Their findings further emphasized that the overall structure of plant ferritin, SFER4, was highly conserved among known structures of ferritins with the unique feature of plant ferritin as its 4-fold symmetry channel.

The occurrence of a unique N-terminal sequence in plant ferritin, which contains an extension peptide (EP) located on the outer surface of the protein might impart special oxidative activities to the protein (Ragland et al., 1990; Lobr´eaux et al., 1992; Zhao, 2010).

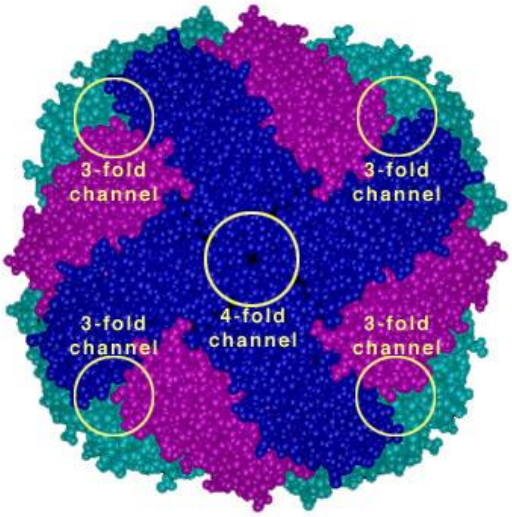

Figure 1: Molecular model of Ferritin with the subunits displayed in the CPK representation. Culled from Casiday and Frey, 2000.

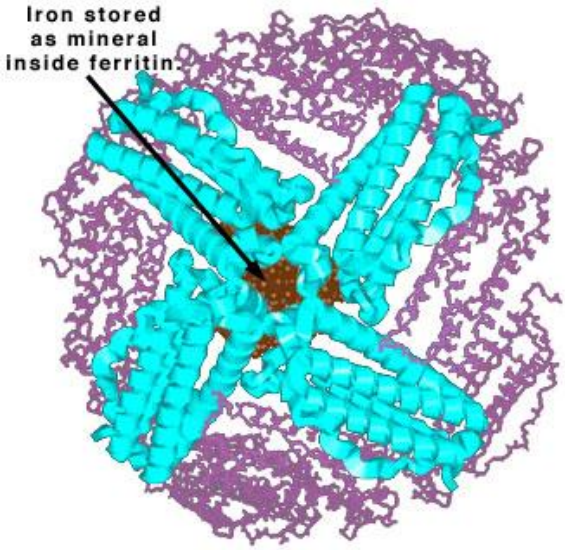

Figure 2: A three-dimensional representation showing ferritin, the iron-storage protein in the human body. Culled from Casiday and Frey, 2000.

2.4 PHYTOFERRITIN

Studies on the functions and regulations of plant ferritin (phytoferritin) and their synthesis strongly indicate that these proteins are responsible for the protection of plants against oxidative stress. Ferritins help plants to cope with adverse situations which could potentially have damaging effects, by buffering iron and exerting a "fine tuning" effect on the quantity of iron metal required for metabolic processes. In seeds, ferritins have been determined to be the major iron-storage form that releases and provides iron to iron-containing proteins after germination in order to exert the maximum benefits effect of iron on plant biomass and seed production (Ravet, 2009; Briat et al., 2010).

2.4.1 Phytoferritin Isolation and Characterization

Phytoferittin was first isolated from pea embryo (*Pisum sativum* L. var. Alaska) (Hyde et al., 1963). The authors developed a procedure for freeing the phytoferritin particles from ribosomes and other cellular components. Prior to the 1963 report, the iron-protein complex, ferritin had only been isolated from animal tissues.

The initial isolation and purification procedures reported in the 1963 report are still being used today, though with modifications. Briefly, the isolation process begins with grinding the pea in distilled water for 1 min at room temperature using a Waring blender and passing the paste through two or three layers of a filtration material (Miracloth) in a basket centrifuge. The filtrate was then centrifuged at 15,000 rpm for 15 min, the pellets were discarded and the supernatant was made to 0.05 M $MgCl_2$ to precipitate ribosomes and phytoferritin, thereby concentrating the phytoferritin. The resultant yellowish brown precipitate was sedimented at 15,000 rpm for 15 min, the supernatant discarded, and the sedimented lipid material was freeze dried. The pellets

were then reconstituted in 10-20 times their volume of distilled water, homogenized, and the denatured ribosome fraction pelleted by centrifugation at 15,000 rpm for 10 min. The result was a slightly turbid yellowish supernatant.

The solution was dialyzed by the addition of bovine pancreatic ribonuclease (5 µg/ml) for 12 hr against 0.01 M phosphate buffer (pH 7.3) to facilitate ribonucleic acid removal. This process was verified by measuring optical density from 200 to 400 nm. Where any turbidity is observed, the sample was subjected to further centrifugation at 15,000 rpm for 10 min. The phytoferritin was purified on a DEAE-cellulose column at 4°C by passing the solution (dissolved in 0.01 M phosphate buffer pH 7.3) from the top of the column where colored proteins formed a broad band. The column was washed stepwise with 0.05 M NaC1 in 0.01 M phosphate buffer, pH 7.3, followed by 0.1 M NaC1 and 0.15 M NaC1 in the same buffer. By the end of the third wash the yellow band had begun to spread down the column. At this point the main phytoferritin fraction studied was eluted with 0.25 M NaCl in the same phosphate buffer. Further fractions containing phytoferritin were obtained by increasing the NaC1 concentration or by adjusting the buffer to pH 8. The eluate was concentrated by spinning for 45 min at 49,000 rpm in the 50 rotor of a Spinco model L ultracentrifuge (Hyde et al., 1963).

These isolation and characterization procedures have been applied and modified over the years resulting in the isolation of phytoferritin in many different plants, some of which include: pea, *Pisum sativum*) and lentil, *Lens esculenta* (Crichton et al., 1978) and in the fungus *Phycomyces blakesleeanus* (Sassen, 1965; Peat and Banbury, 1968; David and Easterbrook, 1971), the leaves of *Phaseolus vulgaris* (Van Der Mark and Van Den Briel, 1985), maize, peas and soya bean seeds (Laulhere et al., 1988), clover seeds, *Trifoliurn subterraneurn* L. cv. Clare (Barcelo et al., 1995), yellow lupine, *Lupinus luteus* var. Ventus (Strozycki, et al., 2003), broad bean *Vicia faba*,

(Yun et al., 2012) and soybean seeds *Glycine max*, var DM 4800 (Galatro et al., 2012). A ferritin-like molecule was also isolated from yeast, *Saccharomyces cerevisiae* D261 (Raguzzi et al., 1988).

2.5 IRON DEFICIENCY ANAEMIA (IDA)

Iron deficiency occurs due to a depletion of iron stores and if left to progress untreated, leads to IDA, which is characterized by a significant decrease in haemoglobin production levels, deformation of red blood cells and impairment of oxygen delivery to tissues. IDA is often multi-factored and manifests in different ways (Glazer and Bilenko, 2010).

IDA is a chronic condition that can sometimes be asymptomatic, but will have impact on the overall quality of health. Physical presentation may include fatigue, pallor, irritation and nausea; and physiologically, has been associated with significant reduction in cognitive functions as well as impaired mental and muscular development in active-growing stages of life.

Severe IDA during pregnancy has been associated with preterm birth, high placental: birth weight ratio, which leads to post-natal complications including neonatal and maternal death and low birth weight (Crowe et al., 1995; Anker et al., 2009). Camaschella (2015) also indicated that IDA may predispose to opportunistic infections and heart failure.

IDA is a global health problem, estimated to be affecting between 2 and 3 billion of the world population (McLean et al., 2009); and the advances in healthcare worldwide have not been able to effectively address the issue (Beck et al., 2014). Kassebaum et al. (2014) reported on the economic burden of the disease over a 20-yr period and concluded that children below the age of 5 yr have the highest prevalence and suffer the greatest long-term effects of IDA. The public

health impact is significant in both industrialized and non-industrialized countries, however, it is more severe in the under developed countries as a result of prevailing factors, including poverty and malnutrition, infections that cause blood loss (such as hookworm and urinary schistosomiasis) and diets which are low in iron content and low availability (Yip and Ramakrishnan, 2002). In developed countries, IDA is often a result of dietary restrictions or choices like vegan and vegetarian meals and abstinence from red meat or the iron-bioavailability problems frequently associated with such diets. IDA can also be due to chronic blood loss resulting from disease conditions like hemolysis or from heavy menstrual flow in women (Heidelbaugh, 2013; Camaschella, 2015).

2.6 TREATMENT OF IDA

Currently, oral application of iron is the preferred method of treatment for IDA in otherwise stable patients; it is affordable and easy to obtain and it is termed an effective treatment (Camaschella, 2015). Various inorganic iron salts are being administered in relatively high doses, taking into account the very low bioavailability and poor absorption. There are reports of serious undesirable side effects using this treatment process (Hyder et al., 2002; Dewey et al., 2002). The severity of side effects has been reported to interfere with treatment and some patients have been reported to discontinue treatment as a result of negative reactions and discomfort. Dietary fortifications are also employed particularly in cereal products because of the rate of consumption in the developed world. Legumes and cereals are relied on to provide most of the dietary energy and protein sources in the industrialized world (Bothwell et al., 1979).

Waldvogel et al. (2012) in a randomized controlled clinical trial involving 154 women (below 50 yr of age) who did not have anaemia, assigned them to an oral treatment of ferrous sulfate or

placebo for one month. They measured before and after the intervention parameters such as level of fatigue, aerobic capacity, mood disorder, quality of life, compliance and adverse events using their analysis of haemoglobin and ferritin as the biomarkers. They concluded that women who were not anaemic did not benefit from the iron supplementation.

Lönnerdal (2009) in another clinical trial, assessed iron absorption from a purified plant protein using healthy, non-anemic women. The work involved administration of iron-free plant protein or $FeSO_4$ followed by iron measurement in red blood cells before and after treatment. They concluded that the there was no significant difference in the regulation and absorption of iron between the groups, and that phytoferritins can serve as a valuable treatment in populations suffering from IDA, since they are well absorbed.

González-Rosendo et al. (2010), conducted a randomized clinical trials involving 35 adolescent girls, who were treated with biscuits fortified with placebo, iron sulfate or heme-iron concentrate developed from haemoglobin of swine. Before and after analyses and results of blood chemistry and hematology suggested that the heme iron was better absorbed (3:4) than the iron sulfate, confirming previous studies on the better uptake of heme iron. They concluded that the iron in the haemoglobin concentrates were well absorbed and tolerated by the study group.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Fourteen different legume seeds were purchased from a grocery store in Winnipeg: Black Beans (BB), Moon Dal Washed (MDW), Red Lentils (RL), Mung Beans (MB), Chick Peas (CP), Green Lentils Whole (GLW), Lima Beans (LB), Soy Beans (SB), Pinto Beans (PB), Black Eyed Beans (BEB), Green Split Beans (GSB), Small White Beans (SWB), Red Kidney Beans (RKB) and Yellow Split Beans (YSB). The use of whole seeds was adopted over isolated protein fractions because it has been suggested that seed hulls are rich in ferritin (Ambe, 1994).

However, a Pea Protein Isolate (PPI) (hull removed) that is commercially produced in Portage la Prairie, Manitoba was also used to determine feasibility of direct use of protein-enriched products.

3.2 OPTIMIZATION OF FERRITIN EXTRACTION FROM LEGUME SEEDS AND PPI

Preliminary research was carried out to optimize the isolation and characterization process of ferritin excitation. This was done by a modification of previously reported protocols with the aim of determining the conditions that give the highest yield of ferritin concentrate from our specific legume samples. Ferritin optimization followed the previously described methods (Lukac et al., 2009; Deng et al., 2010; Li et al., 2009; Punjal et al., 2007). Each condition of isolation was optimized by following the established protocol with variations, but only in the condition being optimized per time. The dry samples were soaked for 12, 20 and 48 hours

respectively, while maintaining all other isolation conditions. The same process was repeated for the soaking temperature of the dry seeds, as well as the temperature at which the slurry was heated after homogenization. The samples were soaked at refrigeration temperature (4^{o}C) and at room temperature (25^{o}C) and then slurry was heated at 55^{o}C for 5, 10 and 15 minutes respectively. All the samples were processed as described in figure 3, until the ferritin concentrate was collected and then the yield was determined for each process.

On the basis of the process which gave the highest yield, we determined the optimized protocol used in this research as follows: Seeds were first soaked in distilled water for 20 hr at 4^{o}C. This was followed by homogenization with 4 volumes of cold (4^{o}C) extraction buffer (10 mM sodium phosphate, 100 mM sodium chloride, 2% polyvinylpyrolidone, pH 7.2) until a smooth homogenate was obtained. For PPI, the protein flour was directly mixed with 4 volumes of cold (4^{o}C) extraction buffer and then homogenized. Each homogenate was heated at 55^{o}C for 5 minutes and then centrifuged (5000xg) for 5 min to remove insoluble materials as a precipitate. The supernatant (contains the solubilized ferritin) was adjusted to 50 mM $MgCl_2$ and stored at 4^{o}C for 30 min after which sodium citrate was added to a final concentration of 70 mM. The added citrate formed a complex with magnesium and prevented the precipitation of ferritin. After citrate has been added, the solution was then stored for 4 hr at 4^{o}C (to promote ferritin aggregation) followed by centrifugation at 12000xg for 30 min. The brown pellet obtained was freeze-dried as the ferritin concentrate. Gross yield of the ferritin concentrate was determined as percentage ratio of the weight of freeze-dried product to dry weight of starting material (seeds or PPI). Initial purity of the ferritin concentrates was compared by determining the iron (420 nm) to protein (280 nm) absorbance ratio of a known ferritin concentration. Higher ratios reflected higher purity of the ferritin-iron preparation.

A flow chart describing the original and the optimized isolation processes are shown in Figures 3a and 3b below:

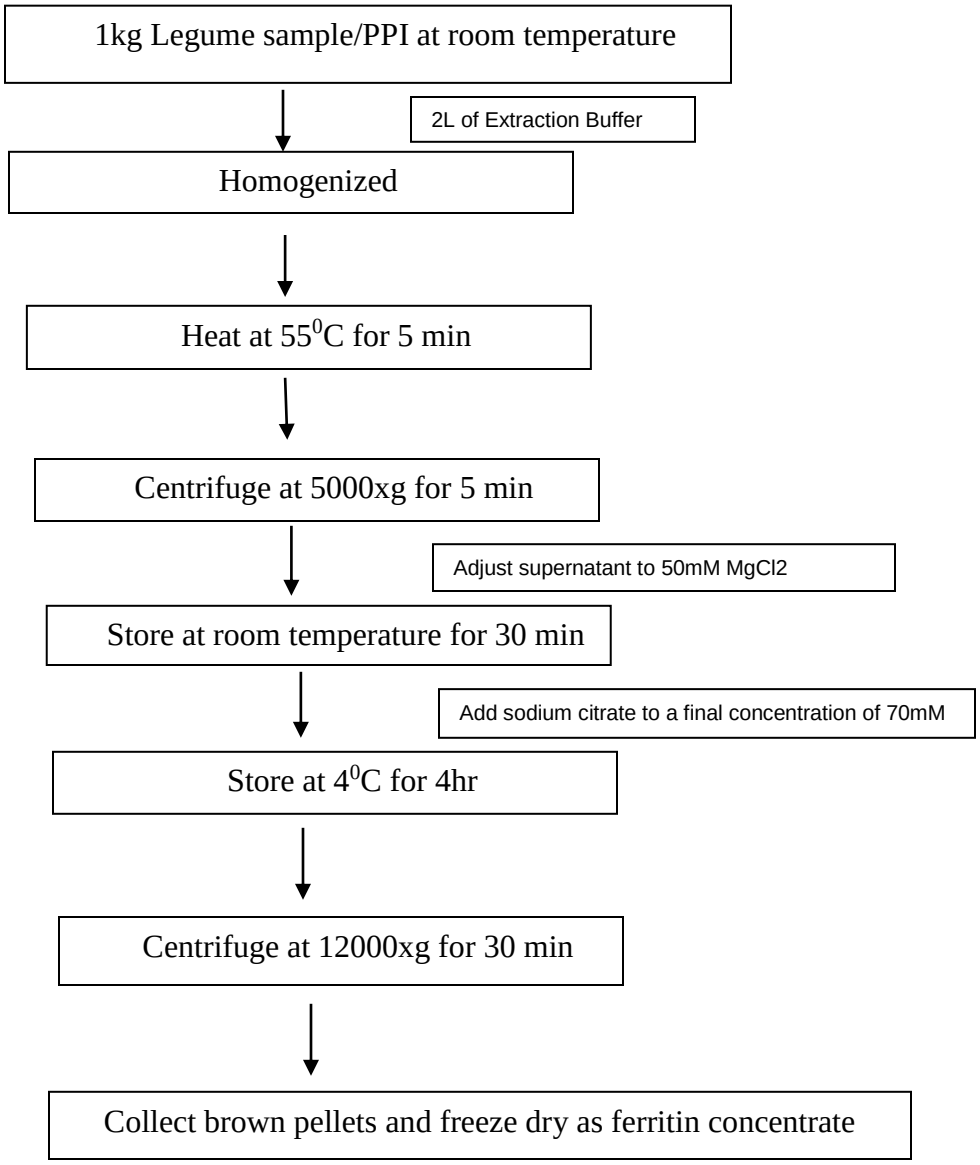

Figure 3a: Ferritin isolation protocol (Li et al. 2009)

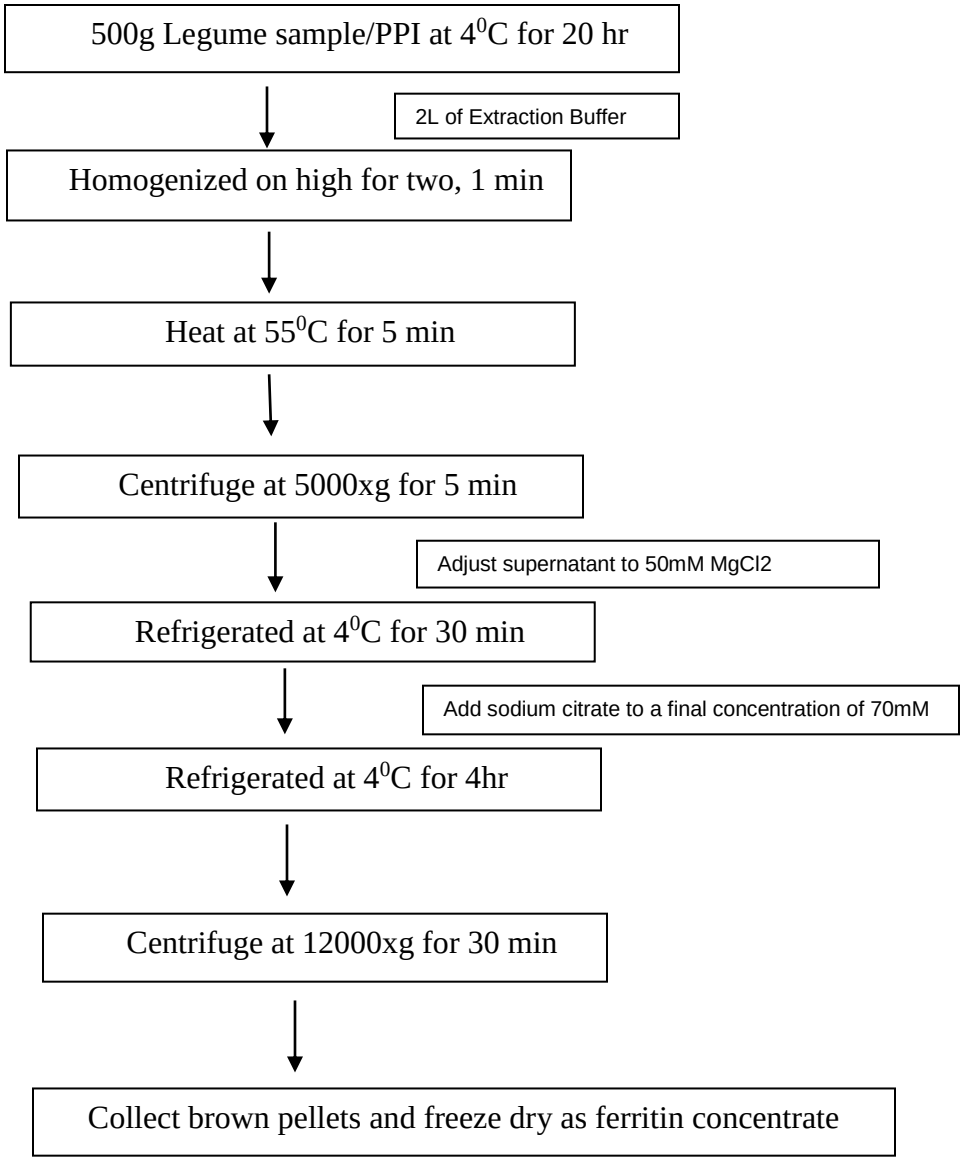

Figure 3B: Ferritin isolation protocol (Li et al. 2009 modified)

3.3 DIGESTION OF FERRITIN ISOLATE WITH EXOPEPTIDASE

Five of the 14 ferritin concentrates obtained were selected for hydrolysis on the basis of their higher iron:protein ratio values (YSB, GLW, CP, MDW and RL). Each of the selected concentrate was hydrolysed by digestion using two exopeptidases: Accelerzyme CPG and Peptidase-R. The aim was to remove outmost segments of the protein cage structure without cage collapse but causing increased iron:protein ratio.

Accelerzyme CPG was obtained from DSM Food Specialties (Parsippany, NJ), while Peptidase-R was obtained from Amano Enzyme U.S.A. Co., Ltd. Both enzymes were stored in the refrigerator (at 4^0C) until needed.

The protocol used for the hydrolysis of the concentrates is described below:

A) Digestion with Accelerzyme CPG:

1. A ferritin slurry was prepared at 5% (w/v) in distilled water and adjusted to pH 6.
2. The ferritin slurry was treated with 2% (w/w; i.e. 2% of the ferritin wt) accelerzyme.
3. Slurry was incubated at 40^{o}C for 4 hr.
4. The digested slurry was cooled to room temperature and passed through a 5 kDa ultrafiltration membrane until about half of the original volume remained.
5. The retentate was freeze-dried
6. The iron/protein absorbance ratio was determined.

The same process was repeated on a second batch of concentrates, following the same protocol but the incubation period was extended to 7 hr. The experiments were carried out in duplicate.

B) Digestion with Peptidase-R:

The same protocol was followed as with the Accelerzyme CPG but instead of digesting at 40^{o}C, digestion was done at 50^{o}C for 4 and 7 hr for the batches.

3.4 DETERMINATION OF PROTEIN CONTENT

The protein content of the legume samples was determined using the Lowry's method (Lowry et al. 1951) as modified by Markwell et al. (1978).

Each concentrate sample (10 mg/ml) was mixed thoroughly with bovine serum albumin (BSA) (10 mg/ml) which is the standard, using a magnetic stirrer. An aliquot (1 ml) of the samples and the standard were prepared in 20-100 μg concentration range. A volume of 3ml of reagent C was added to 1 ml each of the samples and 1 ml each of the BSA, allowed to stand and incubate for 1 h at room temperature. Reagent C consists of reagent A and reagent B. Reagent A consists of 2% Na_2Co_3, 0.4% NaOH, 0.16% Sodium tartrate and 1% Sodium dodecyl sulfate. Reagent B consists of 4% $CuSO_4 5H_2O$ dissolved in distilled water. After the 1 h incubation, 0.3 ml of Folin Ciocalteu phenol reagent (1 part Folin Ciocalteu reagent with 1 part distilled water) was added to the samples and thoroughly mixed using a vortex. The samples were incubated for 45 min at room temperature. Using a spectrophotometer, the absorbance was measured at 660 nm. Triplicate determinations were used to calculate the protein concentrations.

3.5 AMINO ACID COMPOSITION

Amino acid profiles of the ferritin concentrates from different legumes were analyzed in order to determine nutritional value and relationship of amino acid groups (hydrophobic, negatively charged, positively charged and aromatic) with iron-holding capacity. Amino acid compositions were determined using analytical HPLC after samples had been hydrolyzed with 6 M HCl (Bidingmeyer, 1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke et al., 1985) while tryptophan was determined after alkaline hydrolysis (Landry and Delhaye, 1992).

3.6 GEL ELECTROPHORESIS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was determined using the Phastgel instrument and 8-25% gradient gel according to methods already established (Mundi and Aluko, 2012). Reduced samples were prepared by adding 5% (v/v) 2-mercaptoethanol (ME) to the supernatant from the 10% SDS samples. Gels were scanned and densitometry analysis performed using ImageQuant TL software (GE HealthSciences, Montreal, PQ) to determine percentage ratio of 26 + 28 kDa bands (ferritin polypeptides) to other non-ferritin polypeptides. Higher ratios were indicative of increased protein purity of the ferritin preparation.

3.7 SIMULATED GASTROINTESTINAL PROTEIN DIGESTION

Susceptibility of ferritin to structural degradation by gastrointestinal proteases was determined using the method of Miller et al. (1981). Briefly, a mixture of ferritin and pepsin was adjusted to pH 2.0 with dilute HCl solution (simulated stomach digestion) followed by incubation at 37^{o}C for 2 hr. After incubation was complete, the mixture was adjusted to pH 7.0 with dilute NaOH solution and ethylene diamine tetra acetate (EDTA) added to a final concentration of 1 mM to prevent precipitation of solubilized iron at neutral pH. The mixture was then centrifuged (20000xg for 20 min at 4^{o}C) and the supernatant (A) saved. A separate digestion was also carried out with pepsin as previously described, adjusted to pH 7.0, brought up to 1 mM EDTA concentration, treated with pancreatin solution (simulated intestinal digestion) for another 2 hr, centrifuged and supernatant (B) saved. Supernatants A and B were applied to a gel permeation column (Superdex 200) to separate ferritin according to molecular size and determine effects of stomach and intestinal digestion, respectively on structural integrity of ferritin. Untreated ferritin preparation was also run on the gel permeation column and compared (using number and position of protein peaks) with supernatants A and B to determine changes in molecular structure. The Superdex 200 column was attached to a Fast Protein Liquid Chromatography system (GE HealthSciences, Montreal, PQ) and protein separations carried out using phosphate buffer (pH 7.0) as elution buffer according to manufacturer's instructions with protein elution monitored at 214 nm.

3.8 KINETICS OF IRON RELEASE FROM FERRITIN DURING SIMULATED GASTROINTESTINAL DIGESTION

Ferritin concentrate was subjected to pepsin digestion as described above but modified for kinetic studies as follows (Punjal, 2007). Acidity of the ferritin/pepsin mixture was lowered stepwise to mimic the transient changes associated with passage of food through the human stomach. Therefore, digestion was initiated by first lowering to pH 5.0, which simulated the higher pH associated with postprandial buffering effect of a meal in the stomach (Lukac et al., 2009). The acidity was further increased in 3 steps (through addition of 0.1 M HCl solution) to pH 3.5 after 15 min, pH 2.5 after 30 min and pH 2.0 after 60 min. The solution was left for an additional 60 min so that the experiment was terminated after 120 min. All the experiments were performed at 37^{o}C in a shaking water bath kept under argon gas and covered with aluminum foil to prevent iron oxidation. Aliquots of the incubated ferritin/pepsin mixture were taken at time 0, 15, 30, 60 and 120 min, adjusted to pH 7, 1 mM EDTA and a portion was centrifuged (20000xg for 20 min at 4^{o}C) and supernatant saved. The remaining portion was subjected to pancreatin digestion with aliquots taken at time 0, 15, 30, 60 and 120 min. The pancreatin-treated aliquots were also centrifuged (20000xg for 20 min at 4^{o}C) and supernatants saved. Supernatants from 0, 15, 30, 60 and 120 min for each of pepsin and pancreatin treatments were analyzed for ferritin molecular structure by gel permeation chromatography as described above. The supernatants were also analyzed for iron contents using the ferrozine complexation by determining changes in absorbance values of the ferrozine/iron complex at 562 nm in a spectrophotometer (Deng et al., 2010). Higher absorbance values reflect higher iron content in the ferritin sample. It is expected that ferritin protein degradation will increase with time during pepsin or pancreatin treatments and will be accompanied by increased release of iron molecules into the supernatants; therefore,

absorbance at 562 nm will be expected to increase from time zero to 120 min, though not necessarily in a linear manner. Based on previous work by Punjal et al. (2007), most of the iron is usually released during pepsin treatment; therefore, most of the changes in absorbance are expected to be associated with pepsin treatment but not pancreatin treatment. However, the pancreatin treatment is necessary to provide useful information on whether there is release of iron in the intestine in addition to the stomach release and whether these changes are dependent on seed source of the ferritin concentrate.

3.9 MEASUREMENTS OF CIRCULAR DICHROISM (CD) SPECTRA

CD spectra of samples were measured at 25 °C in a J-810 spectropolarimeter (JASCO) using the spectral range of 190 to 240 nm (far-UV) for secondary structure determinations and 250 to 320 nm (near-UV) for tertiary structure according to the method described by Omoni and Aluko (2006). Protein stock solution (10 mg/mL of sample was prepared in 0.1 M sodium phosphate buffer and adjusted to pH 7.0) was diluted to required concentration in 10 mM phosphate and the secondary structure determined using a cuvette with path length of 0.05 cm containing 2 mg/mL protein solution while the tertiary structure was measured in a 0.1 cm cuvette containing 4 mg/mL protein concentration. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of the buffer spectra.

3.10 STATISTICAL ANALYSIS

All the analyses were carried out in triplicates and the results were analysed as the mean values ± standard deviation (SD) after subjecting the averages to ANOVA and Pearson correlation analyses using Statistical Package for the Social Science (SPSS) 16.0. Differences at $p \leq 0.005$ were considered significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 SAMPLE COLLECTION

4.1.1 Gross yield of the ferritin concentrate

The Manitoba legumes used in this research gave their highest yield using the predetermined optimized isolation protocol. The difference in yield from the other conditions was significantly higher ($p \leq 0.05$). This could be as a result of species variation from the legumes used in the previous researches work. It is also possible that the researchers utilized already established protocol without optimizing for maximum yield.

The gross yield of ferritin concentrates from each legume seed and the PPI are shown in Figure 4. Approx. 500g of each sample was used in the isolation process. CP had the highest ferritin gross yield of 19.07g/100g, followed by RL 17.56g/100g. BEB had 15.61g/100g while RKB and GSB had gross yields of 13.33 and 13.22g/100g respectively. LB had the lowest yield of 4.87g/100g, the PPI had second lowest gross yield of 7.90g/100g.

The ferritin concentrate yield of each seed is considerable and gives an indication of potential viability especially for production on a commercial level where profit is a key factor. This will give CP the greater potential, having about 5 times the yield of LB and almost 3 times the yield of PPI. This finding reports higher yields than was reported in the work of Sczekan and Joshi (1987), who reported that the edible parts of plants contain ferritin in varying

concentrations and gave a range of pure ferritin concentration as being between 50-70mg/kg.

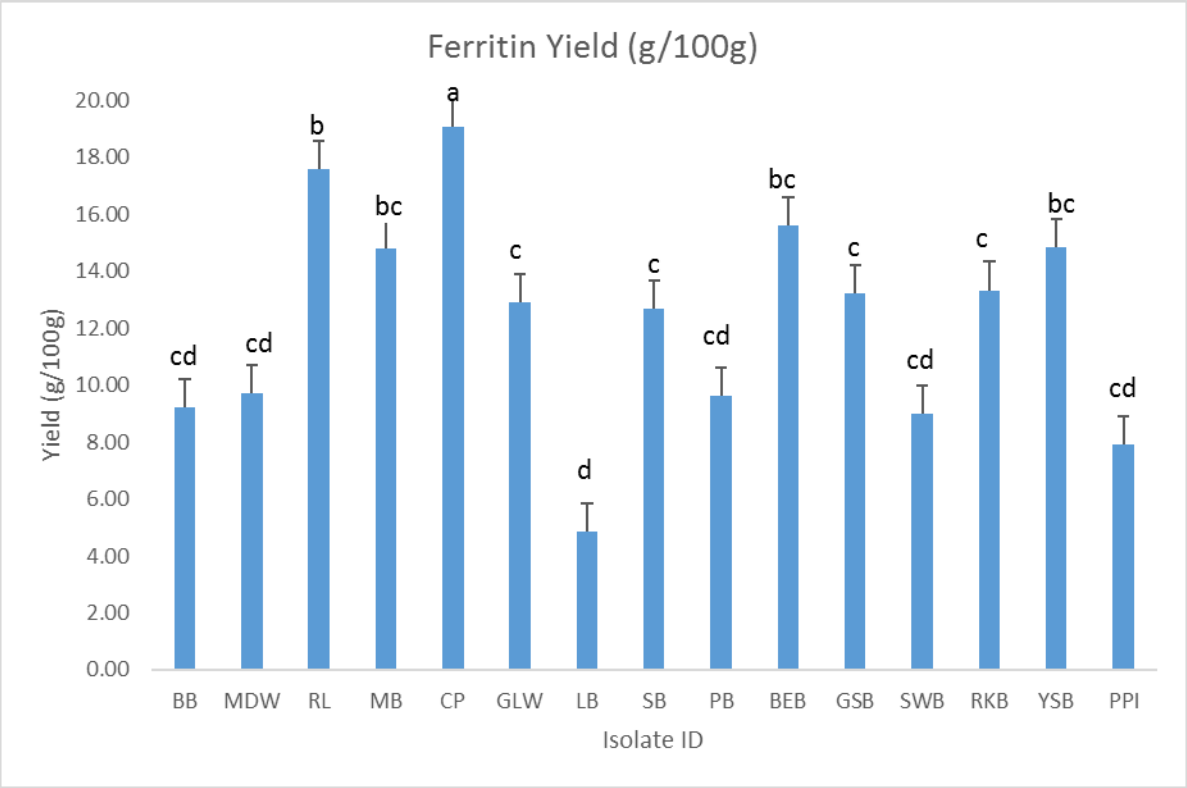

Figure 4: Ferritin Yield (g/100g) extracted from 500g of sample

Key:

Protein Pea Isolate (PPI)
Black Beans (BB)
Moon Dal Washed (MDW)
Red Lentils (RL)
Mung Beans (MB)
Chick Peas (CP)
Green Lentils Whole (GLW)
Lima Beans (LB)
Soy Beans (SB)
Pinto Beans (PB)
Black Eyed Beans (BEB)
Green Split Beans (GSB)
Small White Beans (SWB)
Red Kidney Beans (RKB)
Yellow Split Beans (YSB)

4.2 PROTEIN CONTENT OF THE FERRITIN CONCENTRATES

The results of the protein content assay showed protein content (g/100g) DM as follows:

Hydrolysate of Red Lentil (HRL) - 61.36

Yellow Split Beans (YSB) – 65.86

Green Lentils Whole (GLW) – 70.06

Chick Pea (CP) – 69.57

Moon Dal Washed (MDW) – 72.64

Red Lentils (RL) – 74.28

All the ferritin concentrates had protein content ranging from 74.28 (RL) to 61.36 (HRL). It is interesting to observe the reduction in protein content from RL and its hydrolysate. This confirms the activity of the exopeptidase used in the process of hydrolysis. Exopeptidase have been identified as ubiquitous enzymes that have the capacity to cleave protein bonds and they are extremely non-specific and as such will cleave several different peptide links (Wang et al., 1993). Also the protein from ferritin is a concentration of that from the legume samples. The fact that the concentrates have high protein content is an indication of the high protein contents of the legume seeds form where they were obtained. Pulses are generally consumed for their high protein content as supported by these findings. Ofuya and Akhidue (2005), reported protein contents between 18.4 and 24g/100g DM and Eli et al. (1997), reported protein content in pulses ranging between 192 and 260 g/kg. The range of their protein content is as expected in concentrates of pulses. The similarity in their amino acid composition (Table 6) can further explain the similarity observed in protein content among the ferritin concentrates.

4.3 OPTIMIZATION OF FERRITIN EXTRACTION FROM LEGUME SEEDS AND PPI

4.3.1 IRON:PROTEIN RATIO OF CONCENTRATES

Table 4 shows the iron to protein ratios as an initial screening of all the legume concentrates and Pea Protein Isolate (PPI). Iron:protein ratios ranged between 1.51 and 0.82, with Yellow split beans (YSB) having the highest and Mung beans (MB) having the lowest ratio. The concentrates of Green Lentils Whole (GLW), Chick Peas (CP), Moon Dal Washed (MDW), Red Lentils (RL) and Green split beans (GSB) had 1.39, 1.28, 1.27, 1.12 and 1.11 respectively.

On the basis of their higher iron:protein ratio, these six were selected on for the next stages of experiment:

Sample # 20 Hydrolysate of RL (HRL)
Sample # 21 Yellow Split Beans (YSB)
Sample # 22 Green Lentils Whole (GBW)
Sample #23 Chick Peas (CP)
Sample #24 Moon Dal Washed (MDW)
Sample #25 Red Lentils (RL)

Table 4: Iron:Protein Ratio of Concentrates

Sample	Average Protein (A_{280})	Average Iron (A_{420})	Iron:Protein Ratio
Pea Protein Isolate (PPI)	0.26 ±0.05 [f]	0.28±0.06 [g]	1.07
Black Beans (BB)	0.35±0.15 [d]	0.32±0.09 [fg]	0.92
Moon Dal Washed (MDW)	0.33±0.03 [e]	0.41±0.07 [d]	1.27
Red Lentils (RL)	0.23±0.01 [fg]	0.25±0.06 [h]	1.12
Mung Beans (MB)	0.48±0.08 [a]	0.39±0.04 [de]	0.82
Chick Peas (CP)	0.19±0.14 [g]	0.24±0.14 [h]	1.28
Green Lentils Whole (GLW)	0.32±0.13 [e]	0.44±0.06 [c]	1.39
Lima Beans (LB)	0.35±0.12 [d]	0.36±0.06 [ef]	1.01
Soy Beans (SB)	0.42±0.21 [b]	0.46±0.11 [b]	1.10
Pinto Beans (PB)	0.36±0.15 [d]	0.31±0.08 [fg]	0.85
Black Eyed Beans (BEB)	0.32±0.20 [e]	0.34±0.12 [f]	1.04
Green Split Beans (GSB)	0.35±0.03 [d]	0.39±0.12 [e]	1.11
Small White Beans (SWB)	0.38±0.18 [c]	0.32±0.11 [fg]	0.83
Red Kidney Beans (RKB)	0.38±0.05 [c]	0.33±0.09 [f]	0.86
Yellow Split Beans (YSB)	0.33±0.01 [e]	0.50±0.10 [a]	1.51

Results are presented as mean±standard deviation ($n=3$). For each column, mean values that contain different letters are significantly different at $p<0.05$

4.3.1.1 Iron concentration of six selected ferritin concentrates

The iron concentration of 6 selected ferritin concentrates are shown in Figure 5. Green Lentils Whole (GLW) had the highest concentration of 0.45g/100g and Chick pea (CP) had the lowest of 0.30g/100g. Yellow split beans (YSB) and Hydrolysate of Red Lentils (HRL) have concentrations close to GLW (0.44g and 0.42g/100g respectively). Moon Dal Washed (MDW) and Red Lentils (RL) had 0.35 and 0.34/100g respectively.

It is of interest to note that the same set of samples also had some of the highest gross ferritin yield values. CP and RL had the overall highest yields with YSB and GLW also being very high in the gross ferritin yield.

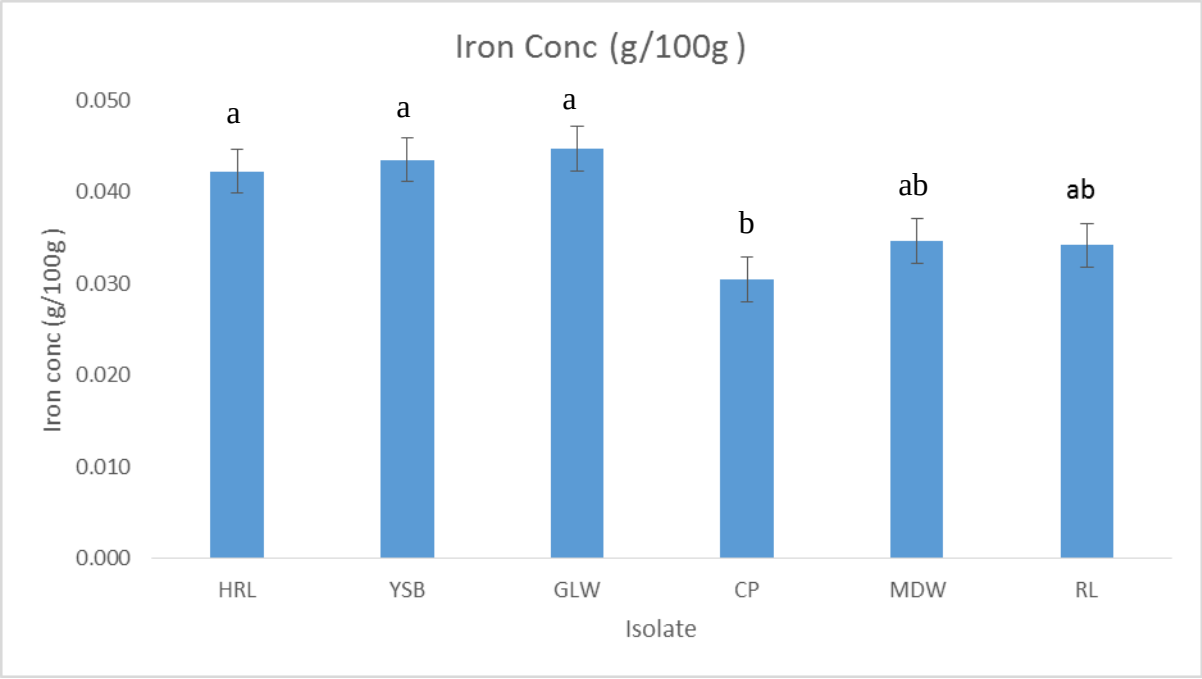

Figure 5: Iron Concentration of selected concentrates

4.3.2 IRON:PROTEIN RATIO OF HYDROLYSATES

The hydrolysates of the six selected ferritin concentrates (HRL, YSB, GBW, CP, MDW and RL), had iron:protein ratio (Absorbance at 420 nm:Absorbance at 280 nm) as shown on Table 5. Hydrolysis was performed using exoproteases to remove some of the protein molecules that are not critical to maintaining ferritin structure and as such increase the iron:protein ratio (purity) of the ferritin preparation. The CP hydrolysate had the highest iron:protein ratio of 0.267 and 0.286 hydrolysed at 40^{o}C with Accelerzyme CPG enzyme, for 4 and 7 hr respectively. The next highest ratio was also the CP hydrolysate hydrolysed with Peptidase R-K enzyme at 40^{o}C for 4 hr (0.185) and 50^{o}C for 7 hr (0.149). The lowest ratio was seen in the hydrolysate of YSB with Accelerzyme CPG enzyme at 40^{o}C for 4 hr. MDW hydrolysate with Accelerzyme CPG enzyme at 40^{o}C for 4 and 7 hr were also low with ratios of 0.037 and 0.044 respectively. The reduced iron:protein ratios suggest that hydrolysis negatively affected the ferritin cage structure, which may have led to iron losses. Hydrolysates have been described as having characteristics that potentially impact the functional properties and food uses of proteins. They are particularly useful in the treatment of patients with impaired nutrient absorption since hydrolysis breaks up protein bonds and make it more readily available (Chiang et al., 1999; Kristinsson and Rasco, 2000). The research did not investigate the bioavailability of the hydrolysates versus the concentrates however, showed a significant increase in iron:protein ratio for some of the samples, which was the purpose of hydrolysis.

Table 5: Iron:Protein Ratio of Hydrolysates

Hydrolysate	Average Iron (A_{420})	Average protein (A_{280})	Iron:Protein
YSB Accel 40C 4hrs	0.003	0.142	0.019
YSB Accel 40C 7hrs	0.008	0.130	0.061
GLW Accel 40C 4hrs	0.017	0.158	0.107
GLW Accel 40C 7hrs	0.005	0.100	0.050
CP Accel 40C 4hrs	0.038	0.141	0.267
CP Accel 40C 7hrs	0.048	0.167	0.286
MDW Accel 40C 4hrs	0.007	0.151	0.044
MDW Accel 40C 7hrs	0.005	0.135	0.037
RL Accel 40C 4hrs	0.004	0.098	0.041
RL Accel 40C 7hrs	0.006	0.126	0.050
YSB Pep-R-K 40C 4hrs	0.021	0.244	0.087
YSB Pep-R-K 50C 7hrs	0.007	0.153	0.048
GLW Pep-R-K 40C 4hrs	0.018	0.172	0.104
GLW Pep-R-K 50C 7hrs	0.017	0.183	0.095
CP Pep-R-K 40C 4hrs	0.034	0.186	0.185
CP Pep-R-K 50C 7hrs	0.023	0.152	0.149
MDW Pep-R-K 40C 4hrs	0.037	0.331	0.112
MDW Pep-R-K 50C 7hrs	0.025	0.297	0.083
RL Pep-R-K 40C 4hrs	0.018	0.189	0.095
RL Pep-R-K 50C 7hrs	0.011	0.149	0.076

4.4 AMINO ACID COMPOSITION

The percent amino acid compositions of the concentrates are as shown in Table 2. All of the concentrates were higher in negatively charged amino acids, aspartic and glutamic acids than all the other amino acids, which is typical of plant proteins. HRL had the highest glutamic acid composition (18.91%) and the second highest aspartic acid content, (12.17%). These values are very close to the aspartic and glutamic acid contents of RL which were 18.17% and 12.19%, respectively making RL the highest in aspartic acid content. The lowest glutamic acid content of 16.22% was obtained from the CP concentrate and YSB had the lowest aspartic acid content of 11.51%. This agrees with the findings of Le Brun et al. (2010) and Bertini et al. (2012), who reported that glutamic and aspartic acids (both negatively-charged amino acids) are stored in the active site of ferritin nanocage from where they intrinsically coordinate the oxidation of iron.

Table 6: Percent Amino Acid Composition

Sample ID / Amino Acid	HRL	YSB	GLW	CP	MDW	RL
Aspartic acid (Asp)	12.17	11.51	11.77	11.83	11.92	12.19
Threonine (Thr)	3.48	3.69	3.73	3.89	3.21	3.53
Serine (ser)	5.79	5.45	5.59	5.67	5.73	5.85
Glutamic acid (Glu)	18.91	17.92	17.22	16.26	18.05	18.17
Proline (Pro)	4.94	5.04	4.90	5.02	4.53	4.69
Glycine (Gly)	3.90	4.03	3.95	4.01	3.51	3.87
Alanine (Ala)	3.45	3.85	3.85	4.04	3.82	3.64
Cysteine (Cys)	0.74	0.94	0.73	1.42	0.42	0.64
Valine (Val)	4.90	4.69	5.16	4.75	4.85	4.91
Methionine (Met)	0.79	0.97	0.99	1.79	1.33	0.75
Isoleucine (Ile)	4.44	4.51	4.48	3.82	3.89	4.28
Leucine (Leu)	8.23	8.25	8.33	8.03	8.19	8.25
Tyrosine (Tyr)	3.44	3.52	3.42	3.04	3.28	3.35
Phenylalanine (Phe)	5.59	5.13	5.53	5.61	6.46	5.51
Histidine (His)	2.72	2.75	2.83	3.16	3.34	2.78
Lysine (Lys)	6.84	8.01	7.18	8.02	7.67	6.89
Arginine (Arg)	8.88	8.88	9.23	8.52	9.01	9.89
Tryptophan (Trp)	0.79	0.84	1.12	1.11	0.80	0.80

4.5 PHYSICOCHEMICAL PROPERTIES OF CONCENTRATES

4.5.1 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The PAGE separations are shown in Figures 6 and 7. Figure 6 shows the result of the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All the fractionated concentrates showed the presence of protein with a molecular weight 50 kDa, being the combined weight value of the ferritin molecules. This agrees with the findings of Laulhere et al. (1989) and Masuda et al. (2001) who reported that phytoferritins are each composed of two subunits of 26.5 and 28.0 kDa, which are referred to as H-1 and H-2, respectively.

Results of the sodium dodecyl sulfate polyacrylamide gel electrophoresis with mercaptoethanol (SDS-ME) are shown in Figure 7. All of the further denatured concentrates showed the presence of polypeptides with molecular weights of 26 kDa and 28 kDa, which is indicative of the presence of both the light (L) and the heavy (H) type subunits of ferritin. The 50 kDa band was not detected under reducing conditions. These findings confirm that the ferritin concentrates (represented by the 50 kDa bands) had their disulfide linkages broken by the mercaptoethanol to release the individual subunits of the ferritin protein.

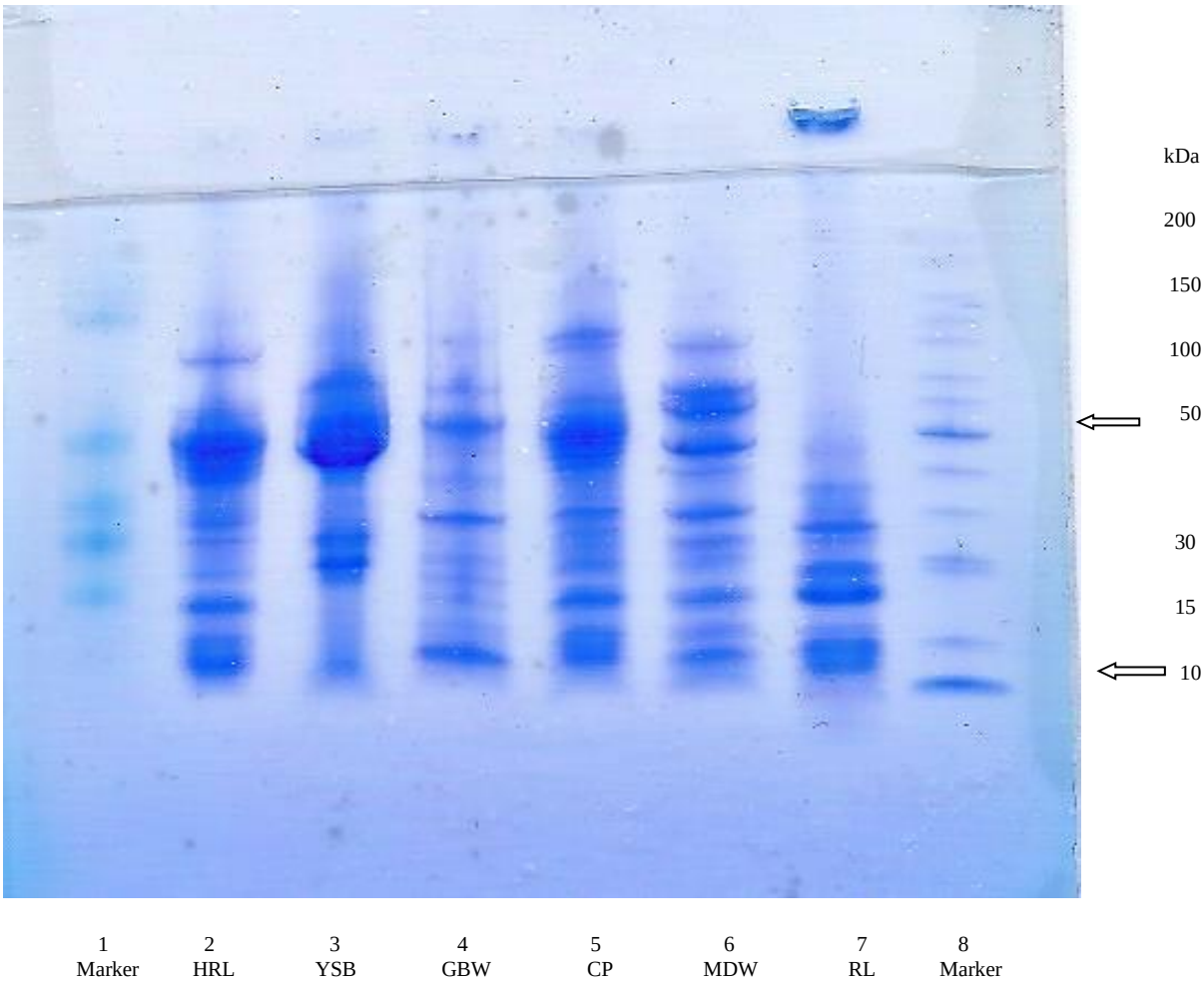

Figure 6: SDS-PAGE without 2-ME

Key:

1 Marker
2 Sample # 20 Hydrolysate of RL (HRL)
3 Sample # 21 Yellow Split Beans (YSB)
4 Sample # 22 Green Lentils Whole (GBW)
5 Sample #23 Chick Peas (CP)
6 Sample #24 Moon Dal Washed (MDW)
7 Sample #25 Red Lentils (RL)
8 Marker

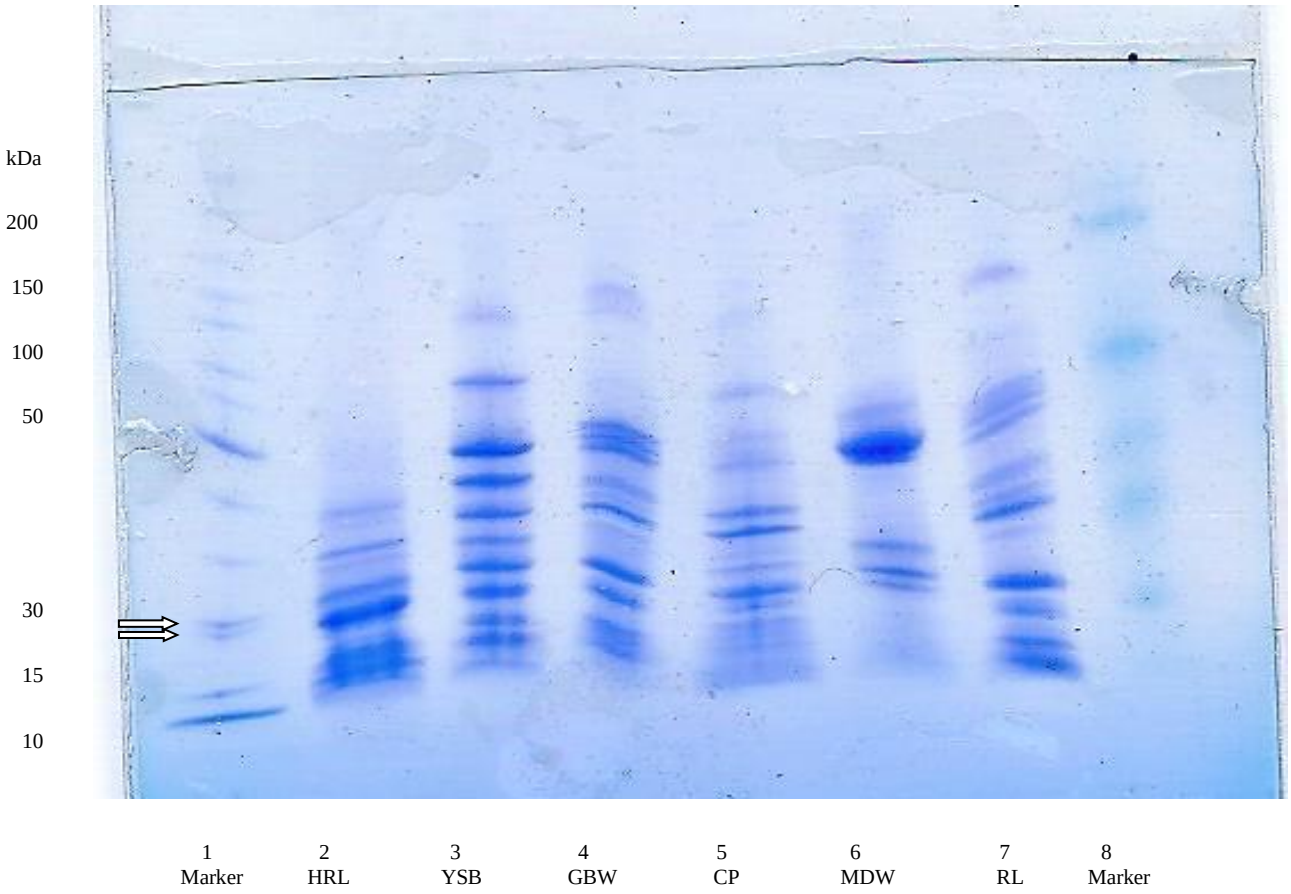

Figure 7: SDS-PAGE with 2-ME

Key:

1	Marker
2	Sample # 20 Hydrolysate of RL (HRL)
3	Sample # 21 Yellow Split Beans (YSB)
4	Sample # 22 Green Lentils Whole (GBW)
5	Sample #23 Chick Peas (CP)
6	Sample #24 Moon Dal Washed (MDW)
7	Sample #25 Red Lentils (RL)
8	Marker

4.6 SIMULATED GASTROINTESTINAL PROTEIN DIGESTION

4.6.1 FPLC OF CONCENTRATES BEFORE SIMULATED GASTROINTESTINAL PROTEIN DIGESTION

Results of FPLC of the selected concentrates before digestion are shown in Figure 8. FPLC chromatograms for each of the concentrates showed one dominant peak, which reflects the main ferritin protein. MDW had the highest elution volume, followed by the hydrolysate of RL, which also showed a minor peak, and RL. CP had the lowest elution volume. GLW and YSB had similar peak lengths, which were just slightly higher than the CP peak.

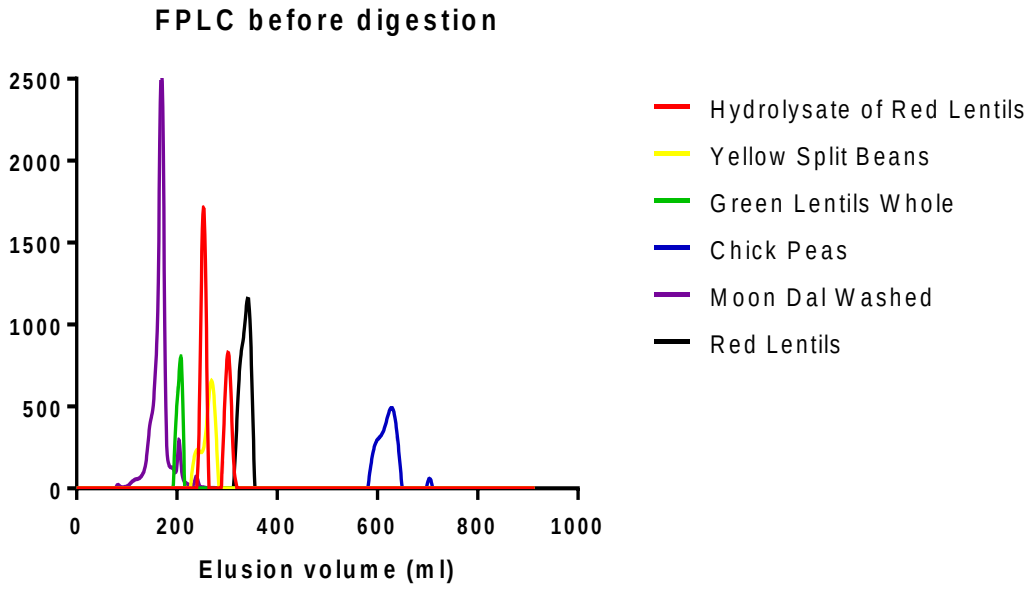

Figure 8: Fast Phase Liquid Chromatography of selected concentrates before digestion

4.6.2 FPLC of Ferritin Concentrates After Simulated Gastrointestinal Protein Digestion with Pepsin

The FPLC results after pepsin digestion is seen in Figure 9. The hydrolysate of RL had the highest elution volume closely followed by MDW and then CP and YSB. GLW and RL had the lowest elusion volumes.

The one dominant peak on each of the FPLC chromatograms, showed much smaller sizes after protein digestion with pepsin, indicating and confirming that the ferritin concentrates are susceptible to the enzyme digestion. This is as expected because the stomach is the primary site of pepsin production and synthesis, being very highly acidic; further facilitated by the fact that gastrointestinal tract temperature range (about 37 °C used in the simulated digestion process), is also optimum for pepsin enzyme activities.

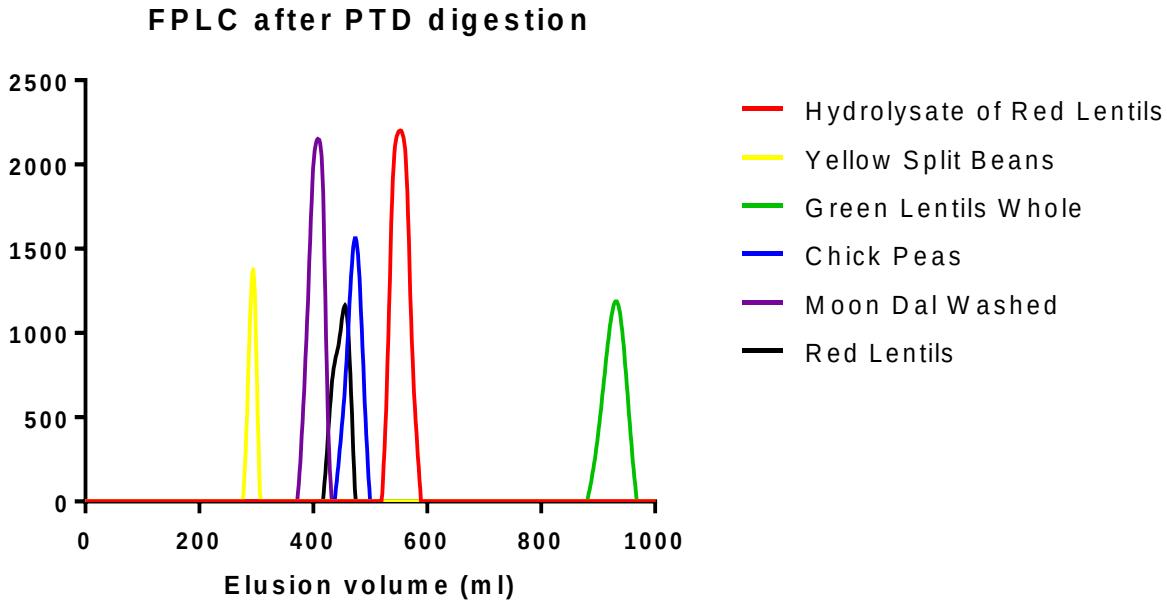

Figure 9: Fast Phase Liquid Chromatography of selected concentrates after pepsin digestion

4.6.3 FPLC of Concentrates after Simulated Gastrointestinal Protein Digestion with Pepsin and Pancreatin

Results of FPLC after consecutive pepsin and pancreatin digestions are shown in Figure 10. All of the six selected concentrates showed similar elution volume peaks with CP being slightly lower than the others. The general peak heights for GLW and HRL was not much different from that observed after pepsin digestion (shown in figure 10), suggesting that the active process of digestion took place during the pepsin digestion. GLW also showed a secondary peak after pepsin-pancreatin digestion. This can be explained by the fact that pancreatin works in the small intestine where it helps in the separation of the protein peptides into smaller peptides and free amino acids.

The pepsin-pancreatin digestion results are in conformity with the stomach digestion process of proteins. The results indicate that the actual enzyme digestion of ferritin takes place in the gastrointestinal track and is carried out by pepsin, where the acid environment assists the enzyme in the breakdown of larger proteins into smaller polypeptides. By the time the polypeptides get into the small intestine, the pancreatin, and other intestinal enzymes then complete the breaking apart of protein polypeptides into small-size peptides and individual amino acids that can be bioavailable to the body.

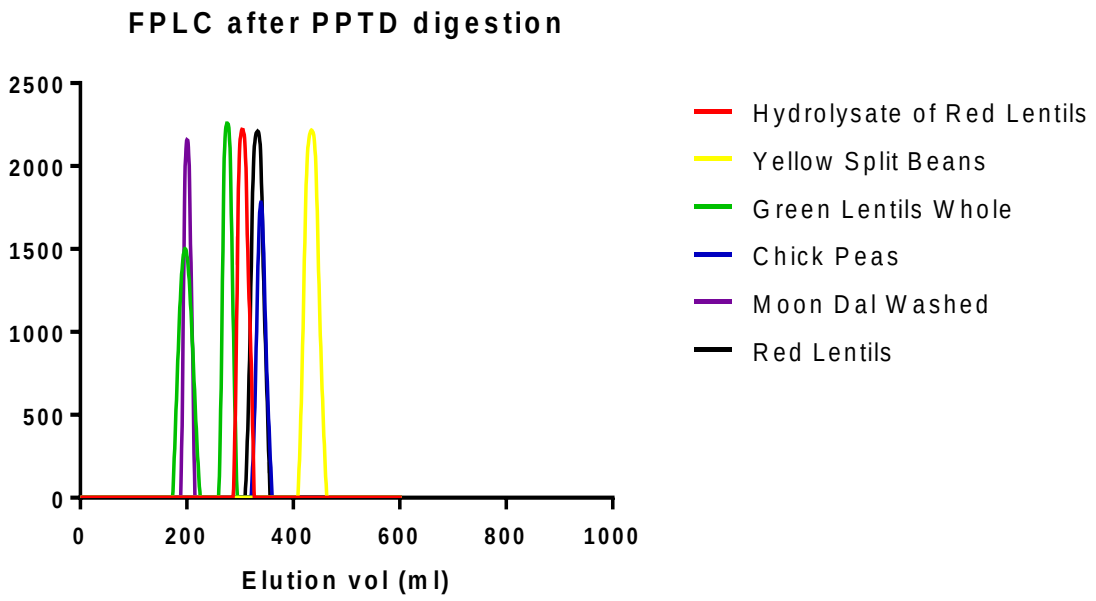

Figure 10: Fast Phase Liquid Chromatography of selected concentrates after pepsin-pancreatin digestion

4.6.4 Fluorescence of Concentrates before Simulated Gastrointestinal Protein Digestion

4.6.4.1 Fluorescence A: 257nm

The fluorescence spectrophotometry (to study the structure of ferritin) was carried out at an excitation wavelength of 257 nm (from phenylalanine) and pH 2 (Fig. 11). The spectra showed only weak elution of the aromatic amino acid, suggesting that the phenylalanine could have been exposed to a hydrophilic environment (while in the buffer) which would have quenched its fluorescence. The MDW showed the highest fluorescence intensity (FI), indicating that it had the highest quantum yield. This was followed by RL, YSB and CP had almost identical fluorescence intensity with GLW following closely after them. HRL had the lowest fluorescence intensity and therefore the lowest quantum yield. Figure 12 shows the FI at 257 nm and pH 3. MDW also had the highest fluorescence intensity, and dropped with a very steep slope; followed by CP. All the other ferritin concentrates had relatively much lower fluorescence intensity with the RL being the lowest. CP and YSB showed a secondary peak with fluorescence intensity relative to their position in terms of their quantum yield. In Figure 13, the FI of the concentrates at 257 nm and pH 4 shows MDW and CP, again, having the highest fluorescence intensity, followed by HRL, YSB, GLW and RL in that order. The last four had relatively lower fluorescence intensities than the first two. Each one of the concentrates showed a secondary peak which were positioned similar to the primary peaks in terms of FI. The fluorescence intensity of the concentrates at 257 nm and pH 5 are as shown in Figure 14. Just like in the other pH values discussed so far, the MDW and CP had the highest fluorescence intensity. These two were followed by HRL, YSB. In this case, unlike was observed at pH 4, RL had the lowest fluorescence intensity, with GLW being next after YSB. Each one of the concentrates showed a secondary peak which were positioned similar to

the primary peaks in terms of fluorescence intensity. Figure 15 describes the results of the concentrates at 257 nm and pH 6. They each had a primary and a secondary peak with MDW and CP, again having the highest FI, followed by HRL, YSB. The primary peaks of GLW and RL had very similar FI, being the two lowest but their secondary peaks were in the order of YSB, RL, GLW and HRL. Figure 16 shows the results of fluorescence spectrophotometry of the concentrates at 257 nm and pH 7. Like the other pH levels used, the MDW had the highest fluorescence intensity, but unlike seen with most other pH values, RL was the next highest, followed by CP, YSB, HRL and GLW which had very similar FI. The results of fluorescence of the concentrates at 257 nm and pH 8 are as shown in Figure 17. MDW, again had the highest fluorescence intensity. It is followed by RL, YSB, GLW, and CP in that order while HRL had the lowest fluorescence intensity. Generally it was observed that all concentrates had less structure at extreme pH values (pH 2 and pH 7), but were more rigid at the median pH values (4, 5 and 6). These findings agree with the findings of Yin et al., (2011) and Malomo and Aluko, 2015, and are critical to the objectives of the research work, since the pH values where the concentrates showed poor structure and were highly unfolded, are the same levels at which digestion takes place in the stomach and small intestine. The FI results also indicate that MDW had the most structural rigidity at all the pH values and was most resistant to pH-induced structure disruptions. Thus the MDW may be less susceptible to enzyme digestion, which is consistent with the FPLC data where this ferritin eluted early, indicating high protein molecular weights in the presence of proteases.

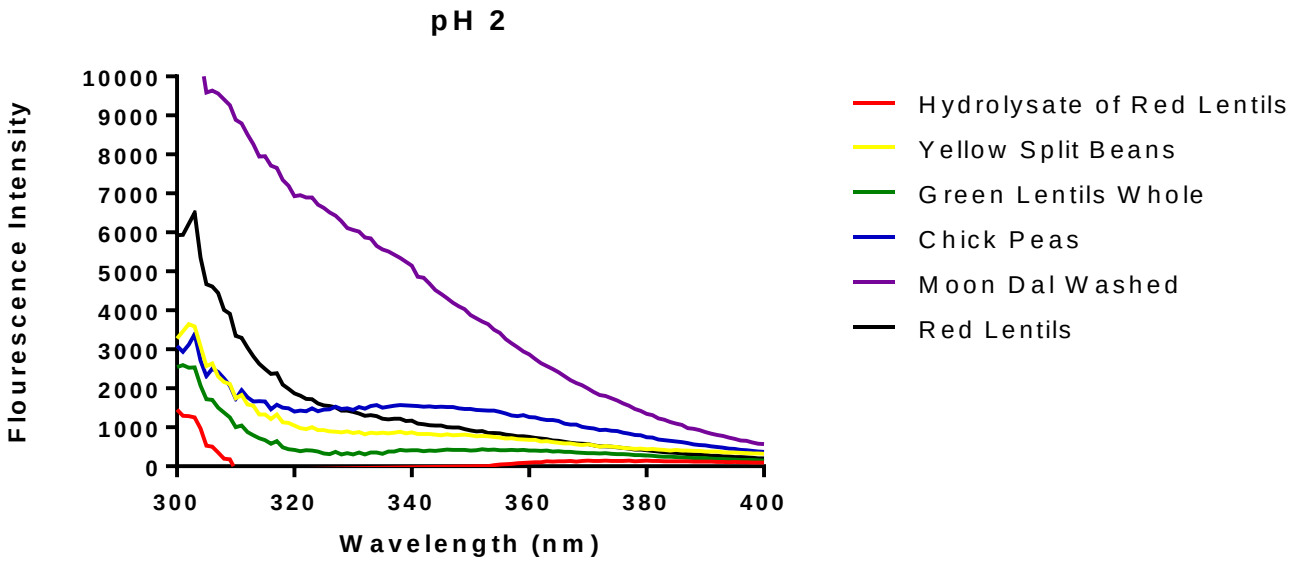

Figure 11: Fluorescence of selected concentrates at 257 nm and pH 2

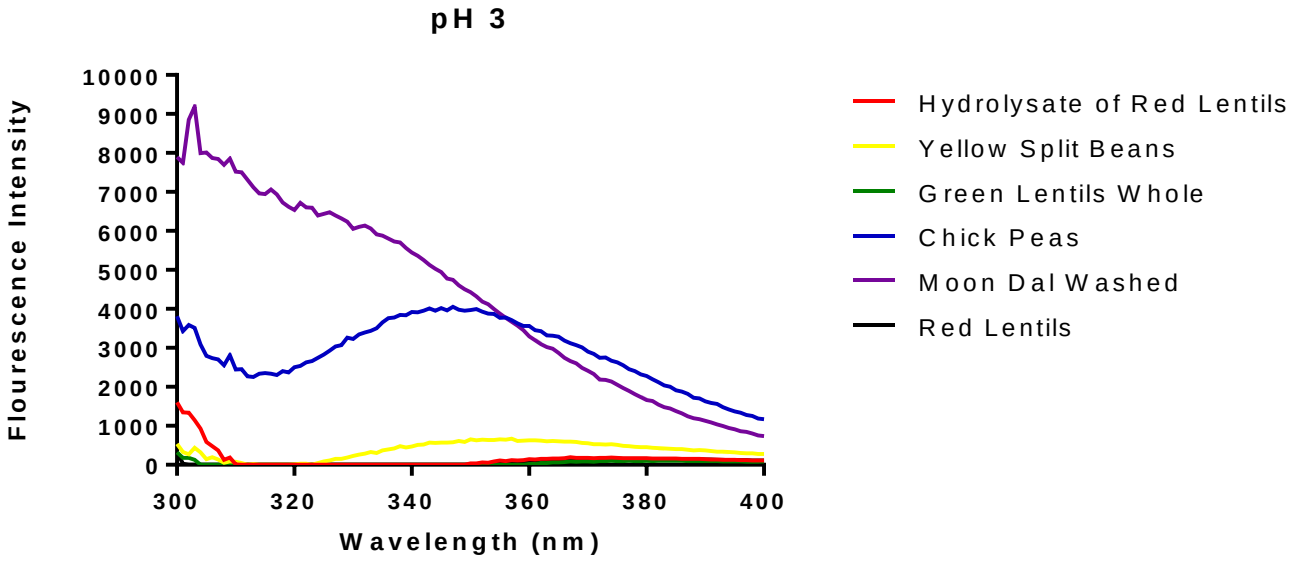

Figure 12: Fluorescence of selected concentrates at 257nm and pH 3

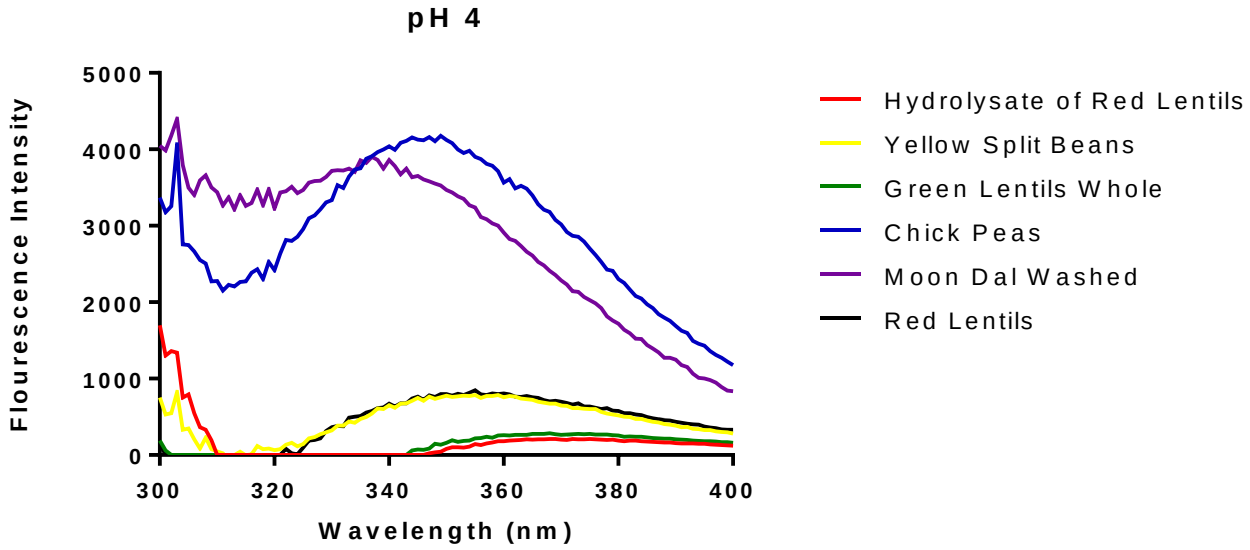

Figure 13: Fluorescence of selected concentrates at 257nm and pH 4

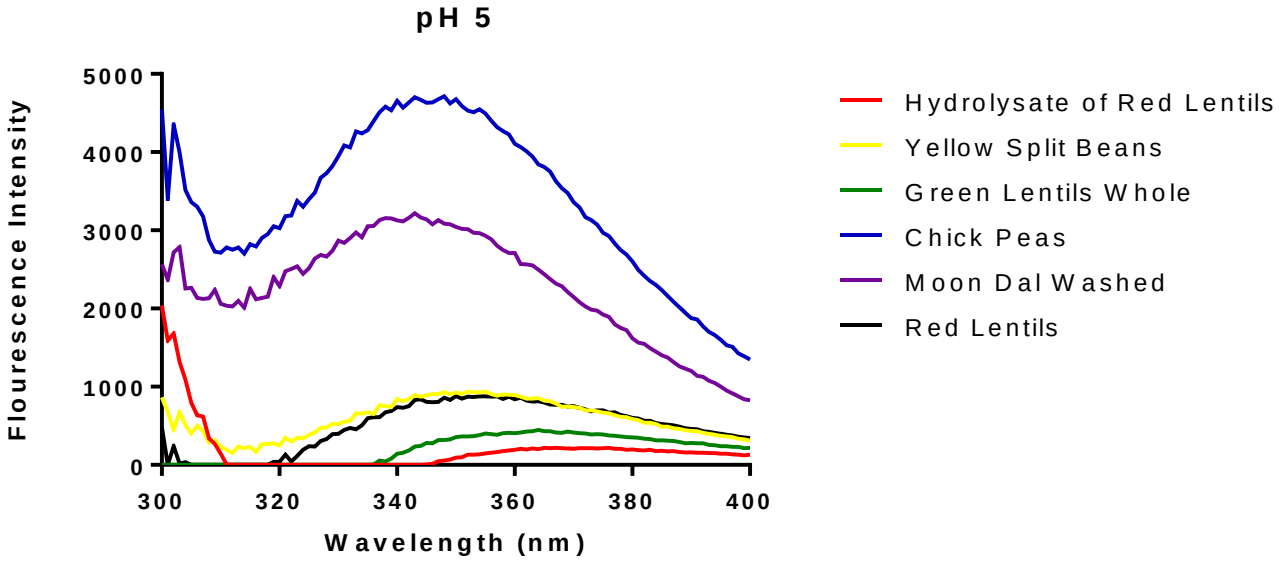

Figure 14: Fluorescence of selected concentrates at 257nm and pH 5

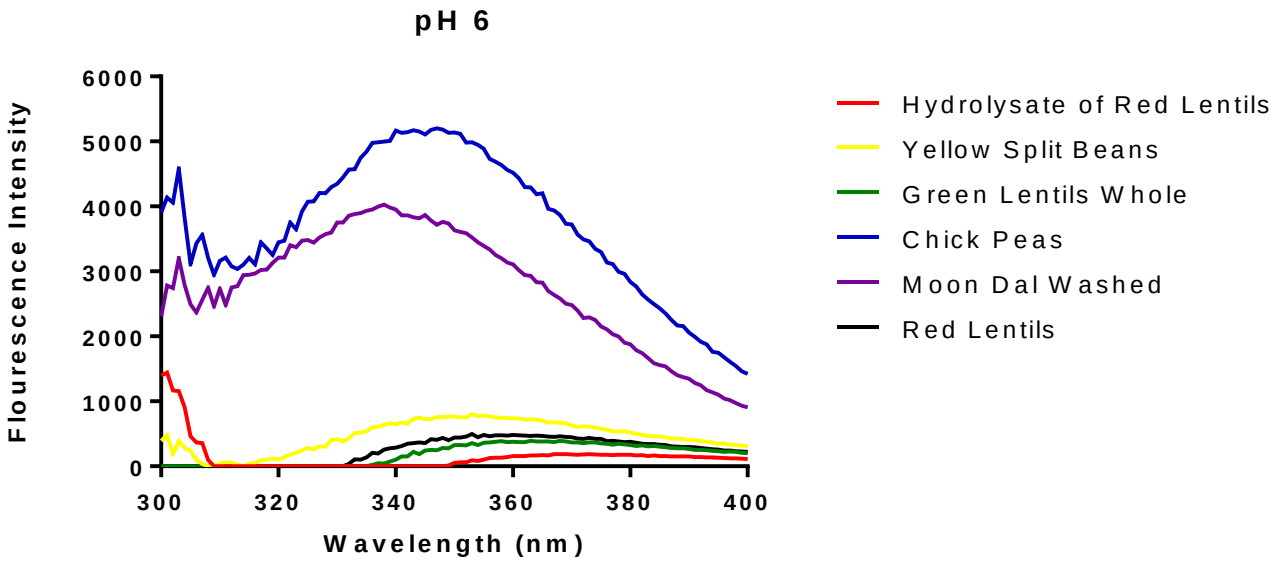

Figure 15: Fluorescence of selected concentrates at 257nm and pH 6

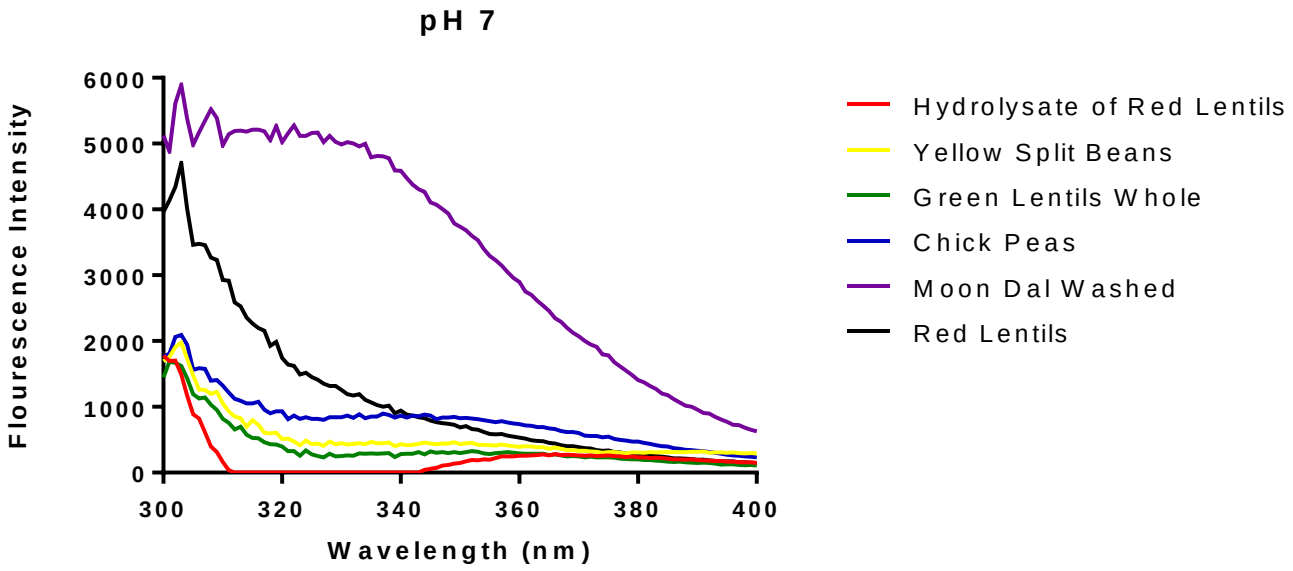

Figure 16: Fluorescence of selected concentrates at 257nm and pH 7

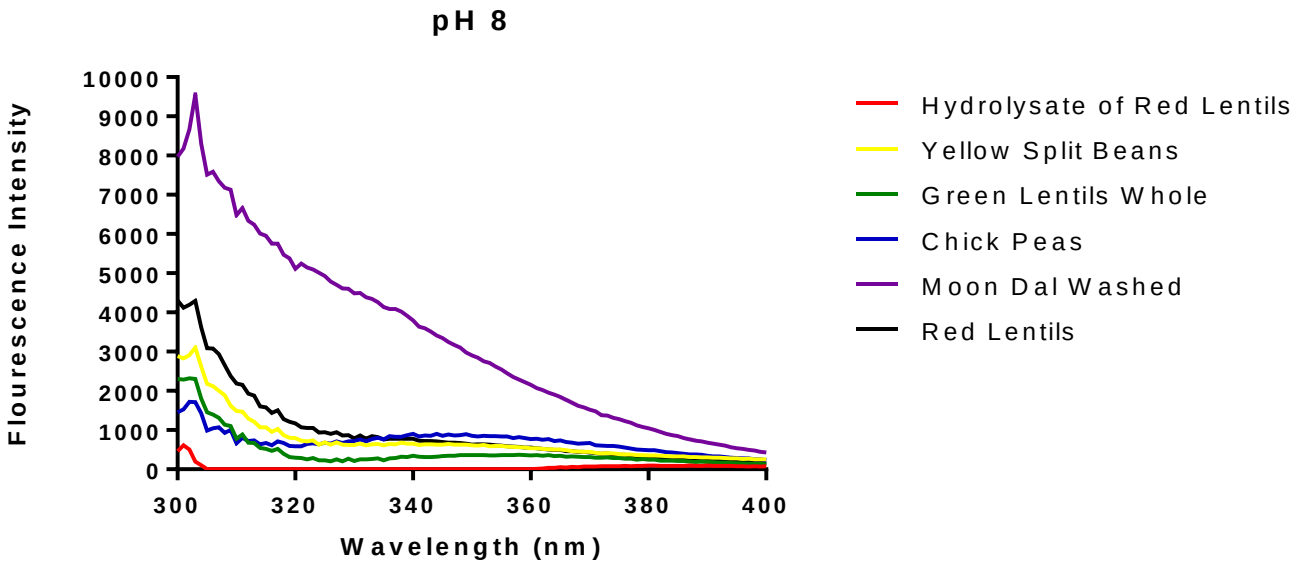

Figure 17: Fluorescence of selected concentrates at 257nm and pH 8

4.6.4.2 Fluorescence B: 275nm

The fluorescence spectrophotometry was then carried out at an excitation wavelength of 275 nm (tyrosine) and pH 2. MDW, like at 257 nm, showed the highest fluorescence intensity, and so, the highest quantum yield. It was followed by the RL, then YSB and GLW and CP were identical and had the next highest intensity. HRL showed the lowest fluorescence intensity as shown in Figure 18. Each of the concentrates had rapidly declining spectra, again, indicating their lack of structure and non-compact protein concentrates which are more readily digestible. The results of fluorescence spectrophotometry carried out at an excitation wavelength of 275 nm and pH 3 is shown in Figure 19. MDW again had the highest fluorescence intensity, and was closely followed the CP, then HRL, YSB, GLW and RL had the lowest fluorescence intensity. CP and showed a secondary peak with fluorescence intensity relative to their position in terms of their quantum yield. Figure 20 is the results of fluorescence spectrophotometry carried out at an excitation wavelength of 275 nm and pH 4. Each of the concentrates showed a primary and secondary peak. CP primary and secondary peaks had the highest fluorescence intensity respectively, followed by the two peaks of MDW and then the primary peaks of HRL, YSB, RL and GLW. The secondary fluorescence peaks from RL and YSB had similar quantum yield and next highest in fluorescence intensity. The secondary peaks of GLW and HRL were very close together and had the lowest fluorescence intensity of this wavelength and pH. The results of the fluorescence spectrophotometry carried out at an excitation wavelength of 275 nm and pH 5 is shown in Figure 21. It closely followed the results previously described at wavelength of 275 nm and pH 4, where all the concentrates had two peaks each and the primary and secondary peaks CP had the highest fluorescence intensity respectively. They were followed by the two peaks of MDW and then the primary

peaks of HRL, YSB, RL and GLW. At pH 5, however, the order of FI secondary peaks was YSB > RL > GLW > HRL.

Figure 22 shows the results of the fluorescence spectrophotometry carried out at an excitation wavelength of 275 nm and pH 6, again like at pH 3, 4 and 5, all the concentrates had two peaks and the first and second peaks from the CP concentrate had the highest fluorescence intensity respectively. They were followed by the two peaks of MDW and then the primary peaks of HRL, YSB, both the first peaks of RL and GLW were identical and the lowest of the primary peaks in fluorescence intensity. The fluorescence intensity of the secondary peak in YSB was higher than, GLW and RL which were identical, and higher than the secondary peak of HRL which had the lowest fluorescence intensity. Figure 23 represents the results of the fluorescence spectrophotometry carried out at an excitation wavelength of 275 nm and pH 7. Again, the MDW had the highest fluorescence intensity, followed by RL, and then CP, GLW, YSB and HRL had the lowest fluorescence intensity at pH 7. The quantum yield of the excitation wavelength of 275 nm and pH 8 is shown in Figure 24. MDW and RL had very similar peak sizes and the highest fluorescence intensity. These were followed by CP, GLW and YSB had similar peaks and were next in fluorescence intensity while HRL had the lowest fluorescence intensity. Excitation at 275 nm also showed that the MDW had the most structural rigidity, which is consistent with the results obtained at 257 nm. The results showed that generally the proteins were highly denatured at pH 2, 7, and 8 due to the absence of a definite peak at 350 nm for tryptophan emission. In contrast at pH 3, 4, 5, and 6, there were distinct tryptophan emission transitions, indicating rigid structural conformation. Tyrosine emission (303 nm) was not very distinct and is an indication that the molecules are very close to tryptophan residues, hence tyrosine emission quenching. This confirms that environmental

conditions are highly influential in protein unfolding, denaturation and subsequent digestion and are similar to the findings of Mundi and Aluko, (2013). Uversky and Dunker, (2010) related the unusual sequencing of the three-dimensional structure of amino acids and their irregular structures to the presence of numerous, negatively charged groups resulting from extreme ribosomal protein P1 values and the absence of hydrophobic amino acid residues.

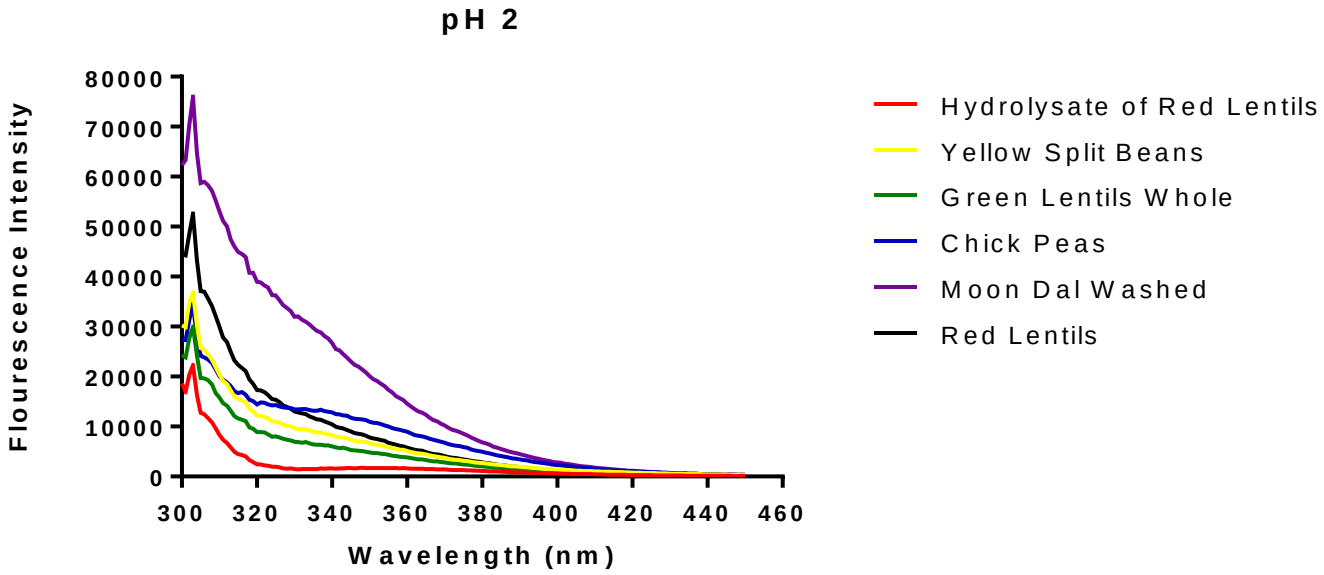

Figure 18: Fluorescence of selected concentrates at 275 nm and pH 2

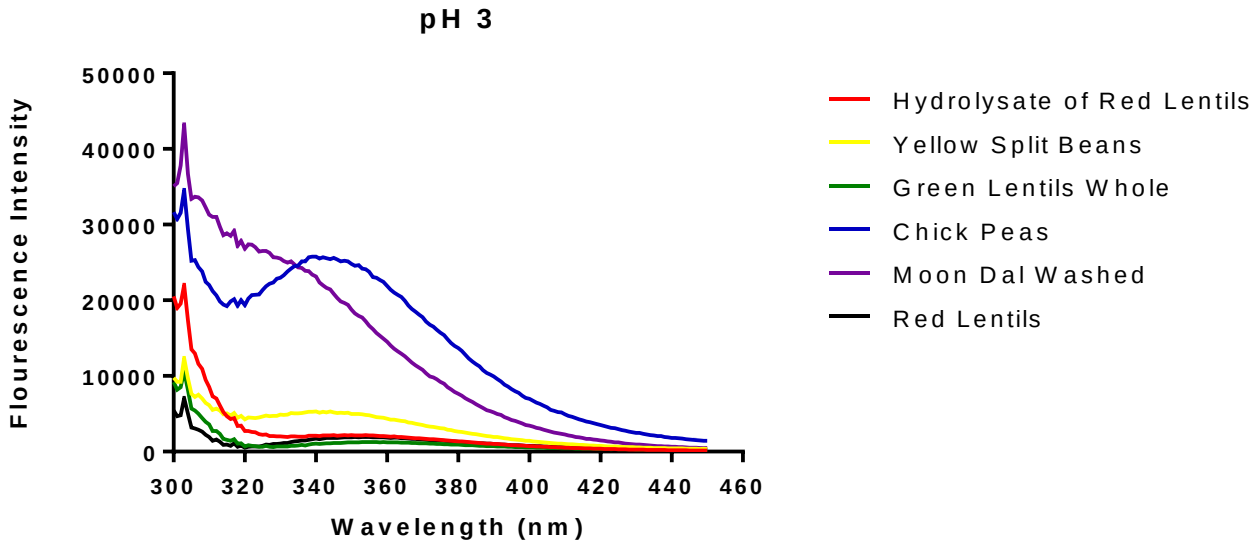

Figure 19: Fluorescence of selected concentrates at 275 nm and pH 3

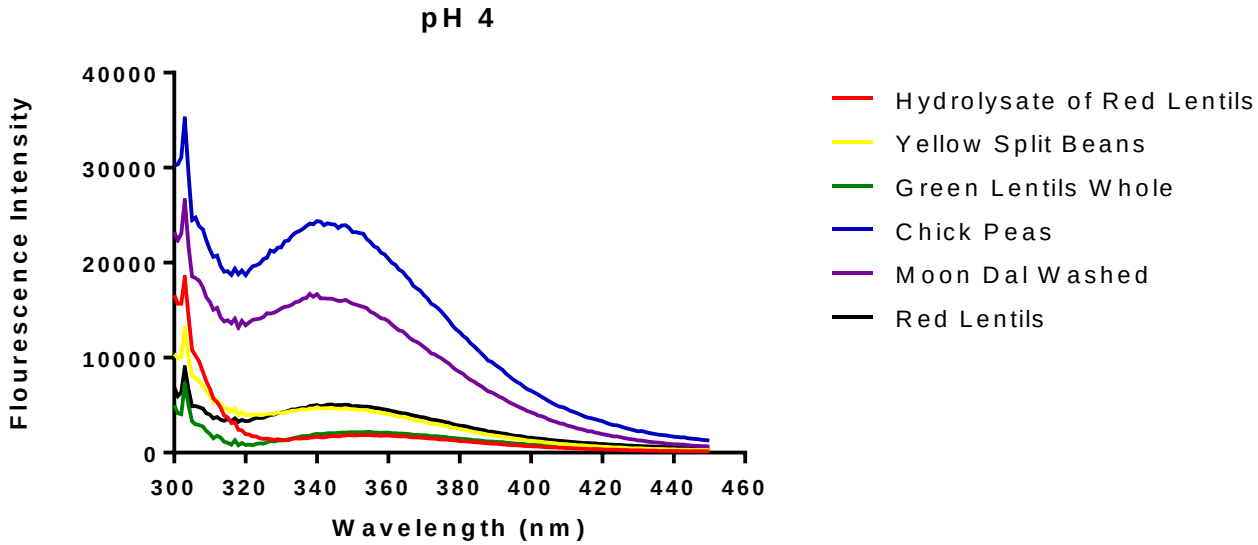

Figure 20: Fluorescence of selected concentrates at 275 nm and pH 4

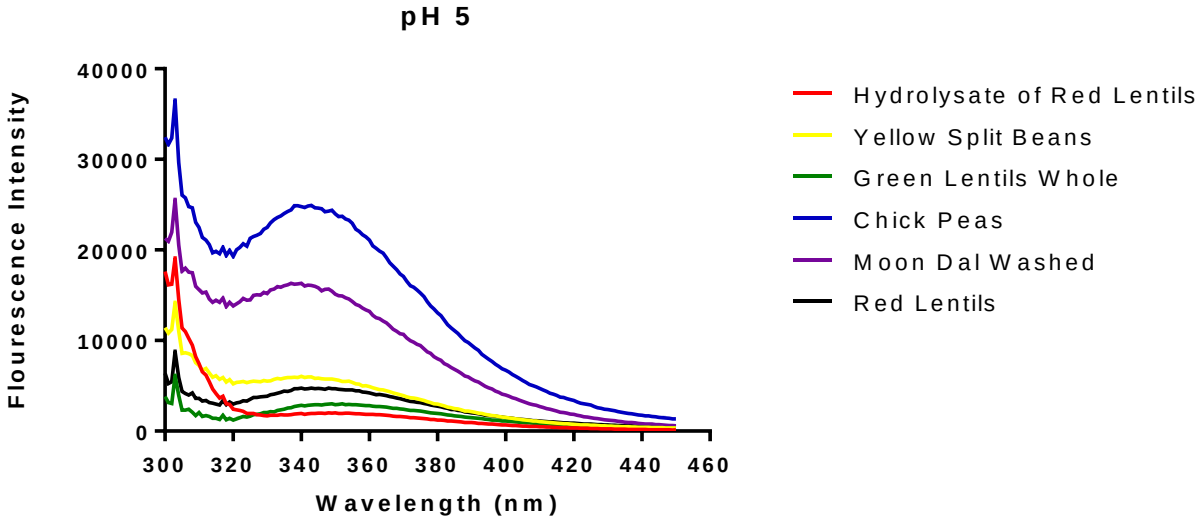

Figure 21: Fluorescence of selected concentrates at 275 nm and pH 5

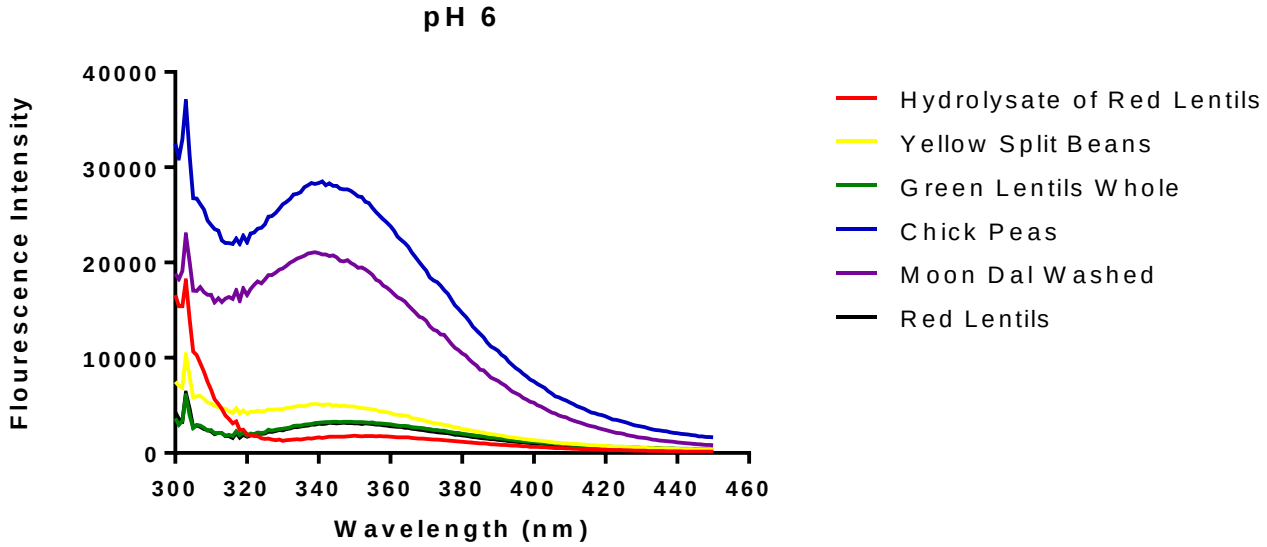

Figure 22: Fluorescence of selected concentrates at 275 nm and pH 6

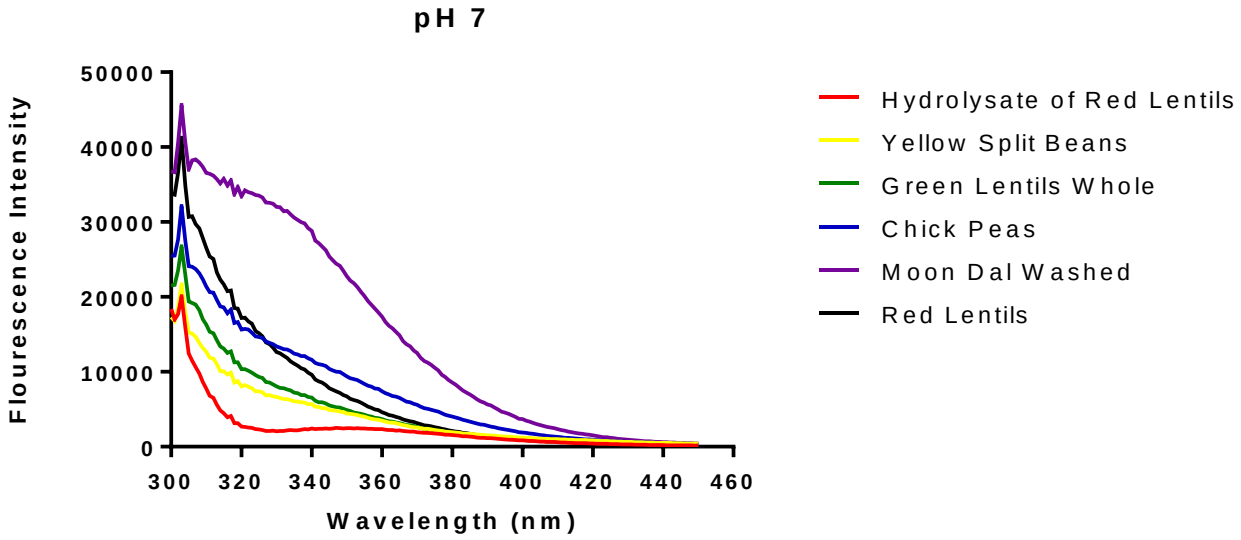

Figure 23: Fluorescence of selected concentrates at 275 nm and pH 7

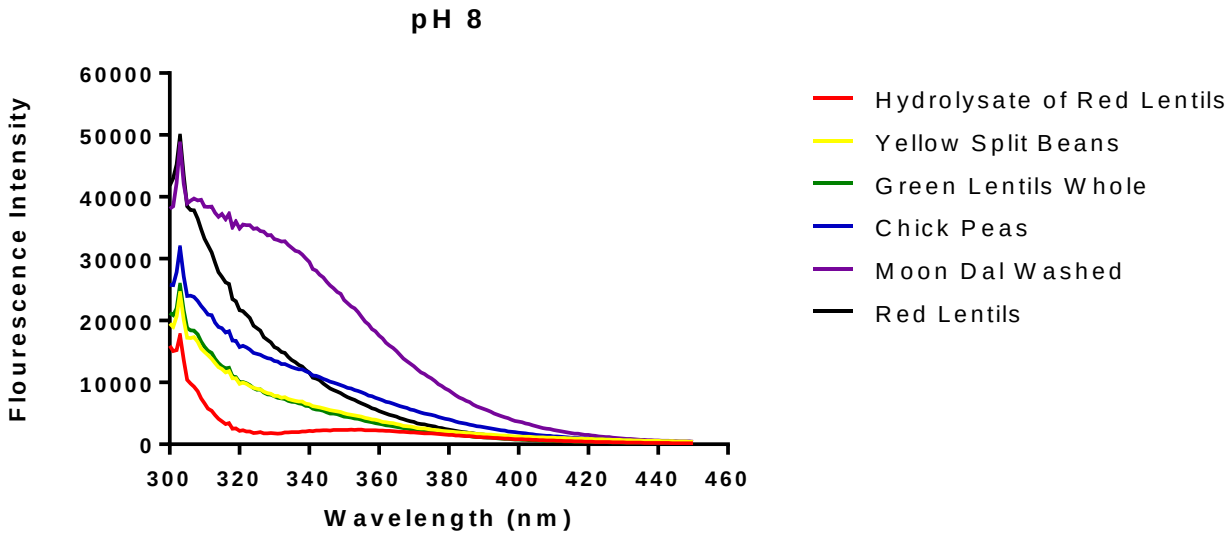

Figure 24: Fluorescence of selected concentrates at 275 nm and pH 8

4.6.4.3. Fluorescence C: 280nm

The fluorescence spectrophotometry was carried out at an excitation wavelength of 280 nm where either or both of any of the aromatic amino acids tyrosine and tryptophan would be excited and could be responsible for the observed FI. Again, all the concentrates were disordered at pH 2, 7 and 8 and had more rigid structure at pH 3, 4, 5 and 6. This will explain the digestibility and bioavailability observed in the kinetics of digestion experiments. At pH 2, CP and HRL showed secondary peaks. Most of the ferritin concentrates had one major peak at 350 nm, which is consistent with tryptophan emission. Like for the spectra at wavelengths 257 and 275 nm, the structure of the concentrates fluorescence emission were without rigidity and structure at pH 2, 7 and 8. This indicates that at the pH levels when they are digested and released for absorption in the human body, the protein concentrates were easily denatured and so will be readily available. MDW had the highest FI, followed by RL and then YSB, CP, GLW and HRL. The secondary peak on CP has FI next to its primary peak, higher than the primary peak fluorescence intensity of HRL. The secondary peak fluorescence intensity of HRL has the lowest fluorescence intensity of the set. This is seen in Figure 25. The fluorescence spectrophotometry at an excitation wavelength of 280 nm and pH 3 is seen in Figure 26. CP, HRL, RL and GLW had secondary peaks. The MDW primary peak, again, had the highest fluorescence intensity. The next highest FI was seen with the primary and secondary peaks of CP, followed by HRL and the secondary peak of YSB. The primary peak of YSB was next followed by the secondary peaks of HRL and RL. The primary peaks of RL and GLW were next and the secondary peak in GLW had the lowest fluorescence intensity. Shown in Figure 27, is the fluorescence spectrophotometry at an excitation wavelength of 280 nm and pH 8, with all the concentrates having primary and secondary peaks. The highest fluorescence intensity was

observed in the CP primary and secondary peaks and MDW primary and secondary peaks were the next highest, followed by primary peak of HRL, YSB primary and secondary peaks, RL secondary peak, secondary peaks of HRL and GLW, while the primary peak of GLW had the lowest quantum yield of the group. The fluorescence spectrophotometry at an excitation wavelength of 280 nm and pH 5 is seen in Figure 28. The spectrum was very similar to that of wavelength of 280 nm and pH 4. All the concentrates have primary and secondary peaks, with the highest fluorescence intensity seen in the CP primary and secondary peaks. This was followed by MDW primary and secondary peaks as the next highest, followed by primary peak of HRL, YSB primary and secondary peaks, RL secondary peak. However, at pH 5, the secondary peak of GLW had a slightly higher FI than HRL, while the primary peaks of GLW and RL had the lowest quantum yield of the group, having the lowest fluorescence intensity. Figure 29 shows results of the fluorescence spectrophotometry at an excitation wavelength of 280 nm and pH 6. The spectrum was very similar to that of wavelength of 280 nm and pH 4 and 5. Again, all the concentrates have primary and secondary peaks, with the highest fluorescence intensity seen in the CP primary and secondary peaks. This was followed by MDW primary and secondary peaks as the next highest, followed by primary peak of HRL and just like in the case with pH 4, YSB primary and secondary peaks were next highest, followed by the GLW and RL secondary peaks. HRL had the next highest peak intensity, while the primary peaks of GLW and RL again, had the lowest quantum yield of the group, having the lowest fluorescence intensity. Figure 30 shows results of the fluorescence spectrophotometry at an excitation wavelength of 280 nm and pH 7. Of all the concentrates, only HRL showed two peaks at pH 7. MDW had the highest fluorescence intensity, followed by RL, CP, GLW, YSB and the secondary peak of HRL. The primary peak of HRL had the lowest fluorescence intensity and the lowest quantum yield of

the group. The results of the fluorescence spectrophotometry at an excitation wavelength of 280 nm and pH 8 show, like at pH 7, only HRL showed two peaks. RL had the highest fluorescence intensity, closely followed MDW, then CP, and GLW is closely followed by YSB. The next highest quantum yield is seen in the primary peak of HRL, while the secondary peak of HRL had the lowest fluorescence intensity. This is seen in Figure 31.

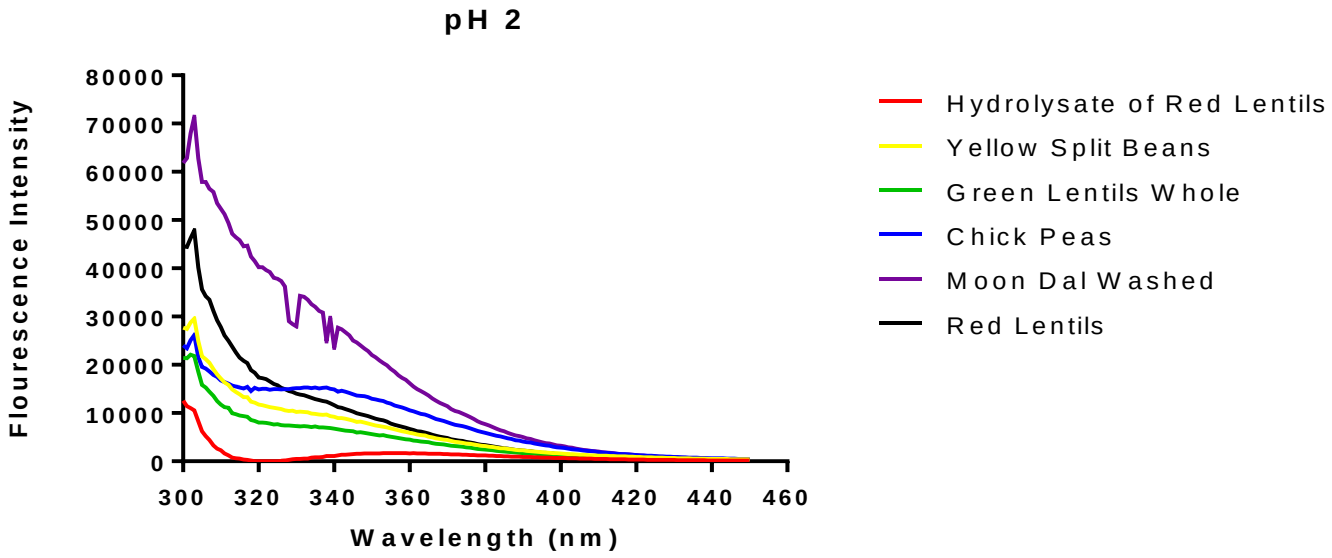

Figure 25: Fluorescence of selected concentrates at 280 nm and pH 2

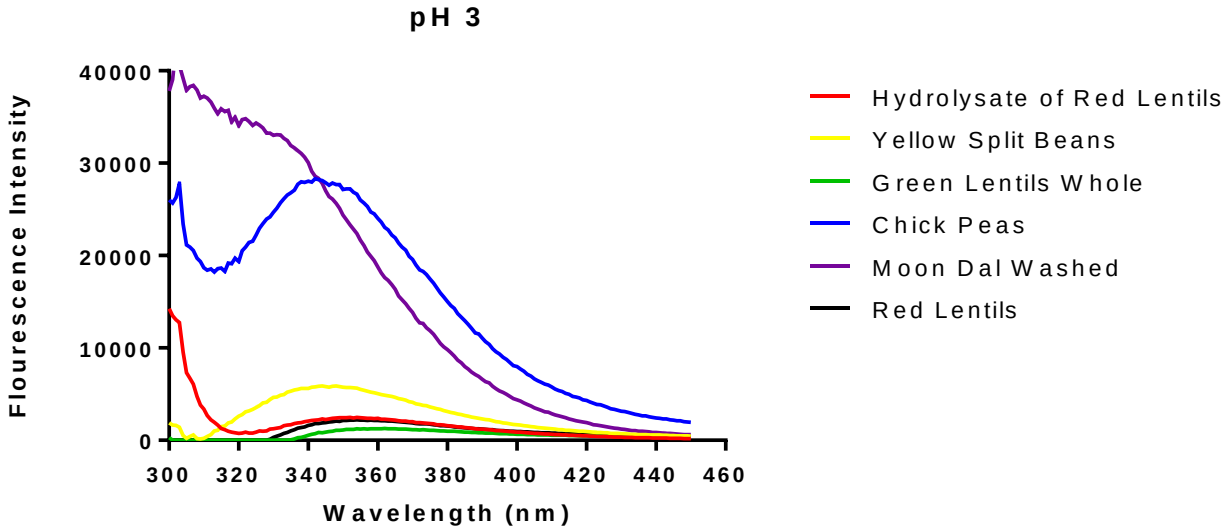

Figure 26: Fluorescence of selected concentrates at 280 nm and pH 3

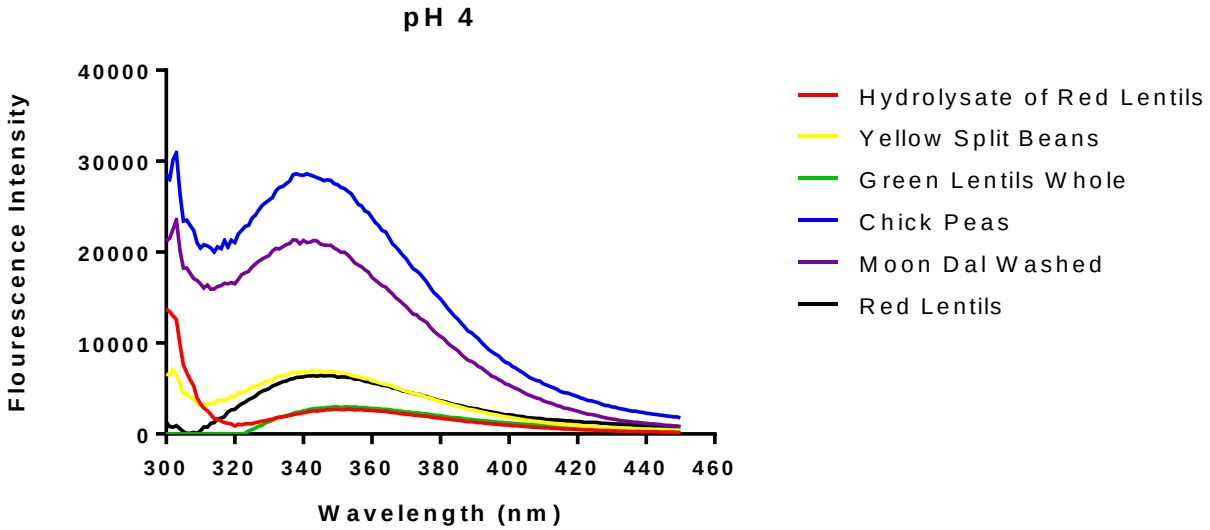

Figure 27: Fluorescence of selected concentrates at 280 nm and pH 4

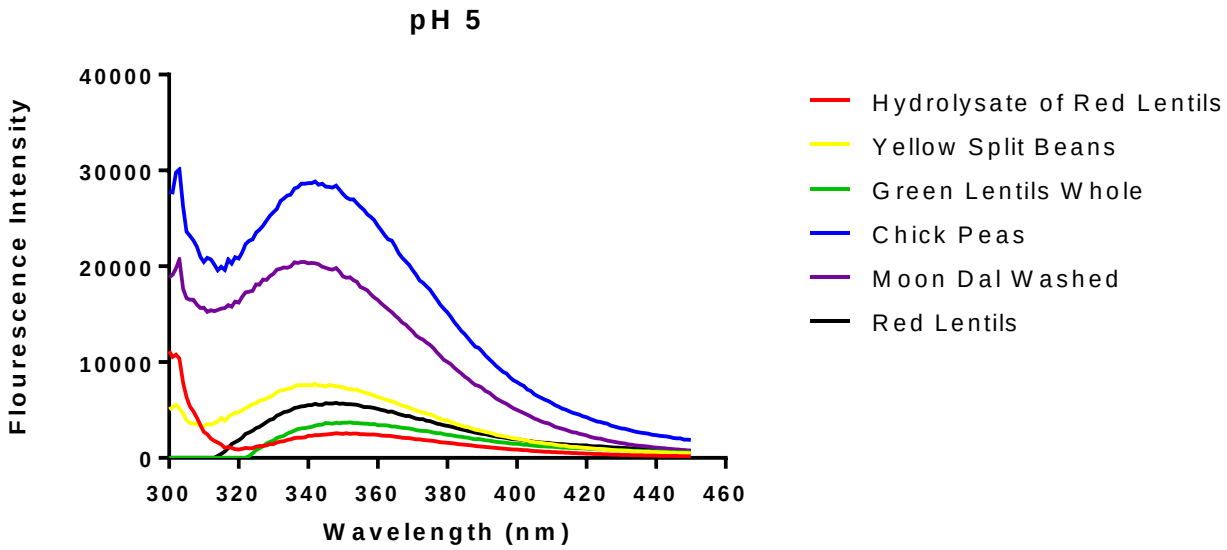

Figure 28: Fluorescence of selected concentrates at 280 nm and pH 5

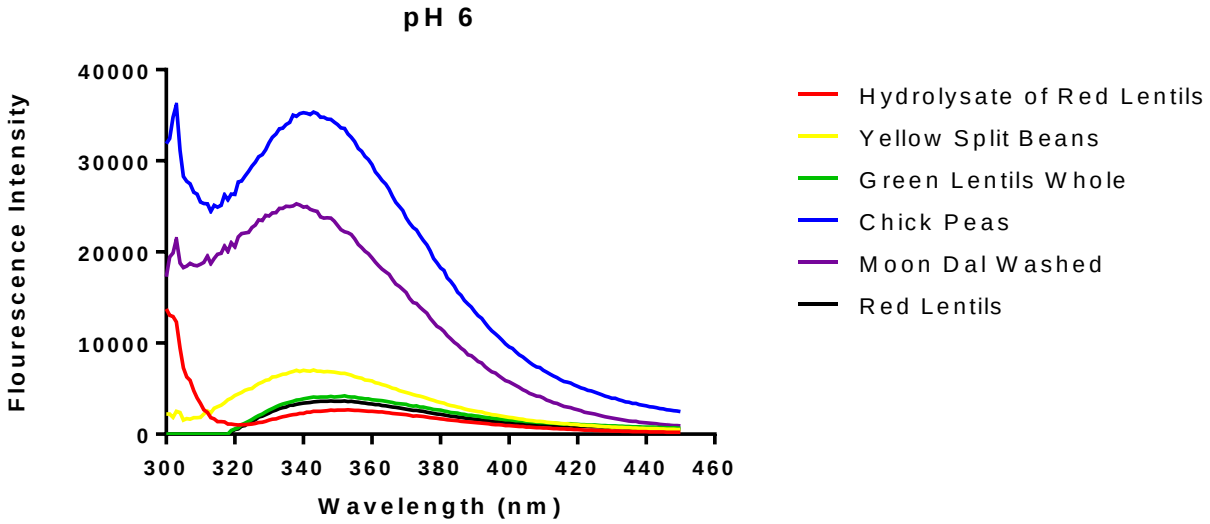

Figure 29: Fluorescence of selected concentrates at 280 nm and pH 6

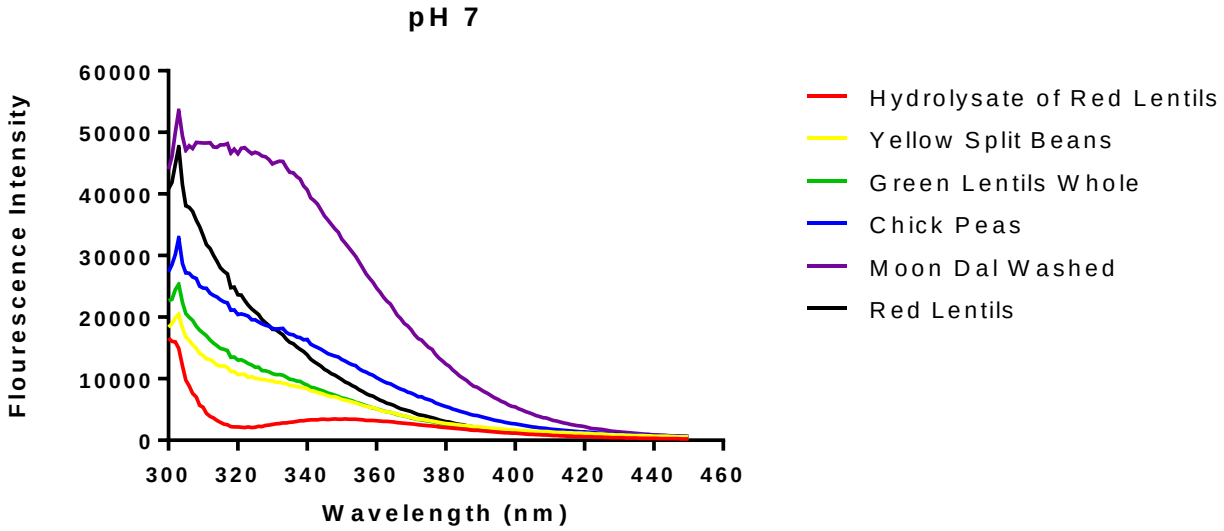

Figure 30: Fluorescence of selected concentrates at 280 nm and pH 7

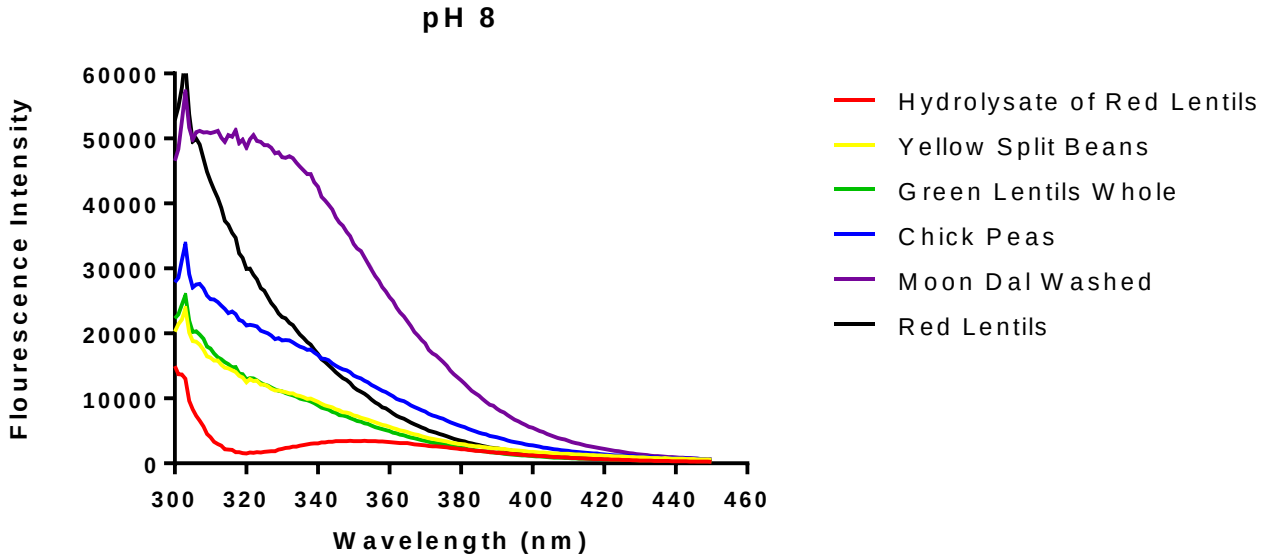

Figure 31: Fluorescence of selected concentrates at 280 nm and pH 8

4.6.4.4. Fluorescence D: 295 nm

The excitation wavelength of 295 nm, selectively excite tryptophan (Trp) only (Schmid, 1989). The fluorescence spectrophotometry was carried out at 295 nm and pH 2, as shown in Figure 32. MDW showed a double peaked primary intensity which had the highest quantum yield, way higher than its tertiary fluorescence peak which was closely leveled in height with RL; they were the next highest in fluorescence intensity. YSB, CP followed next, and GLW and HRL were the next highest in fluorescence intensity. The secondary peak intensity of MDW showed the lowest quantum yield. Figure 33 shows the results of the fluorescence spectrophotometry carried out at 295 nm and pH 3. At this wavelength and pH, we did not detect any positive peaks in any of the concentrates. This could have been due to the proteins being exposed to a hydrophilic environment, within the buffer. The results of fluorescence spectrophotometry carried out at 295 nm and pH 4 is shown in Figure 34. MDW again showed the highest fluorescence intensity, and therefore, the highest quantum yield. It is followed by HRL and the first peak from CP. The second fluorescence peak from CP and RL peak are the next highest in fluorescence intensity. GLW was next while YSB had the lowest fluorescence intensity. Figure 35 shows the results of fluorescence spectrophotometry carried out at 295nm and pH 5. Here, CP, HRL and MDW had the highest fluorescence intensity, in that order. The secondary peak from CP was the next highest, followed by RL, then GLW and YSB which had the lowest fluorescence intensity. Fluorescence spectrophotometry was also carried out at 295nm and pH 6, as shown in Figure 36. The spectrum looked very similar to that at pH 5. However, HRL had the highest fluorescence intensity and was followed very closely by CP. The next highest was, again, the secondary peak from CP. It was followed by GLW and RL, YSB, again, had the lowest fluorescence intensity. Figure 37 shows the results of fluorescence

spectrophotometry was also carried out at 295nm and pH 7. RL had a double peaked primary fluorescence intensity and was the highest. The first peak from MDW was the next highest and was followed by CP and the secondary intensity peak from MDW, then HRL, YSB while GLW had the lowest fluorescence intensity and quantum yield. The fluorescence spectrophotometry carried out at 295nm and pH 8 is shown in Figure 38. MDW showed a double primary peak and had the highest fluorescence intensity. This is followed by RL which had multiple peaks and then the secondary peak of MDW. The next highest fluorescence intensity is seen in the peak from CP, followed by YSB and GLW. HRL had the lowest fluorescence intensity and quantum yield.

Vivian and Callis, (2001) reported that the fluorescence peak wavelength of tryptophan in proteins is predicted by the charges (negative or positive), hydrophilic environment and the α-helix and β-strands of the protein in question.

Just like for the other wavelengths measuring excitation of the aromatic amino acids, the spectra at 295 nm pH 2 showed characteristics of unordered protein concentrates, which are unfolded and unstructured, making them potentially easily digestible in the human body. The kinetics of digestion at pH 7, shown in Figures 8 and 9, is in tandem with these findings showing the digestion and bioavailability of each of the concentrates during the simulated digestion.

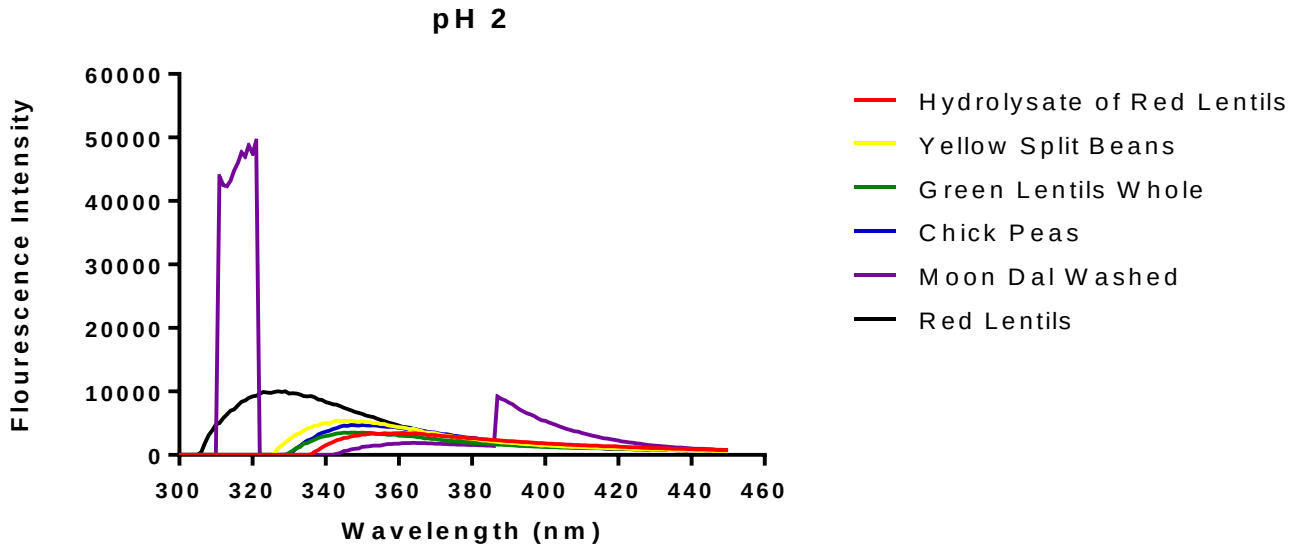

Figure 32: Fluorescence of selected concentrates at 295 nm and pH 2

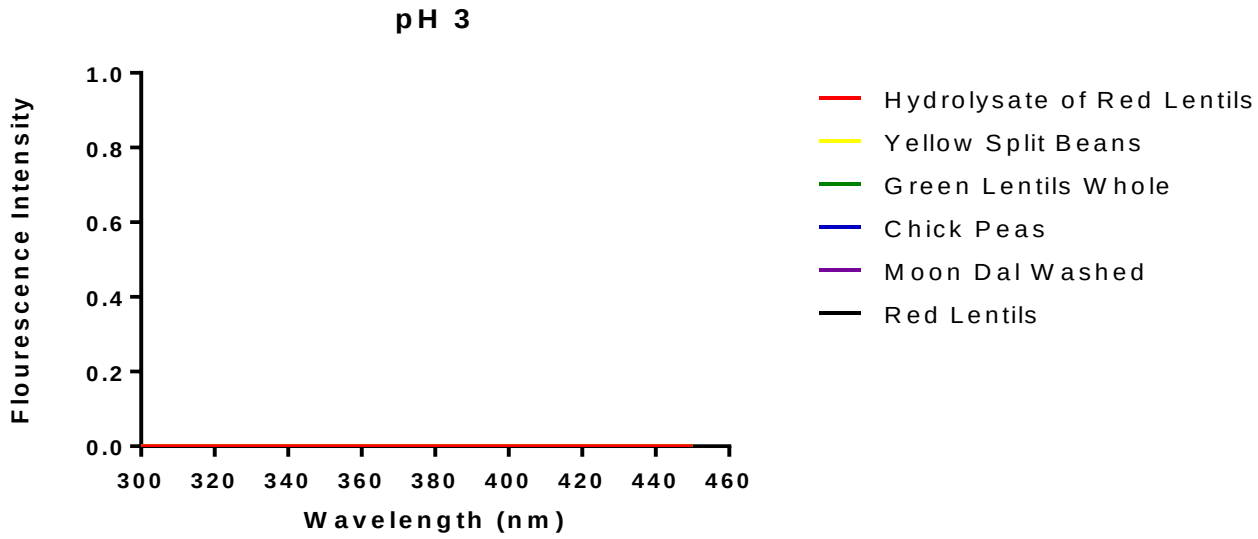

Figure 33: Fluorescence of selected concentrates at 295 nm and pH 3

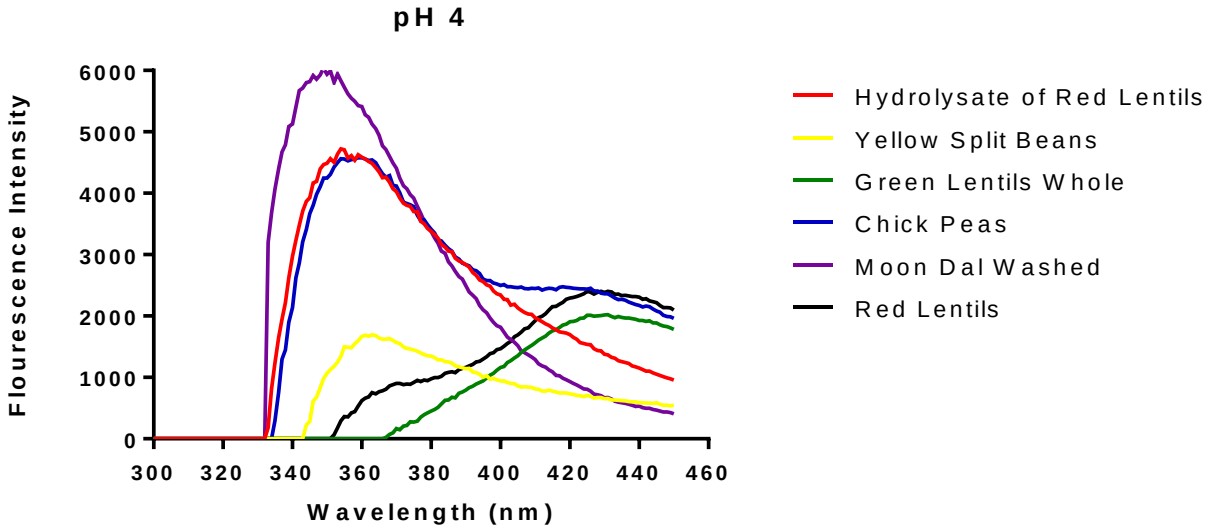

Figure 34: Fluorescence of selected concentrates at 295 nm and pH 4

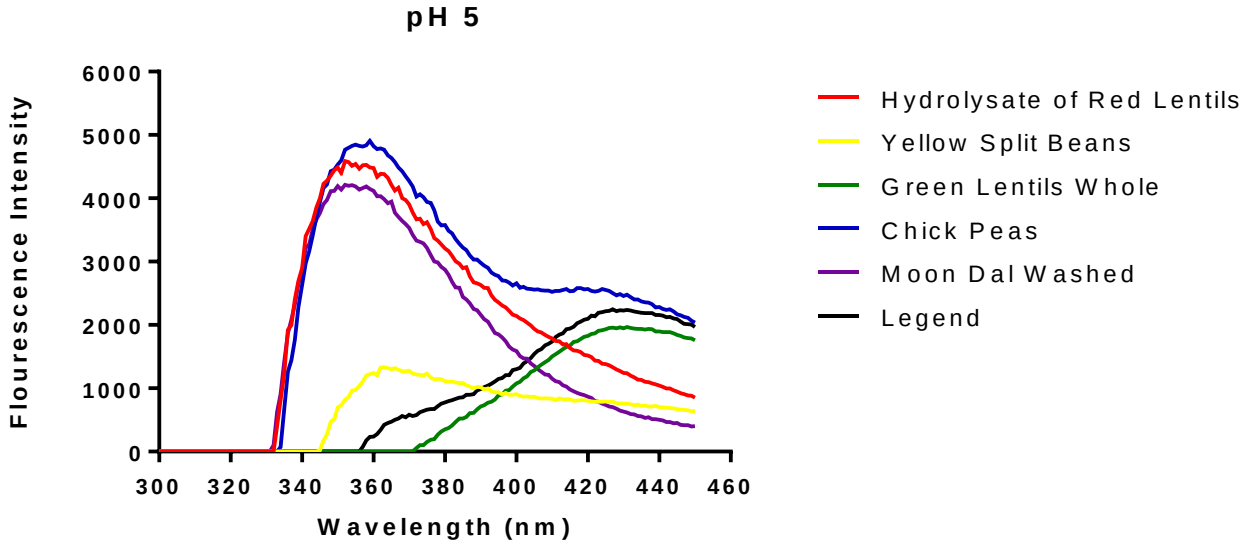

Figure 35: Fluorescence of selected concentrates at 295 nm and pH 5

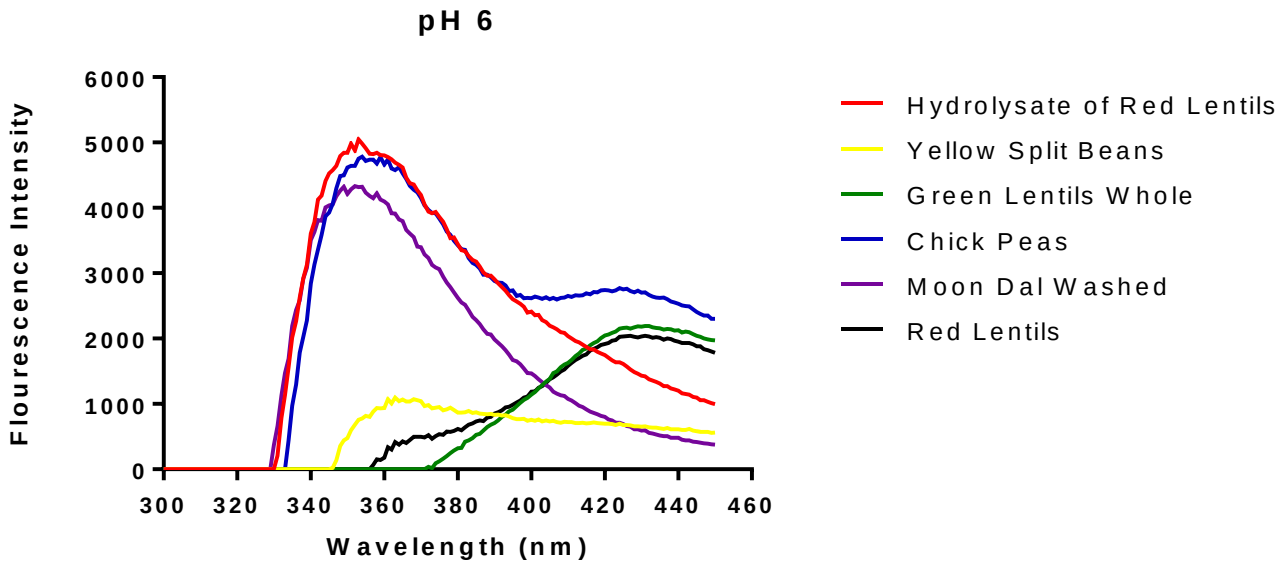

Figure 36: Fluorescence of selected concentrates at 295 nm and pH 6

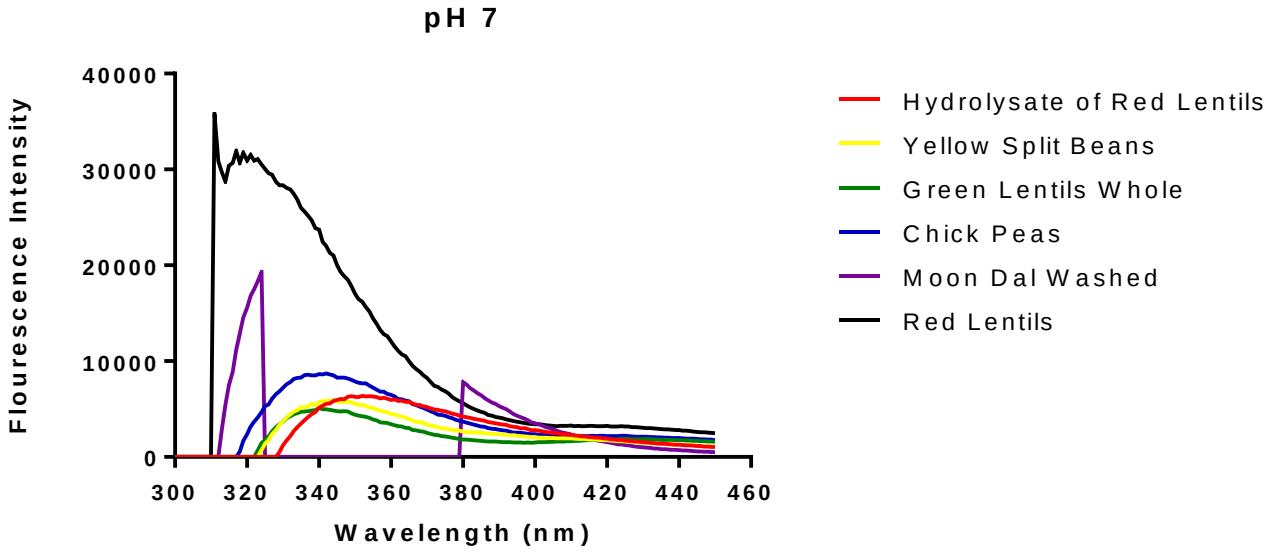

Figure 37: Fluorescence of selected concentrates at 295 nm and pH 7

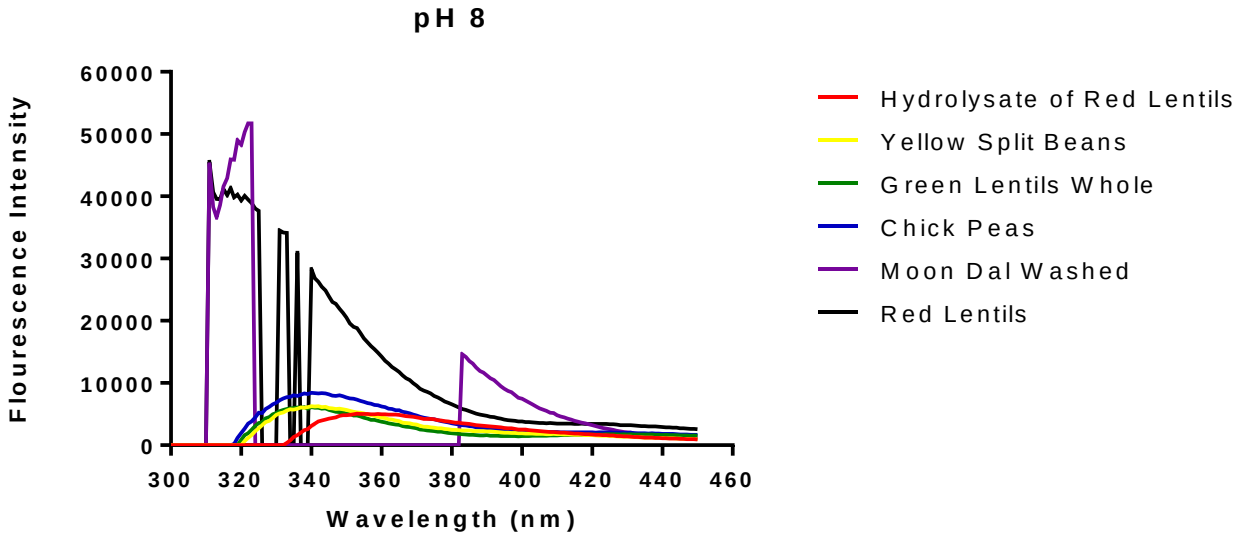

Figure 38: Fluorescence of selected concentrates at 295 nm and pH 8

4.7 CIRCULAR DICHROISM (CD) SPECTROSCOPY

The results of Circular Dichroism (CD) spectroscopy which was used to monitor changes in protein folding and changes in conformations (to examine the structure of proteins in solution) is shown in Table 7, and describes the fine details of the aggregated proteins. It shows mean α- Helix 1 ranging between 0.4 and 1.4 with YSB having the lowest and RL having the highest α- Helix 1, MDW had a mean value of 1. α- Helix 2 ranged between 3.9 (YSB) and 4.9 (RL and CP). MDW had a mean value of 4.3. β- Strand 1 had mean values between 23.1 and 25.45. The highest mean value was seen in the CD of GLW and the lowest in CP. β- Strand 2 mean value ranged very closely together, being between 11.95 and 12.15.

Overall, the CD data indicates that all the ferritin concentrate had very similar secondary structures. This is supported by the results of the fluorescence spectrophotometry at pH 7, where all the concentrates showed similar unordered protein structures. The kinetics of digestion, as well, showed concentrates that are readily digested and available for absorption, which is characteristic of unfolded and denatured aromatic amino acids.

Near-UV spectra in Figure 39 show the ellipiticity values at pH 7.0 for all the concentrates. MDW showed the highest ellipticity. It increased steadily, peaking at 270 nm wavelength and then declined steadily as well, reaching equilibrium at about 305 nm. The RL had the next highest ellipticity, followed by GLW. YSB, CP and HRL showed much lower ellipiticity values in that order, indicating that, even with the same pH treatment, quite unlike the results observed in the far-UV, the concentrates showed distinct tertiary structures. This suggests that there are factors other than pH which affect the shape, structural transition and magnitude of the near-UV spectra of proteins. The peaks seen in MDW, RL and the GDW are indicative of the presence of more compact and highly structured protein concentrates. This, however, does

not support the florescence spectra at pH 7 applying different wavelengths, which showed unordered and unfolded protein structure. Kelly et al. (2005) reported other factors including the number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability) and their spatial disposition in the protein.

Table 7: Circular Dichroism (CD) Spectroscopy, Showing Near-UV and Far-UV of the Concentrates

Sample	α- Helix 1	α- Helix 2	β- Strand 1	β- Strand 2	Turns	Unordered
Yellow Split Beans (YSB)	0.4 ±0.1	3.9±1.3	23.45±1.35	11.65±0.65	18.55±0.25	42.05±0.85
Green Lentils Whole (GLW)	1±0.7	4.05±1.15	25.45±1.25	11.95±0.65	18.9±0.8	38.6±0.8
Chick Peas (CP)	0.9±0.7	4.9±1.5	23.1±2	12.15±0.65	19.6±0.2	39.4±0.2
Moon Dal Washed (MDW)	0.8±0.4	4.3±1.4	23.85±1.85	12.15±0.75	19.05±0.15	39.85±0.95
Red Lentils (RL)	1.4±1	4.9±1.5	23.55±2.15	12.05±0.75	19.25±0.15	38.85±0.55
Hydrolysate of RL	1.15±0.85	4.05±1.35	24.6±1.3	12±0.7	19.55±0.95	38.6±0.7

Results are presented as mean±standard deviation (n=2)

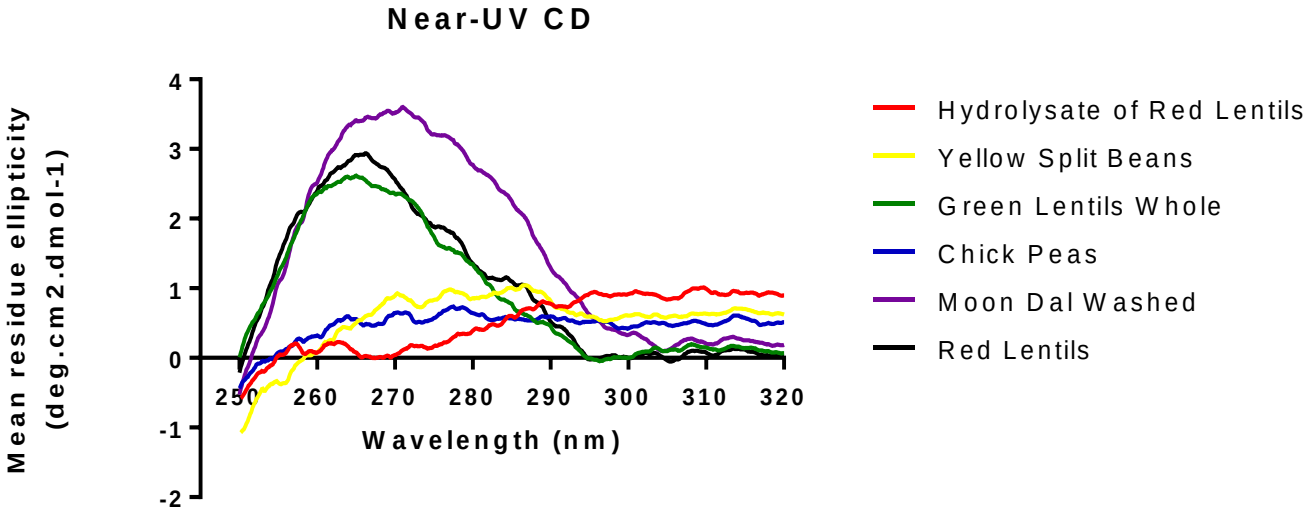

Figure 39: Near-UV circular dichroism spectra of selected concentrates

4.8 KINETICS OF IRON RELEASE FROM FERRITIN DURING SIMULATED GASTROINTESTINAL DIGESTION

4.8.1 KINETICS OF IRON RELEASE DURING PEPSIN DIGESTION

The results of kinetics of iron release from ferritin during simulated gastrointestinal digestion with pepsin are shown in Figure 40. HRL showed minimal release within the first 20 min and then increased slightly all the way to the 120th min. This is because after food is consumed, there is a postprandial period before enzyme secretion and subsequent digestion which will explain the lag in commencement of the digestion process. The release of YSB and HRL increased, albeit slightly, but consistently from 0 to 120 minutes which is a slower release rate than the MDW, which was more rapidly released. This can be an indication that they are a little bit resistant to digestion, or it could be related to the FPLC results described above, which indicated that at pH 2 and pH 7, all the concentrates had unstructured spectra, suggesting that they had already been denatured and did not require the pepsin addition for their bioavailability, through digestion. The result of the FPLC is further corroborated by the samples RL, GLW and CP, which all maintained the same level of release from 0 to 120 min of simulated digestion as a result of the ferritins being already denature at the stomach digestion pH. The greatest reaction was observed in the MDW where there was a lag period from 0 to just over 20 min, after which there was a spike in release up till the 60th min and then there was a decline all the way till the end of 2 hr of the simulated digestion process. The initial lag is consistent with a rigid structure that was measured in the fluorescence assays. However, as time progressed, the rigid structure was overcome by the proteases, which led to iron release. The rapid, steady decline in iron release observed after 60 minutes could be an indication that the iron supply had been exhausted. This is consistent with the action of pepsin

which works to hydrolyse the polypeptides into peptides. The findings of the kinetics of release agree with the fluorescence spectrophotometry and the CD data, all indicating that the unfolded and unordered structure of the protein concentrates used in this study was susceptible to pepsin digestion in a simulated experiment, making it potentially bioavailable. These findings are in synch with the results obtained at pH 7 in the fluorescence spectra, which showed unordered and unstructured proteins concentrates as well.

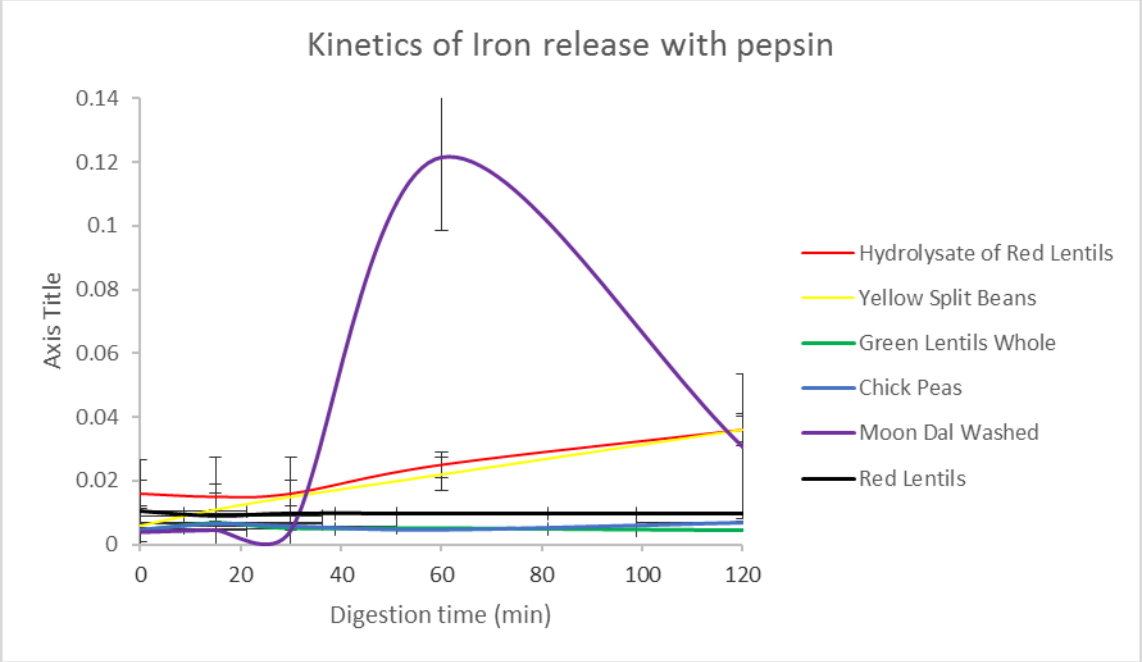

Figure 40: Kinetics of iron release during pepsin digestion

4.7.2 Kinetics of Iron Release during Pepsin-Pancreatin digestion

Figure 41 shows the results of kinetics of iron release from ferritin during simulated gastrointestinal digestion with pepsin-pancreatin digestion. During this simulated process, all the concentrates except MDW showed a lag phase pf release from 0 min of the experiment and they retained that stage all the wat till the end of the process at 120 min. The MDW concentrate, on the other hand, reacted very similarly to the kinetics of iron release in MDW described during simulated gastrointestinal digestion with pepsin, where there was a lag period from 0 min to about 30 min, after which there was a spike in release up till the 60th min and then there was a decline all the way till the end of 2 hr of the simulated digestion process. These results show that the unstructured proteins, which were readily digestible in the simulated stomach are bioavailable and so, can be absorbed during intestinal digestion.

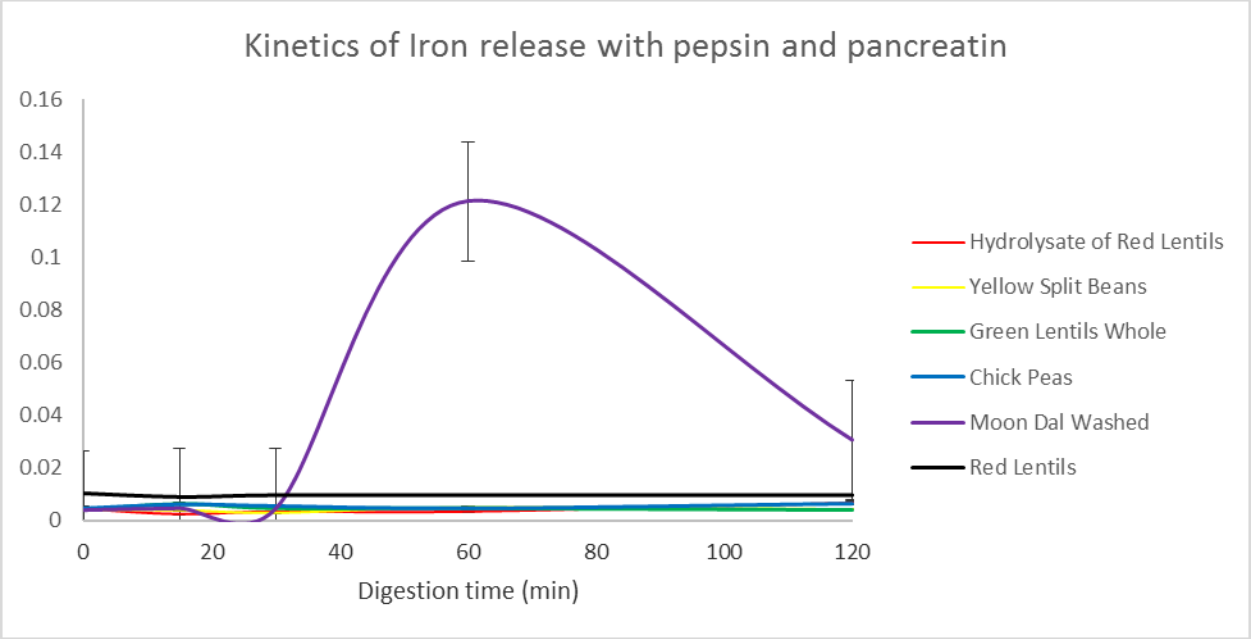

Figure 41: Kinetics of iron release during pepsin-pancreatin digestion

4.8 PEARSON CORRELATION ANALYSIS

Statistical analysis showed very strong correlation (the possible linear association between continuous variables) between the iron content, percent protein content and iron to protein ratio in all the sample. This is supported by the findings observed in Figure 5 and Table 4 where all the concentrates had the highest iron:protein ratio and showed no significant differences in their iron concentration. The concentrates with the highest iron concentrations also had the highest iron:protein ratios of the samples, agreeing with the reports of Zielinska-Dawidziak & Siger (2012), who reported that ferritin iron is proportional to the total iron concentrations of the seed source. They obtained iron concentrations of between 50 and 60% of the total iron concentration.

The correlations were significant at $p \leq 0.05$ for all the variables as shown in Table 8. This indicate that iron in each sample correlated with percent protein content, as well as the iron:protein ration. This supports our selection of samples which had the highest protein ration to further our research work on, since higher iron:protein ration tends to mean higher iron concentration. It is of advantage that the selected concentrates also had the higher ferritin yield of all the samples.

Table 8: Pearson correlation analysis between Iron content, percent protein content and iron to protein ratio of the concentrates

Correlations

		Iron	Percent protein	Ratio
Iron	Pearson Correlation	1	-0.664	0.905*
	Sig. (2-tailed)		0.150	0.013
	N	6	6	6
Percent protein	Pearson Correlation	-0.664	1	-0.715
	Sig. (2-tailed)	0.150		0.111
	N	6	6	6
Ratio	Pearson Correlation	0.905*	-0.715	1
	Sig. (2-tailed)	0.013	0.111	
	N	6	6	6

*. Correlation is significant at the 0.05 level (2-tailed).

CHAPTER FIVE

5.0 GENERAL DISCUSSION AND CONCLUSION

The World Health Organization (WHO) has recognized IDA as the single, most prevalent nutrient deficiency in the world. Several researchers have also identified the fact that there are populations that suffer IDA as a result of poverty, and the subsequent insufficient consumption of iron. There are also populations, especially in the developed world, which suffer from IDA in spite of the fact that their food intake contain adequate dietary iron and should supply same. Scientists have identified the presence of substances in plant diets (example, phytate and polyphenols), which act as anti-nutrients and block the absorption of dietary iron in the human body. This is especially a problem in vegetarian diets.

WHO has, therefore, recommended that the use of organic forms of the micronutrient be encouraged and made to replace the inorganic forms of iron which are not bioavailable and are absorbed only minimally even when taken as concentrated supplements.

This study examined the production of ferritin concentrates that can be used as ingredients to formulate nutraceutical products for IDA treatment, with the aim of such a concentrate being prepared in the form of pills or tablets for industrial production.

Even though meat products contain the heme iron (the more readily available form), the choice of phytoferritins (non heme iron, a less readily available form) for this study stems from the fact that legumes are relatively inexpensive when compared to meat products. As well, they are readily and easily available in large quantities in Canada, with Manitoba and Alberta being the largest producers.

Previous reports have indicated the presence of purified ferritin contents in legumes, albeit, in relatively small quantities. In this research, ferritin concentrates were extracted and characterized from 14 different legumes and a commercial pea protein concentrate.

The potential of phytoferittin was carefully measured according to standard methods. Briefly, the isolation process was optimized and the resulting concentrates were subjected to digestion with exopeptidase with a view to reducing the protein content while enhancing the iron level. Amino acid composition was determined, and the fractions of the concentrate were separated by gel electrophoresis. They were further subjected to a simulated digestion process to observe the action of stomach enzymes on the digestion process of ferritin and its bioavailability. The kinetics of iron release was also studied, as well as measurement of circular dichroism (CD) to study the secondary and tertiary structures of the concentrates.

Results of the findings clearly revealed the presence of ferritin in the concentrates and the 26 and 28 kDa bands were observed in the electrophoresis gel. The digestion process revealed that the protein polypeptides were broken down into smaller peptides, showing the digestion and breakup of the protein cage of the concentrates and the release of individual amino acids which can be readily available for absorption. Iron was released at the stomach pH and temperature, after an initial lag phase. Florescence spectrophotometry showed that the concentrates were subject to digestion and CD showed the presence of secondary structures, which were similar in all the concentrates. It also revealed the presence of tertiary structures which showed unique structural transition in each of the concentrates.

Overall, the findings of this work prove that the optimized protocol produced ferritin concentrates. These concentrates obtained also showed loose structures at pH 2 and 7, and are

susceptible to proteolysis when treated with gastrointestinal proteases. This is important for stomach and intestinal digestion and subsequent bioavailability

In conclusion, we have ferritin concentrates from legumes show all the intrinsic characteristics required for easy iron release, digestion and absorption as characterised by their structures. It can potentially play a critical role in the treatment and prevention of IDA, as a more bioavailable form of iron.

CHAPTER SIX

6.0 FUTURE RESEARCH

From the findings of this research, ferritin has all the parameters of being a better iron supplement than inorganic iron during treatment of IDA, as shown during in vitro experiments.

Further research will need to be conducted to determine the effect of the concentrates in vivo.

The first stage will be animal trials where rodents with IDA will be treated with various concentrations of the ferritin concentrates and their therapeutic effects as well as toxicity determined. If the findings are promising, the next step will be randomized clinical trials among susceptible groups of patients to determine their reaction to treatment as well as the dosage required for optimum iron levels, without the dangerous side effects of iron overload.

Since eventual commercialization is one of the objectives of the project, viability of production will need to be assessed in terms of a proper cost analysis to maximize profit while delivering the expected outcome in terms of IDA alleviation. In view of the fact that there is a ready market, it is hoped that, after a series of positive clinical trials, the concentrate can be produced industrially as pills and tablets or as an additive in foods.

Finally, the mechanism of the treatment process will need to be determined.

CHAPTER SEVEN

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