# EFFECT OF HIGH PRESSURE PROCESSING ON SELECTED PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF YELLOW LENTIL PROTEIN CONCENTRATE

By

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ABSTRACT OF THE THESIS

EFFECT OF HIGH PRESSURE PROCESSING ON SELECTED PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF YELLOW LENTIL PROTEIN CONCENTRATE

By Sawali Navare

Thesis Director: Professor Mukund V. Karwe

There is a growing interest in utilizing plant proteins as functional ingredients in foods, in an effort to address the consumer demand for 'clean label' or only natural ingredients in foods. Plant proteins can be used as emulsifiers, foaming agents and fat binding agents in order to replace synthetic food surfactants such as Sodium Tri-polyphosphate. High Pressure Processing (HPP) can bring about structural modifications in the plant proteins and increase their functionality. The aim of this project was to study the effect of High Pressure Processing parameters on selected physicochemical and functional properties of yellow lentil protein concentrate.

Yellow lentil protein concentrate (YLPC) solutions (10% w/w) were subjected to high pressure processing, followed by freeze drying. Experiments based on Box- Behnken (BBD) design were carried out, with Pressure (MPa), time (min), and pH of the protein solution as the three independent factors. The freeze dried samples were then analysed for emulsification capacity, foaming ability, solubility, fat binding capacity, surface hydrophobicity, and surface zeta potential. These analyses were carried out in two different pH systems, namely, pH = 3 and pH = 7. Unprocessed yellow lentil protein concentrate was used as the control

It was observed that lower pressures and intermediate treatment times at neutral pH (=7) protein solutions were most effective in improving the functional properties of the protein. The solubility of the protein in a pH 7 system increased from 40% ± 3% for unprocessed control to 50% ± 5% for the sample processed at 150 MPa. The emulsifying ability of the protein decreased at higher pressures of 350 MPa and 550 MPa. In the pH 3 system, the droplet size of the dispersed phase of the emulsion stabilized by the protein sample processed at 550 MPa increased to 24 µm ± 0.2 µm from 16 µm ± 0.5 µm for the unprocessed control. Similarly, in the pH 7 system, the droplet size increased from 8 µm ± 1.5 µm for the emulsion stabilized by the unprocessed control to about 11 µm ± 2 µm for emulsion stabilized by the protein sample treated at 550 MPa. However, samples processed at these pressures imparted higher stability to the emulsions- around 89% ± 2% compared to 82% ± 3% for the emulsions stabilized by the unprocessed control. Foaming ability was not significantly affected by the HPP treatment in both systems: pH 3 and pH 7. The surface hydrophobicity increased from $H_0$ = 2800 ± 228 for unprocessed control to $H_0$ = 6400± 51 for the sample processed at 350 MPa Surface zeta potential was not significantly affected by the three independent factors studied. Different factors or combination of factors played a significant role in the changes in each of these functional properties.

In conclusion, pressure treatments at lower pressures (150 MPa, 10 min) at neutral pH were found to be better at improving the functional properties compared to higher pressures and longer times at lower or neutral pH values.

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# 1. INTRODUCTION

High pressure processing (HPP) has been extensively studied as a cold pasteurization technique, as well as in combination with mild heat (40 ºC - 50 ºC) treatment to inactivate micro-organisms in food. This technique is now being explored for its potential in the development of foods or ingredients with enhanced functionalities due to structural changes that occur in the food constituents during this process. The aim of this study was to evaluate the effect of HPP on the functional properties of a plant protein, specifically yellow lentil protein. The goal was to create natural functional ingredients that can potentially replace artificial additives currently used in processed foods.

## 1.1 Proteins

Proteins are biologically active molecules that play a variety of roles in the plants and animals. They make up the tissues that provide for structure and the enzymes that control its function. In food, proteins are essential for texture, structure, functionality, and nutrition. These properties are due to hydration, solubility, gelation, coagulation, surface activity of proteins.

1. The ability of proteins to interact with water (hydration and solubility)

Hydration is the first and most important step in imparting functionality to proteins and other functional properties usually are dependent upon how well the protein can hydrate. The properties of water bound by proteins are different from those of free water. Swelling, water holding capacity and gelling are dependent on the level of protein hydration. Protein water interaction takes place at specific sites on the protein molecule. Polar amino acids on the protein molecule usually bind water. Many factors contribute

towards this interaction, both structural and environmental. The conformation of the protein molecule, the nature and total number of the available binding sites are the structural factors that determine protein-water interactions. If the protein undergoes conformational changes from a compact to a more open, unfolded state, previously buried amino acids are exposed, which results in altered protein binding with surrounding molecules. In addition, the pH of the environment, salt concentration, temperature, and mechanical disruption dictate protein hydration because of changes in the structure and consequently the solubility of the protein increases or decreases (Pace et al., 2004).

2. Interaction with other proteins causing them to gel, precipitate, and coagulate

Protein water interaction causes an increase in the viscosity of the solution due to protein swelling. Heat and acidity can denature viscous protein solutions to form reversible/irreversible gels. Protein coagulation is usually irreversible because disulfide bond formation is involved in the process, for example, egg albumen coagulated by heat. Protein-protein interaction within gels leads to the expulsion of water and a stronger gel. Usually greater than 10% concentration of proteins is required for the formation of a gel network. This network is stabilized mainly by disulphide bonds and a balance of attractive and repulsive forces on the protein structure. If there are a greater number of disulphide linkages stabilizing a gel, it is irreversible (coagulation). Thus, gel formation usually involves the immobilization of water by proteins (Schmidt, 1981).

3. Surface properties which enable them to form emulsions and foams

Adsorption of proteins onto air-water and oil-water interfaces is due to the amphiphilic nature of proteins. Proteins interact with non-polar molecules like fat/ air and form stable foams and emulsions. High solubility in the continuous phase and the ability to form an interfacial layer around the dispersed phase leads to the formation of foams/emulsions. Repulsion between the proteins covering the air bubble/oil droplet keeps the dispersed phase from coalescing (Chou, et al., 1979).

Some examples of the role of proteins in foods:

- Milk proteins play a structural role in ice cream. When the ice cream mix is homogenized, the milk proteins emulsify the fat phase and keep the fat from coalescing into a distinct phase. This is because some proteins get adsorbed onto the interface between fat and water. During the whipping stage, the proteins entrap air which leads to the formation of a stable foam. Here, proteins get adsorbed onto the air-water interface. Proteins that do not get adsorbed onto these interfaces increase the viscosity of the ice-cream mix, which leads to an improved texture and reduces the formation of large ice crystals detrimental to the quality of the ice cream (Goff, 2016).

- Egg white proteins, on application of shear in the form of whisking, can bind and entrap air in their structure, which causes them to form a strong foam (Pernell et al., 2002).

- When the pH of milk is lowered by addition of acid, the casein milk proteins coagulate. When the pH of the milk approaches its isoelectric point, the net negative charge on the casein molecules decreases, which leads to a decrease in the electrostatic repulsion between casein molecules. They start interacting via hydrophobic interactions which causes the formation of a three dimensional network of casein molecules in the form of

chains and clusters. This imparts the necessary structure and texture to yogurt (Yogurt made simple, Washington State University Extension Program).

- The Maillard reaction between amino acids and reducing sugars in cakes/breads imparts the brown colour to these foods due to the formation of colored compounds (Manley,2011).

### 1.1.1 Structure of proteins

The structure of proteins is what imparts them their biological activity and functionality. Manipulation of the environment they are in has a significant effect on their structure, and consequently, on their activity and functionality. This environment includes pH, ionic strength, temperature, pressure. These factors affect the protein folding and unfolding, which determines which functional groups are exposed to the surroundings and which are buried in the interior of the structure. This in turn influences the affinity of the protein to polar/ non-polar compounds (Kinsella et al., 2009).

Proteins consist of amino acids. An amino acid is a compound which contains an amino group, a carbonyl group, and a side chain. The twenty different amino acids that exist in nature are distinguished by the functional group on the side chain.

When two amino acids undergo a condensation reaction to lose a molecule of water, they lead to the formation of a peptide linkage. Chains of different sequences of amino acids held together by –CO-NH- or peptide linkages are called proteins. These sequences held together by peptide linkages constitutes the primary structure of the protein. Each

chain of amino acids thus has two ends- the amino (NH) terminus and the carboxyl (-CO-) terminus.

The functional groups on amino acid side chains are either polar or non-polar and can participate in various chemical interactions with each other and with the environment. These include hydrogen bonding, van der Waal interactions, hydrophobic bonds, and ionic interactions. Due to these interactions, proteins undergo folding, which leads to the formation of unique stable structures such as ά-helix, ß-sheet, loops or turns. Secondary structures are usually associated with hydrogen bonding between the primary amino acid sequence chains. Several such patterns of hydrogen bonding interactions will lead to the formation of the secondary structure of protein. This pattern describes the folding that occurs due to interaction of neighbouring residues in a segment of a polypeptide chain.

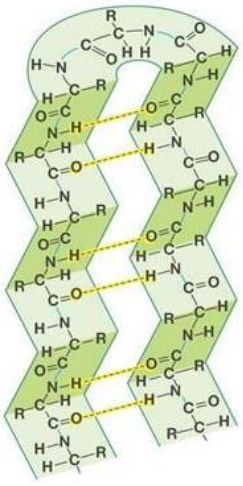


Figure 1. β-sheet conformation- one of the secondary protein structures (http://cbm.msoe.edu/teachingResources/jmol/proteinStructure/secondary.html)

Such stable secondary structures interact with each other through ionic interactions such as formation of salt bridges or disulphide bonds. This leads to a tightly packed, compact structure in which the hydrophobic pockets are buried in the interior and the hydrophilic/polar regions orient themselves towards the surface. This is called the tertiary structure of the protein. It describes the three dimensional structure of protein- how it is arranged with respect to different segments and residues within fragments in space (Lehninger et al., 1970).

When such tightly packed subunits of proteins with two or more polypeptide chains interact through the same non-covalent interactions as the tertiary structure, the resulting three dimensional arrangement is known as the quaternary structure of proteins. Proteins that have just one polypeptide chain do not have quaternary structures. The quaternary structure describes how two or more chains are spatially arranged with respect to each other.

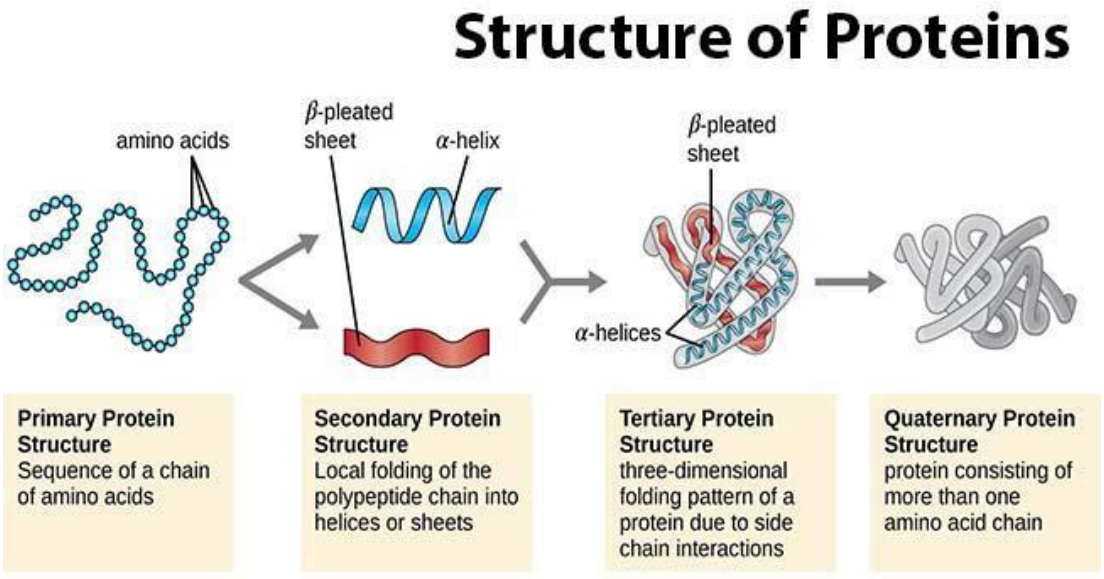


Figure 2. Differences in primary, secondary, tertiary and quaternary protein structures (https://www.biosciencetimes.com/study-notes/protein-structure/169/).

Based on their shapes, proteins can either be fibrous or globular. Fibrous proteins are characterized by long, narrow strands that are generally insoluble in water. They usually have a structural role in the plant/animal body. For example, the collagen, fibrin, keratin proteins that make up the muscles and tissues are fibrous proteins. Globular proteins have a compact, spherical shape and are generally soluble in water. They are sensitive to the environment they are in- the temperature, pH, ionic concentration and are usually play functional roles in the plant/animal body. Enzymes are mainly globular proteins (Lehninger et al., 1970).

## 1.2 Physiochemical properties of proteins

### 1.2.1 Surface hydrophobicity

Surface hydrophobicity of a protein surface is a measure of its non-polar character. It dictates the interaction of the protein with non-polar solvents or other non- polar components in its environment. This is important in determining the efficacy of the protein as a functional ingredient. Consequently, surface hydrophobicity and solubility usually follow an inverse relationship as solubility is enhanced with an increase in the surface charge (Hayakawa et al., 1985). An optimum balance in the hydrophobic and hydrophilic character thus might be ideal for the protein to possess good surface activity. Since the hydrophobic groups of amino acids are buried in the interior of the protein structure, partial unfolding of the structure leads to an increase in the surface hydrophobicity of the protein (Yin et al., 2008). Thus, measuring surface hydrophobicity can give an insight into the

changes in protein conformation. This measurement is done using a fluorescent dye which can bind to the hydrophobic regions and emit fluorescence in apolar environments. Measuring the intensity of the emitted fluorescent signal can give an estimation of the hydrophobicity of the surface. A study by Yin et al. (2008) on the effect of high pressure processing on the physicochemical properties of red kidney bean isolate found that the surface hydrophobicity of red kidney bean isolate increased with an increase in the pressure treatment.

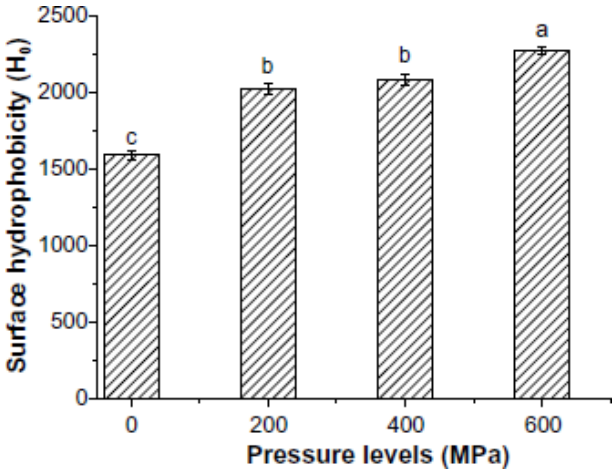



Figure 3. Increasing surface hydrophobicity of Red Kidney Bean Isolate treated at different pressure levels, for 10 min in a pH 7 solution (Yin et al., 2008)

### 1.2.2 Surface zeta potential

There is a development of a net charge on the protein surface when it is dispersed in water, due to the dissociation of functional groups of amino acids. Another reason for the development of this charge is ion adsorption at the surface. This leads to the formation of a layer of balancing opposite charges near the surface. The inner layer of charges

immediately surrounding a protein particle is called the ‘Stern layer’ and the layer of opposite charges is called the ‘Diffuse layer’ (Salgin et al., 2012). The potential difference between the diffuse layer and the surrounding liquid is called the zeta potential. Thus, it is an important indicator of conformational changes such as folding and unfolding of a protein molecule. It could also be an indicator of the solubility of a protein as a higher net charge implies greater interaction with polar solvents. At the isoelectric pH of a protein, the net charge and consequently, the zeta potential of a protein is zero. Since pH affects the charge distribution on a protein, it also affects the zeta potential. Processing treatments like heating or high pressure processing will cause partial or complete denaturation of a protein which will have an impact on the zeta potential. Zeta potential is measured using the speed of mobility of ions towards oppositely charges electrodes (Hunter, 1981).

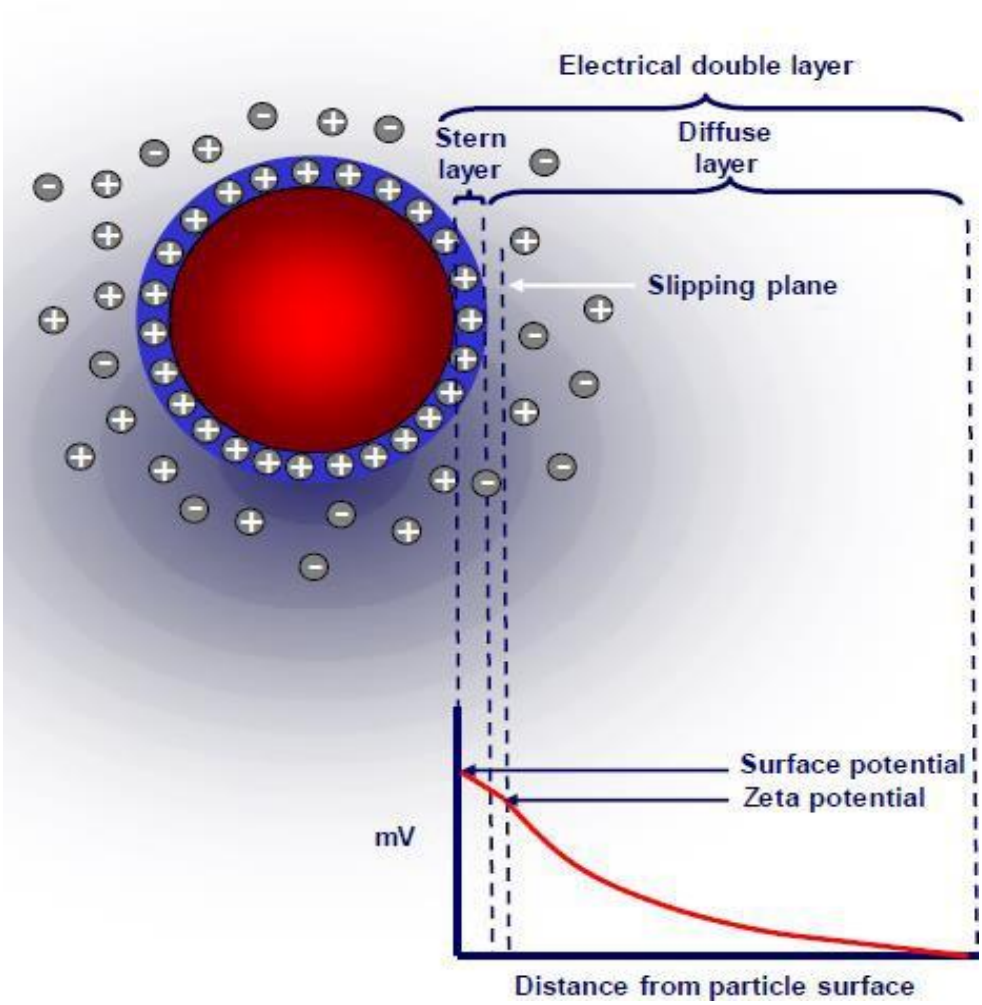



Figure 4. Schematic representation of surface zeta potential and electric double layer surrounding a dispersed particle in solution (Garcia et al., 2014).

## 1.3 Functional properties of proteins in food systems

Proteins can perform various functions in food due to their physicochemical properties such as surface charge and surface hydrophobicity, and due to their structural properties like conformation, flexibility and molecular shape and size. These properties affect the interactions of proteins with other components of the food system and/or the environment surrounding the food system, such as water, air, fatty acids or triglycerides,

other proteins and polysaccharides. These interactions can be useful for specific attributes of a food system. For example, strong interaction of proteins with air helps to form stable foams. The functional properties that were studied in this project are described below.

### 1.3.1 Emulsification

Stable emulsions are usually homogenous two-phase systems involving a dispersed phase (usually in the form of droplets) and a continuous phase. For example, fat globules in milk are the dispersed phase and water is the continuous phase. A variety of processed foods exist as emulsions, such as milk based beverages, sauces, salad dressings, ice-cream, imitation meat products. Smaller the droplet size of the dispersed phase, higher is the stability of the emulsion.

Amino acids in a protein structure can be hydrophobic/non-polar or hydrophilic/charged. They can also have charged/uncharged side chains. This property makes them amphiphilic in nature. Proteins can stabilize emulsions by adsorption at the interface of the two liquids, due to the protein surface activity. This surface activity of proteins is due to the hydrophobic amino acids/side chains of amino acids in the protein structure. These hydrophobic parts bind to the non-polar phase (oil droplets), forming a viscoelastic/sticky monolayer around the droplet. The repulsion between two such sticky oil droplets is what keeps them from combining or coalescing into one larger drop. This helps lower the tension/energy at the interface, which in turn helps reduce the size of the dispersed phase droplets. Since less energy is now required for the formation of a droplet,

a greater number of droplets can be created. Greater number and a smaller size of droplets means longer time for them to come together and phase separate (Zayas, 1997).

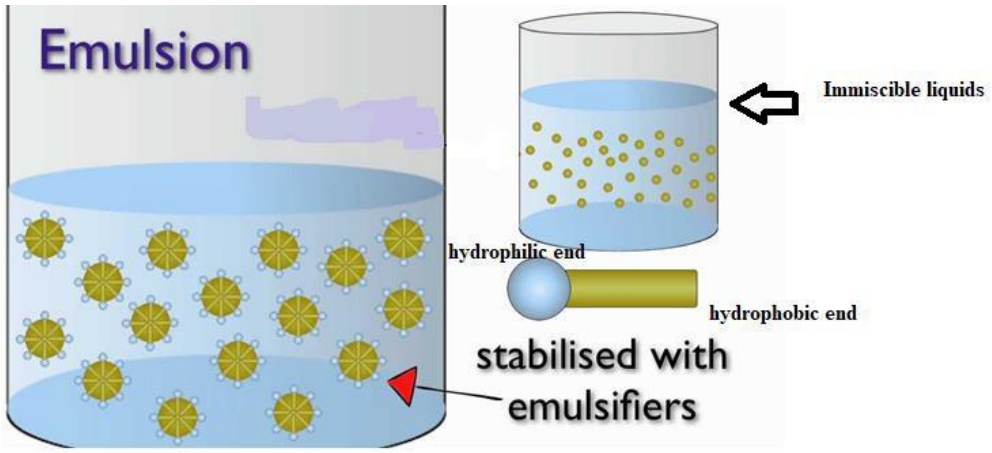



Figure 5. An oil in water emulsion (modified from http://www.molecularrecipes.com/emulsions/)

### 1.3.2 Foaming

When air is trapped by the protein in the food system, a foam is formed. Foams are important components of whipped creams, ice creams, meringues, leavened breads, etc. For example, beer foam is stabilized by hop proteins (such as the Avenin-like protein ALP in barley). The entrapment of air bubbles works much the same as emulsion droplet formation. When air is made to enter the solution/food system by the action of whipping or homogenization, the proteins diffuse to the interface of the air and the liquid, lowering the surface tension. Thus, a large number of tiny bubbles are created with a viscoelastic protein layer surrounding each. The hydrophobic amino acid groups interact with air through hydrophobic interactions and the charged moieties orient towards and form

hydrogen bonds with the water in the system. This process requires the protein molecules to undergo slight denaturation on reaching the interface, which involves unfolding of the quaternary structure of proteins into tertiary structure in such a way that the final conformation has the least free energy. Higher the surface activity of the protein involved, greater is the speed with which this transition occurs. Electrostatic repulsion between the films surrounding adjacent air bubbles keeps them from coalescing. Thus, the speed of migration of the proteins towards the interface, the surface activity, and the flexibility of the protein molecule dictate foam formation. Whereas, the size of the bubbles (smaller, better foam formation), polarity and conformation of the protein at the interface will determine the stability of the foam (Zayas, 1997).

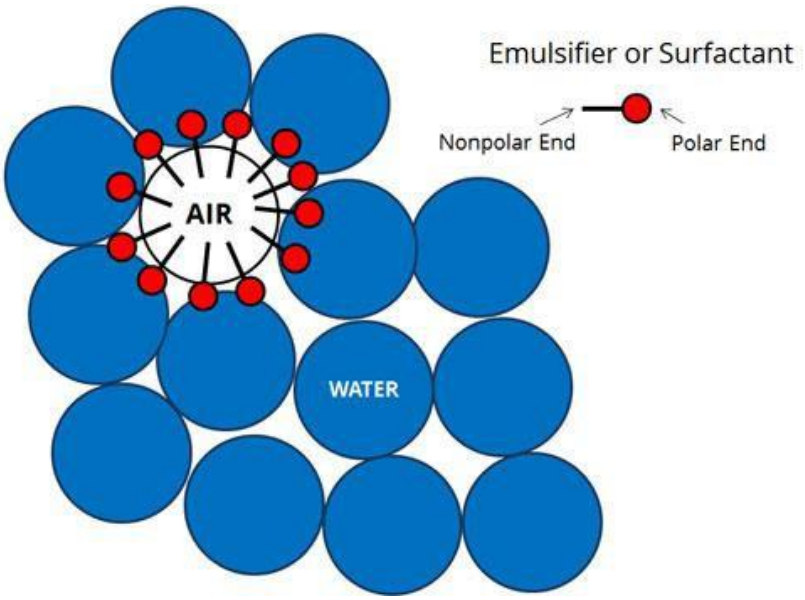



Figure 6. Air entrapped in a liquid to form a foam and stabilized by a surface-tant (http://www.molecularrecipes.com/foams).

### 1.3.3 Solubility

In a two-phase solid-liquid food system, solubility of a protein can be defined as the ratio of the amount of protein in liquid phase to the total amount of protein in the liquid and the solid phase. Solubility of a protein is the primary functional attribute which governs other functional properties of that protein. A highly soluble protein is a better emulsifier or foaming agent as compared to a protein that is not as soluble in aqueous phase. Solubility depends on surface charge, surface hydrophobicity, and state of a protein (native/denatured). More the number of charged or polar groups on the protein surface, higher is the capacity of the protein to hydrogen bond with water, which makes it more soluble in water. Conversely, higher the surface hydrophobicity, lesser is the solubility of the protein. The state of a protein depends on the properties of the system it is in, such as pH, temperature and salt concentration. The surface charge of the protein also depends on the pH of the food system. The distribution of the positive and negative charges as well as the nature of the charges (positive/negative) is affected by the system pH. At the isoelectric point of the protein (pI), the net charge on the surface of the protein is zero. Although positively and negatively charged groups exist on the protein surface at its pI, the net charge is zero and the protein-protein interactions increase, which leads to the formation of insoluble aggregates which precipitate out of the solution. Proteins undergo unfolding at extreme pH values (3.5-6.5), which leads to the exposure of hydrophobic groups usually buried in the protein core, decreasing solubility (Hayakawa et al., 1985).

### 1.3.4 Fat binding capacity

Fat binding capacity can be described as the amount of fat that can be bound by a known weight of the protein. This binding can be due to hydrophobic interactions between the fat molecules and the protein molecules. It is an important property in foods like plant based meat products for texture and flavor enhancement, since many flavor compounds are fat soluble. It is also indicative of the ability of proteins to act as emulsifiers and stabilizers. Fat binding is influenced many factors such as the source of the protein, the protein conformation, conditions of processing, the temperature, and the particle size of the protein. Smaller protein particle size helps in entrapping more fat (Zhang et al., 2013).

## 1.4 Plant Proteins

There is a growing interest in replacing synthetic additives in processed foods with natural ingredients due to increased consumer awareness and demand for clean labels. Plant proteins have been explored in recent years due to their surface properties, low cost, and low allergenicity. The structure of plant proteins is the first parameter that needs to be studied in order to determine its potential as a functional ingredient. Commonly used plant proteins are from peas, beans, chickpeas, lentils, lupine, and soybeans. Various isolated protein flours made from Faba beans, peas, chickpeas. Lima beans have been explored for their emulsifying properties in systems of different pH. The proteins perform better as functional ingredients away from their isoelectric pH of around 4-5. The methods employed to extract protein from these legumes and pulses affect their functional properties. There have also been various attempts at increasing the functionality of plant

proteins using methods like heating, chemical modification, however, achieving the balance between the exposure of hydrophobic and hydrophilic groups is difficult to control (Arntfield et al., 2011).

### 1.4.1 Yellow lentil protein

Figure 7. Dehulled and split Yellow lentil (*Lens Culinaris*) (http://ufenal.ru)

Yellow lentil (*Lens Culinaris*) is the oldest known pulse crop with a seed protein content of between 20.6% and 31.4%. Out of these, ~16% are albumin proteins, ~70% are globulin proteins, ~11% are glutenins, and ~ 3% are prolamins. The globulins, which comprise the major fraction in lentil proteins, are made of both vicilin (7S) and legumin (11S)-like fractions. The legumin group consists of six pairs of polypeptides with a molecular weight of 320 kDa – 380 kDa. Vicilin proteins exist as trimers of protein subunits that have glycosylated functional groups in their structure, with a molecular weight of 50 kDa - 60 kDa (Jarpa-Parra, 2017). The isoelectric point (pI) of these proteins is around 4-4.5. Native lentil protein solubility varies from around 15% to 35% at pH 2. The protein is the most soluble around pH 8 (98%) and least soluble around the isoelectric

point (2%). The emulsifying activity index, which is a turbidimetric measure of the ability of a protein to form an emulsion, for vicilin is around 54%. The emulsion stability index, which is a turbidimetric measure of how well a protein stabilizes an emulsion, for vicilin is around 52%. The foaming capacity and foam stability of lentil protein is around 63% and 62% respectively in a pH 7 system (Joshi et al., 2012). These inherent functional properties of lentil protein make it a good candidate for high pressure processing based modifications, which could improve the protein functionality to make them commercially usable.

## 1.4 High Pressure Processing

High pressure processing is a non-thermal processing technique in which food is subjected to extreme pressures, in the range of 100 MPa to 700 MPa. This serves to reduce or eliminate the microbial load of the food, while maintaining the organoleptic and nutritional quality of the food. The fresh-like flavor is retained because high pressure cannot break covalent bonds, like heat treatment can. Conventional processing technologies such as pasteurization, which employ heat as the lethal agent have the disadvantage of not retaining the fresh-like sensory characteristics and also lead to destruction of thermally labile nutrients. They also need to have an additional rapid cooling step after heating the food (Balasubramaniam et al., 2015). A variety of pressure treated foods are available in the market today, ranging from meat products to fruit juices and guacamole. High pressure can be used to treat both soft solid and liquid food products and can also be used in combination with heat treatment. This is called ‘Pressure assisted thermal treatment’ (Wimalaratne et al., 2008).

Figure 8. High Pressure Processing unit at Rutgers University, New Brunswick, NJ.

### 1.5.1 History and Background

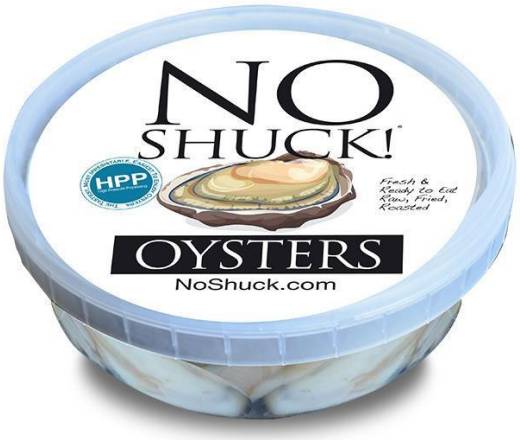
In the later part of the nineteenth century, the development of equipment like steam engines that were capable of withstanding pressures up to a few megapascals and vessels for holding gaseous chemicals that could withstand up to 70 MPa, was the first step towards the development of this technology. The first attempt to use high pressure to inactivate food spoilage microbes was made by Burt Hite at West VirginiaUniversity, Agricultural Experimental Station in 1894, when he was developing pressure vessels that would be able to withstand 700 MPa of pressure .The development of flexible polymer packaging and high pressure resistant pumps, seals and vessels during the early to middle part of the twentieth century provided a base for the safe use of high pressure equipment. Research

was then carried out to test the effect of high pressure treatment on meat product tenderization in the 1970s. It was also found that spores of microorganisms could not be inactivated by high pressure, unless used in combination with other treatments.

With increasing consumer awareness about the use of chemical preservatives in packaged foods, the demand for fresh and minimally processed food began to increase in the 1980s. High pressure studies were carried out on a variety of products, while varying the magnitude of pressure, the holding time and the effect of pressure cycling on the pressure sensitivity of pathogenic strains. Validation studies were conducted with the aim of commercializing this technology because the existing packaging and labelling regulations for frozen foods would be applicable to high pressure treated foods. The first high pressure treated food with extended shelf life was Guacamole from fresh avocados (Balasubramaniam et al., 2015). Since the early 1990s, many pressure treated food products have been introduced in the American and Japanese market, such as meat products and fresh fruit juices like ‘evolution’ orange juice, sold by Starbucks. High pressure equipment manufacturers such as Hyperbaric (Spain), Avure (USA), Stansted (UK), Engineered Pressure systems (USA), have since then started large scale manufacture of high pressure vessels and other equipment.



(https://www.evolutionfresh.com/juice/) (https://www.amazon.com/WHOLLY-GUACAMOLE-)




(www.noshuck.com)

Figure 9: Example of currently available products treated by High Pressure Processing.

### 1.5.2 Governing Principles of HPP

#### 1.5.2.1 Isostatic principle

According to the isostatic (Pascal's law) principle, hydrostatic pressure is instantaneously and uniformly distributed regardless of the geometry, size of the food package or its position in the pressure chamber. Since the volume of the food decreases, the intermolecular interactions such as Van der Waals forces, hydrogen bonds, hydrophobic, and electrostatic interactions are affected, since their strength depends on the distance between the entities involved. This means that covalent bonds are not affected since the distance between the entities cannot be reduced any further by increasing pressure. However, the shape of food products which contain air pockets will change, because of the unequal compression of air and water. Thus, this principle is what gives high pressure processing the unique advantage of the retention of flavour compounds, pigments, nutrients and the 'fresh-like' taste, since their preservation depends on the integrity of covalent bonds (Balasubramaniam et al., 2015).

#### 1.5.2.2 Le Chatelier's Principle

According to Le Chatelier's principle, when a variable in any system at equilibrium changes, the system will adopt a new equilibrium which compensates for or tries to undo the change. Thus, a corresponding variable will change in the direction that tries to undo the change. For high pressure processing, the equilibrium shifts in the direction of a decrease in volume. Thus, if pressure increases, the new equilibrium will be towards the direction that will reduce the volume (Balasubramaniam et al., 2015).

### 1.5.3 Equipment and Processing

High pressure processing is a batch processing operation. A typical high pressure system consists of a thick walled stainless steel vessel, which has closures at the top and bottom, a yoke to seal the pressure vessel while it is in operation, a pump to generate and maintain pressure, a process control system to monitor the pressure level and a recording system for keeping a track of process variables. This is a type of indirect pressurization system. There are direct pressurization systems in which a piston is used to compress the pressurizing medium on inserting the piston inside the closed vessel.

During the HPP process, the food which is packaged in flexible polymer packaging, is loaded into the pressure vessel. The pressure vessel is then sealed shut using the top closure. Pressure transmitting fluid (usually water), is pumped into the vessel and pressure is built up in the vessel until the set process value is reached. Pressure is continuously recorded and monitored by a process control system, and more water is pumped into the system if the pressure falls below the set value. During the process, the water transmits the pressure to the food and there is volumetric compression of water by about 15% when pressurized up to 600 MPa. The food returns back to its original volume when the pressure is released. Due to compression heating of the food product, its temperature rises by about 3 ºC/100 MPa for high moisture foods and by (8-9) ºC/100 MPa for high fat foods. This temperature decreases because this heat is rapidly lost to the colder walls of the vessel and the temperature falls back to the initial state or even lower at the end of the process. Typical process time at high pressure is about 5-10 min. Pressure cycling is the process of holding the vessel at a specific pressure for a finite period of time, depressurizing the vessel and repressurizing without emptying the pressurizing fluid or the contents of the vessel. This

can be repeated two or more times continuously if the application requires it. Pressure cycling can also be done if the application requires it.

### 1.5.4 Stages of High Pressure Processing

Come-up time: After the vessel is filled with water, additional water is pumped in the vessel in order to build pressure inside it. The time taken by the vessel to reach the desired pressure is called the come up time (CUT). This can be typically between 1 and 5 min.

Pressure hold time: The vessel is held at the desired high pressure for a specified, pre-programmed period of time to achieve the purpose of High Pressure Processing. This is generally between 1 to 15 min for most applications, for the process to be economical.

Depressurization: The additional water pumped into the vessel is now released, which reduces the pressure on the vessel. This is the fastest stage in the process taking up to 5 seconds to complete.

Figure 10 depicts a typical HPP cycle, showing variation of pressure and temperature inside the vessel as a function of time.

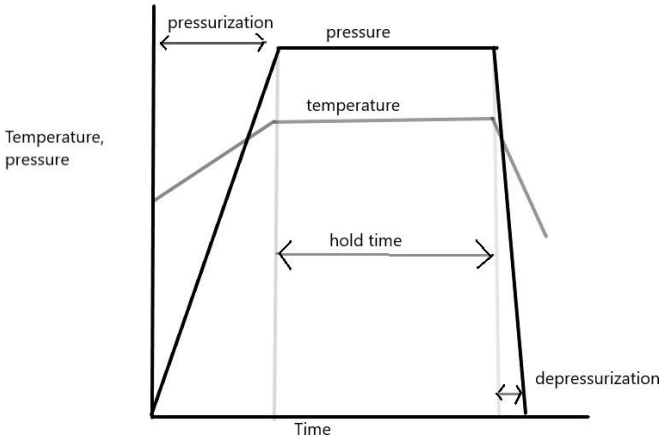



Figure 10: The schematic of the variation of pressure and temperature inside the HPP vessel during the different stages of HPP as a function of time, in a typical cycle.

### 1.5.5 HPP units used for this study:

Two different units were used for the study because the HPP unit at Rutgers University was not functional during the period that this study was conducted. A Box-Behnken Design (BBD) of experiments was carried out. Experiments were also carried out to isolate the effect of pressure on the properties of yellow lentil protein concentrate.

The HPP unit used for BBD experiments was located at Washington State University's Department of Food Engineering at Pullman, WA. The unit was manufactured by Engineered Pressure Systems, Massachusetts, USA and capacity of this unit is 1.5 L. It is temperature controlled and the pressurizing medium used is water at room temperature. The maximum pressure that this unit can reach is 600 MPa. The experiments isolating the effect of pressure on the properties of Yellow lentil protein concentrate (YLPC) were conducted at University of California, Davis, in the Department of Food Science and

Technology. Their unit was manufactured by Avure (Ohio, USA) and the capacity of this unit is 2 L. Maximum operating pressure for the unit is 600 MPa.

## 1.6 Effect of High Pressure Processing on proteins

The stability of the native protein structure can be attributed to a balance between specific environmental conditions such as pH, salt concentration and its interaction with the solvent. On being subjected to high pressure, this balance is disrupted and the protein structure undergoes changes such as partial or complete unfolding of the polypeptide chain (Figure 11). Based on the degree of unfolding, the protein can exist in any one of three states-the native structure, the partially unfolded or molten globule state or the completely unfolded state or denatured state. The polypeptide chain gains some mobility during this progression from the native state to the fully unfolded state. In the unfolded state, the degree of randomness is the highest and the protein has no defined tertiary structure. In the partially or fully unfolded state, the different polypeptide chains can interact with each other as well as the solvent, and can even form new bonds to impart some stability to the structure. An example of this is the formation of disulphide bonds after the protein has unfolded. Non-covalent interactions are more sensitive to pressure than covalent interactions. Because of this, the tertiary and quaternary structures are more sensitive to the effects of high pressure as compared to the secondary and primary structures. Irreversible denaturation generally occurs after 300 MPa of pressure has been exceeded. A change in the structure results in a change in the activity of the protein. These changes can be negative or positive and a careful selection of processing parameters is essential in order to modify the protein structure to increase protein functionality (Balny et al., 2009).

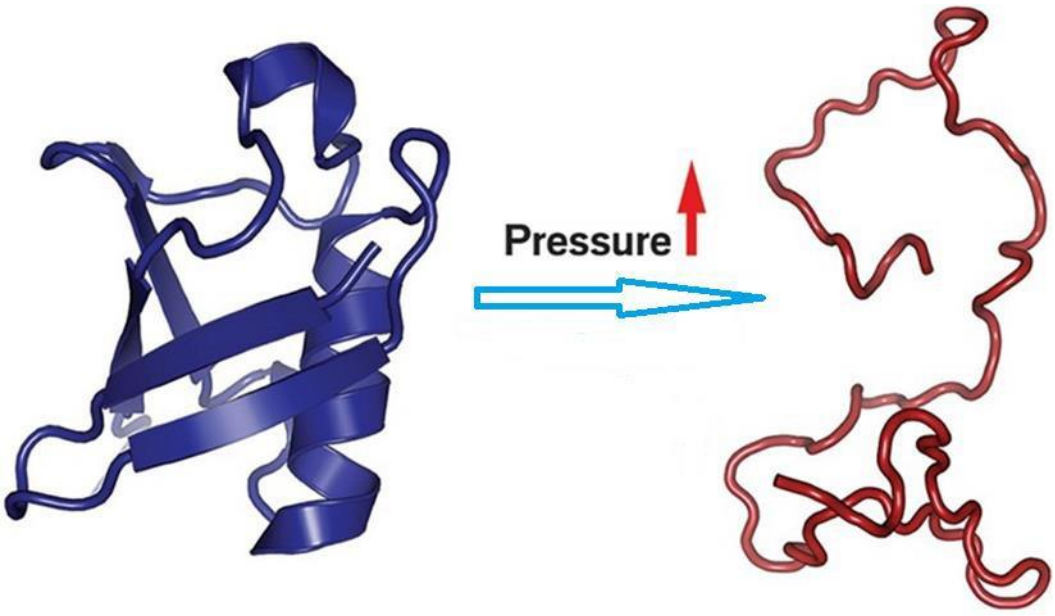



Figure 11: Protein unfolding under high pressure (www.chemlibretexts.org).

### 1.6.1 Change in volume

This is mainly due to the reduction in the volume of the internal cavities in the protein structure. When the protein unfolds, it allows the solvent to access the gaps or cavities in its interior, thus leading to the loss of volume. However, a more significant contribution to the change in volume is because of increased solvent interaction after unfolding. The breaking of intramolecular non-covalent bonds under pressure leads to formation of non-covalent bonds between the protein and the solvent (Muntean et al., 2016).

### 1.6.2 Change in protein solubility

Various studies on the effect of high pressure on protein solubility have yielded variable results with some studies reporting an increased solubility after treatment, some

reporting a significant decrease in solubility and some reporting no observed changes in protein solubility. These variable results might be a result of the many possibilities that exist in terms of how the protein unfolds, which depends in its native structure, the pH of the environment in which it is pressurized, the concentration of the protein in the solvent, and the intensity of the treatment. Depending on all these factors, a protein can unfold in such a way that more hydrophilic groups interact with water leading to an increase in the solubility, or it can unfold in such a way that the increased surface hydrophobicity can cause increased protein-protein interaction, leading to the formation of insoluble aggregates. It was observed by Yin et al. (2008) that red kidney bean isolate, on being subjected to pressures under 200 MPa, formed insoluble aggregates. However, when the pressure was increased beyond 200 MPa, the insoluble aggregates broke down into smaller, soluble aggregates which led to an overall increase in solubility beyond 400 MPa. These smaller aggregates could be stabilized by newly formed disulphide bonds. In contrast, protein solubility decreased when the pressure was increased beyond 200 MPa in the case of lupin proteins. This disparity in the effect of increased pressure can be attributed to the different major protein fractions in each of these proteins. Red kidney bean protein is constituted mainly of vicilin while lupin proteins have albumin as their major fraction. The different structures of these two fractions and their conformations might explain the difference in their behaviour under high pressure.

### 1.6.3 Emulsification

An improved emulsification capacity or emulsion stability can be a result of improved solubility or increased amphiphilic character of the protein. Increased surface hydrophobicity due to unfolding under high pressure can lead to increased amphiphilicity

because of the ability to bind apolar compounds as well. In the study by Yin et al. (2008) the emulsion activity index (EAI) and the emulsion stability index (ESI) of red kidney bean isolate, both improved significantly under pressure. The surface hydrophobicity also significantly increased with an increase in pressure up to 600 MPa. In a study by Molina et al. (2001) the emulsifying capacity of soy protein isolate significantly improved under pressures of up to 200 MPa, but decreased significantly after 400 MPa. This can be due to the increased hydrophobic character, which does not allow interaction with water, leading to phase separation.

The pH of the protein solution while it is being treated under high pressure plays an important role in determining the emulsifying capacity of the treated protein. This is because the net charge of the protein under given conditions will determine the interactions between the polypeptide chains when they unfold and their interactions with the solvent. In a study by Puppo et al. (2005) it was observed that soy bean protein isolate, when treated under the same pressure and hold time, but at different pH, exhibited improved emulsification when treated at pH 8, but decreased emulsification capacity when treated at pH 3. This also means that the pH of the system which is being tested also influences how the protein will perform as an emulsifier in that system.

### 1.6.4 Foaming

The unfolding of the protein molecule under high pressure and the subsequent loss of the tertiary and/or the tertiary structure of the protein imparts increased flexibility to the protein molecule. The mobility of the protein molecule in a solution is altered due to

increased protein -protein interaction. The solubility of the protein and its surface hydrophobicity also affect the speed and efficacy with which the protein can move to the interface and form stable foams. These are in turn affected by the intensity of the treatment, the pH of the solution when it is under high pressure and also on the pH of the test food system. It was found by Plancken et al. (2006) that for egg white proteins that were treated with pressure above 450 MPa, foams with highest volume and stability were obtained in a food system with a pH 8.8. The study could also correlate the solubility of the pressurized protein with its foaming ability and foam stability. Pressurized proteins that were more water soluble exhibited a higher foaming ability and those that were less water soluble could form more stable foams. However, they found no correlation between protein surface hydrophobicity and foaming ability or stability. The improved foaming properties were attributed to the exposed SH groups and higher solubility. However, not all proteins have the tendency to become more water soluble under high pressure, as has been demonstrated by several studies. If the protein forms large insoluble aggregates under certain high pressure processing conditions, the mobility of the protein is hampered and its diffusion to the interface slows, resulting in a reduced availability to form stabilizing films around air bubbles (Balasubramaniam et al., 2015).

### 1.6.5 Fat binding capacity

There have been few studies which evaluated the effect of high pressure on the fat binding capacity of proteins. It is clear that fat binding properties are related to the increased exposure of hydrophobic groups when a protein is denatured. In a study by Nakai et al. (1985) on the denaturation of beef proteins by heat and its effect on their fat binding

capacity, it was observed that increased denaturation led to high surface hydrophobicity and increased sulfhydryl content. This correlated well with the observed fat binding properties of the protein, which followed the same trend.

## 1.7 Principles of instruments used in the study

### 1.7.1 Mastersizer 3000: Droplet size analysis for quantifying emulsifying ability and emulsion stability

This instrument works on the principle of dynamic light scattering. A laser beam is passed through a dilute emulsion. The intensity of the laser beam fluctuates based on how it is scattered by the emulsion droplets. The larger the hydrodynamic radius of the emulsion droplet, the greater is the scattering of the laser beam and the higher is the fluctuation of the light intensity. These fluctuations in intensity and angle of scattering are measured and correlated to the diameter of the emulsion droplets. This correlation is given by the following equation:

$RH = kT/6\pi\eta D$……..(1)

Where, RH = the hydrodynamic radius of the emulsion droplet (m) k = Boltzmann constant = 1.3807 x 10 -23 (J. K -1 )

T = Temperature (K)

$\eta$ = Solvent viscosity ( Pa s )

D = Translational diffusion co-efficient (m2/s) obtained from dynamic light scattering data For a fixed temperature and solvent viscosity, smaller droplets are more mobile in

Brownian motion. They cause fewer fluctuations in measured light intensity and scatter light at a smaller angle. Thus, their diffusion co-efficient is lower.

D4,3 -Volumetric mean diameter

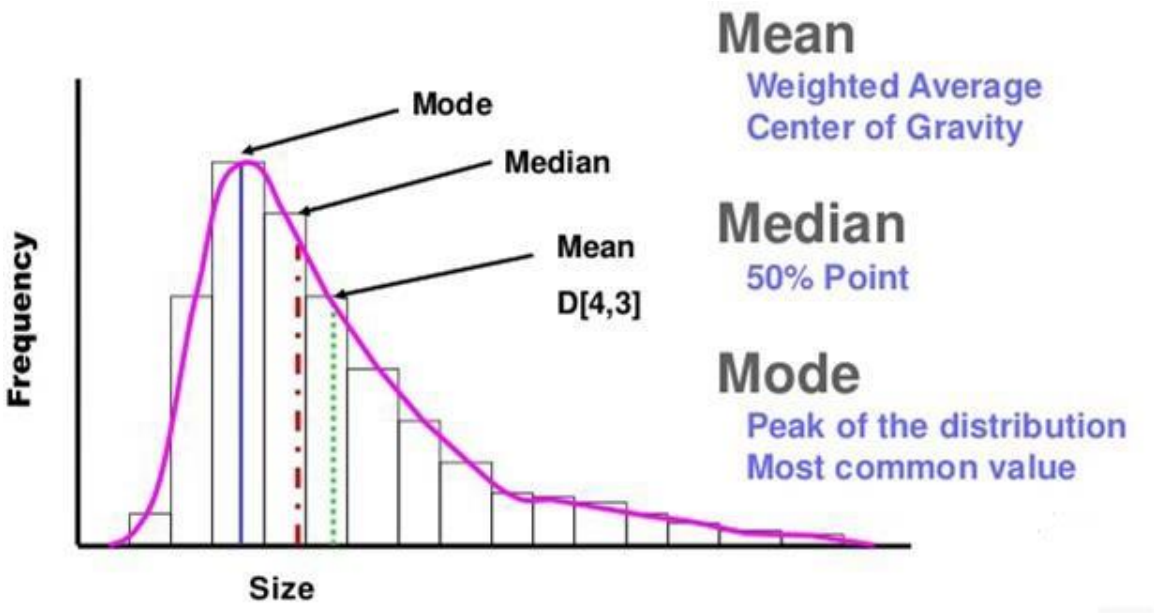



Figure 12. A representation of the mode, median, mean and volumetric mean in a particle size distribution (Malvern Paranalytical, UK).

It is the mean droplet size of the emulsion droplets calculated in the basis of volume. It is defined as follows:

$D4,3 = \Sigma D4 / \Sigma D3$……(2)

Where

Di = mean diameter of droplets = square root of (highest*lowest diameters).

If the diameter of a droplet equals D4,3, the volume of that droplet multiplied by the total number of droplets would equal the total volume of the emulsion.

### 1.7.2 Protein solubility using Sigma Aldrich's BCA assay kit

Bicinchoninic acid is used for quantifying the total protein content of a sample. The peptide backbone in proteins as well as certain amino acids like cysteine, tyrosine and tryptophan can reduce cuprous ions Cu+2 to cupric ions Cu+1 in an alkaline solution.

This is accompanied by a color change to purple. The intensity of the color is dependent on the concentration of cupric Cu+1 ions formed, which depends on the concentration of proteins (peptide backbone or certain amino acids) in the solution. This is measured using a spectrophotometer and the protein solubility of the unknown sample is quantified using a standard graph of known protein concentrations.

### 1.7.2 Zetasizer: measuring zeta potential of protein solutions

The velocity of a particle in a specific medium under the influence of an applied electric field is measured and correlated to its surface charge. Higher the surface charge, the greater is the particle velocity of the particle in travelling towards the oppositely charged electrode. This relation is given by the equation:

UE = Vp/Ef

where

UE = electrophoretic mobility (V/m2s) Vp = particle velocity (m/s)

Ef = electric field strength (V/m).

The zeta potential is related to the electrophoretic mobility by the following equation: UE = 2ε Zf(ka)/3η…….(3)

where

Z = zeta potential (V)

f(ka) = Henry's function (depends on the polarity of the medium; ka= ratio of particle radius to double layer thickness)

ε = dielectric constant

Ƞ = viscosity of the medium (Pa. s)

### 1.7.3 Surface hydrophobicity

Surface Hydrophobicity measurement was performed according to the method described by Yin et al (2008). ANS (8-Anilino-1-naphthalenesulfonic acid) is an anionic dye which on binding to hydrophobic sites on the protein surface, gives an increased fluorescence emission, which can be spectrophotometrically measured. To make this measurement, a series of known increasing protein concentrations in appropriate buffer solutions are prepared. The fluorescence emission of these is measured with and without ANS (to account for background peaks) and the fluorescence intensity of these is plotted against the protein concentrations. The slope of the graph thus obtained is called the Hydrophobicity index (HO). Figure 13 is an example of calculation of surface hydrophobicity from fluorescence intensity measurements:

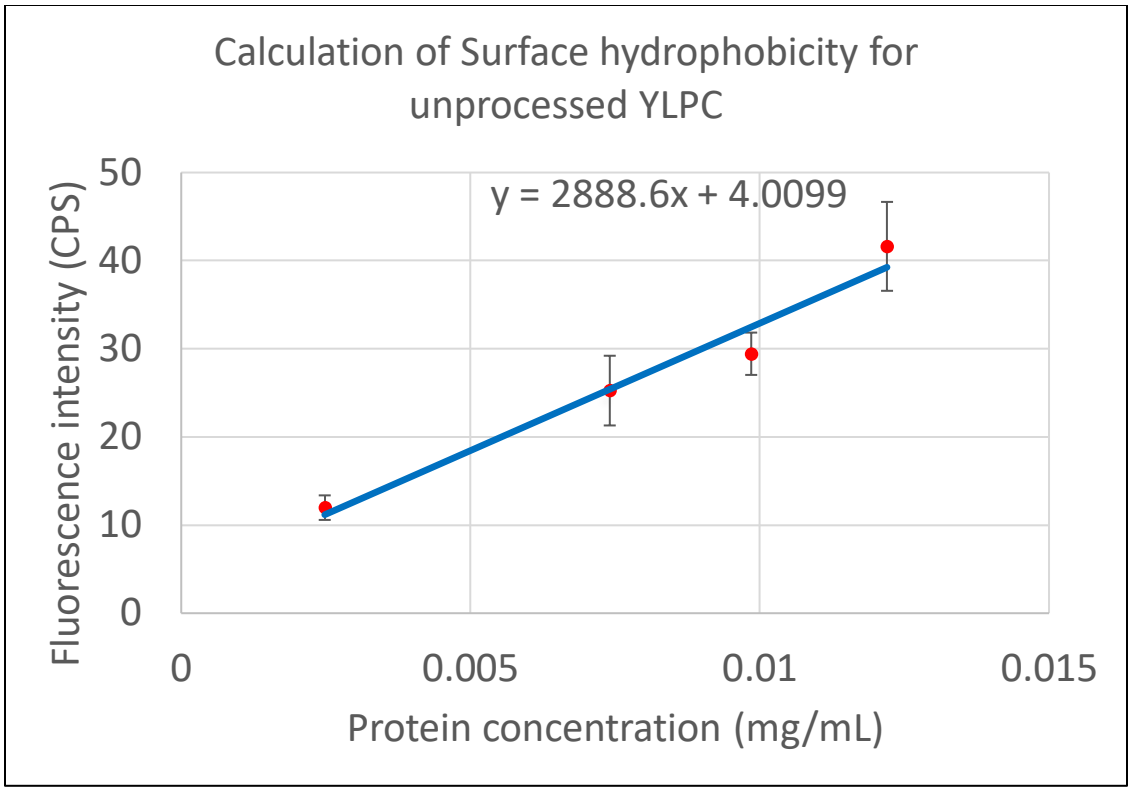



Figure 13: Calculation of Hydrophobicity index (Ho) using slope of the graph of Fluorescence intensity vs protein concentration.

For the above example, slope = 2888.6, H0 = 2888.6

## 1.8 Surfactants used in packaged food:

Surfactants are amphiphilic compounds that contain hydrophobic as well as hydrophilic moieties in their structure. Sodium tripolyphosphate, polysorbate 80, salts of mono and diglycerides are some examples of surfactants used in packaged food to stabilize emulsions, foams and extend the period for which the food quality is maintained.

Sodium tripolyphosphate:

Tripolyphosphate (Figure 14) is an inorganic salt known as E451 used as an emulsifier and humectant additive in many packaged foods and also used as a meat and sea food preservative. In this study, it was used as a control to compare the functional properties of pressure treated YLPC with a known standard.

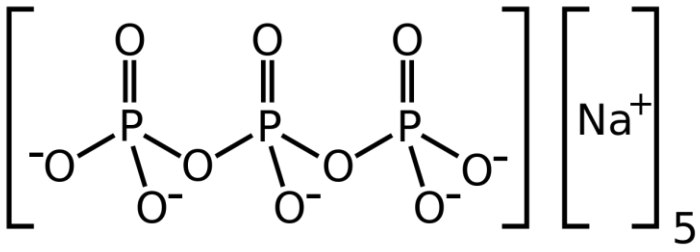



Figure 14: Structure of Sodium Tripolyphosphate

## 1.9 Rationale and significance of this research

Almost all the packaged food sold in the market contains one or more additives such as emulsifiers, foaming agents, stabilizers, binders. They are used as processing aids and for achieving a desirable texture and mouthfeel that can be retained until the end of the products shelf life. Increased consumer awareness about the ingredients and additives in packaged food has led to a demand for natural ingredients and additives. and minimally processed food. Synthetic food additives thus need to be replaced with natural, functional ingredients without compromising the quality and sensory characteristics of the food product. The inherent surface activity of proteins makes them a good alternative to chemical additives. Plants like legumes are rich sources of protein and they also have the added benefit of nitrogen fixing the soil in which they are planted. However, to replace

synthetic additives, these proteins need to be effective functional ingredients. In their native state, this requirement is not fulfilled. Thus, it becomes necessary to modify (not chemically) them to increase their functionality. High pressure processing has been explored for its ability to modify the structure of proteins such as milk proteins, egg white proteins, and soy proteins. However, these sources pose the problem of allergenicity. Thus, it is essential to look at other sources such as legume proteins for this purpose. Previous research (section 1.6) on plant proteins has demonstrated that high pressure can enhance the functional properties of these proteins by bringing about a change in their physicochemical properties.

This research evaluates how high pressure processing parameters affect the functionality of Yellow Lentil Protein (YLPC). This will add to the existing knowledge about the effects of high pressure on proteins and present a possible alternative to replace artificial additives in packaged food.

## 1.10 Hypothesis

It was hypothesized that high pressure processing would enhance the functionality of yellow lentil protein concentrate (YLPC) by bringing about a change in its physiochemical properties because of a change in the structure of the protein (unfolding).

## 1.11 Research objectives

The primary objective of this research was to evaluate the effect of high pressure processing parameters such as pressure (MPa), time of treatment (min), and pH of Yellow Lentil protein concentrate (YLPC) solution on:

1. Surface hydrophobicity of YLPC
2. Surface zeta potential of YLPC
3. Emulsifying properties of YLPC
4. Foaming properties of YLPC
5. Fat Binding properties of YLPC
6. Solubility of YLPC
7. Fat binding properties of YLPC

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Yellow lentil protein concentrate (YLPC)

Vitessence Pulse flour 2550 was obtained from Ingredion Incorporated, Bridgewater, NJ. It is made from yellow lentils (Lens culinaris). It has a protein content of 55%, 14% dietary fibre, 8% moisture and 4% starch (w/w). It is made by milling the pulse followed by air classifying to remove the dense starch or fiber.

### 2.1.2 Citrate phosphate buffers

Citrate phosphate buffers at pH 4 and pH 7 were used. Dibasic sodium phosphate and citric acid from Sigma Aldrich were used to prepare 0.2 M and 0.1 M solutions of each respectively. These were then combined in the ratios shown in Table 1 and diluted with water to make the two buffers (Essential Biochemicals for Research, Biochemicals sourcebook, EMD Millipore).

| **pH** | **Amount of 0.2 M dibasic sodium phosphate (mL)** | **Amount of 0.1 M citric acid (mL)** |
|---|---|---|
| 3 | 10.2 | 39.2 |
| 5 | 25.7 | 24.3 |
| 7 | 43.6 | 6.5 |

Table 1: Preparation of Citrate-Phosphate buffers.

## 2.2 Instruments used for emulsion droplet size analysis, surface zeta potential measurement, surface hydrophobicity measurement and solubility measurement

Malvern's Mastersizer 3000 was used to measure the emulsion droplet size in order to measure the emulsification ability and stability. Malvern's Zetasizer was used to measure the zeta potential, surface hydrophobicity as measured using fluorescence spectrophotometry using RF 5301 PC spectrofluorometer. The protein solubility was measured using the BCA kit by Sigma Aldrich. The principles of these were described earlier in previous sections.

## 2.3 Processing

### 2.3.1 Preparation of buffer solutions:

Citrate phosphate buffers were used to prepare protein solutions of pH 3 and 7. Citric acid from Sigma Aldrich was used to prepare a 0.1 M solution and Dibasic sodium phosphate from Sigma Aldrich was used to prepare a 0.2 M solution. For 100 ml of the pH 3 buffer, 10.2 ml of 0.2 M dibasic sodium phosphate and 39.8 ml of the 0.1 M citric acid solution was combined and the solution was diluted with 50 ml deionized water. For 100 ml of pH 7 buffer, 43.6 ml of 0.2 M dibasic sodium phosphate was combined with 6.5 ml of 0.1 M citric acid and diluted with 50 ml water. Buffers were stored in amber coloured bottles and appropriately labelled.


Figure 15: Vitessence Pulse 2550 (Ingredion Inc, Bridgewater, NJ)- Yellow Lentil Protein Concentrate.

2.3.2 Sample preparation for HPP experiments

The yellow lentil protein concentrate powder (Figure 15) was suspended in the buffer prepared as described above. The suspension was stirred on a magnetic stirrer Thermolyne Nuova at 1000 rpm for 1 h (Figure 16). Two hundred (200) ml of the dispersed protein solution was then filled into flexible pouches and vacuum sealed (Figure 17). Protein solutions were prepared within an hour prior to the HPP runs.

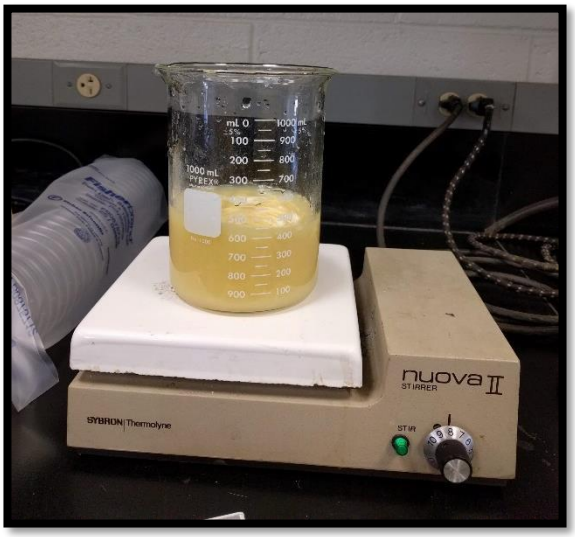



Figure 16: Dispersal of Yellow Lentil Protein Concentrate in Citrate-Phosphate buffer using a magnetic stirrer.

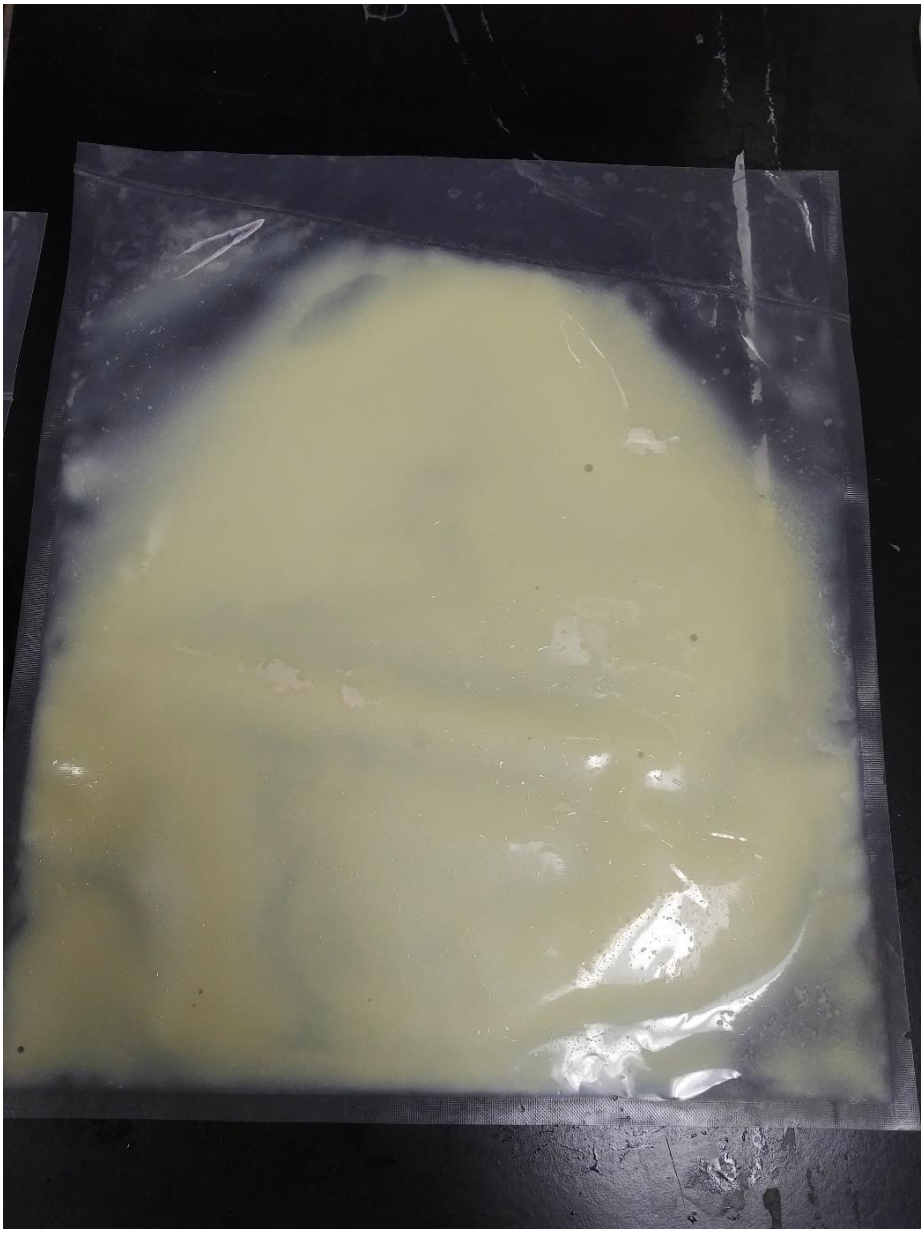


Figure 17: Vacuum YLPC solution.

### 2.3.4 High pressure processing

High pressure processing experiments were performed in a 1.5 L high pressure unit (manufactured by Engineered Pressure systems) at Washington State University (Pullman, WA, USA) (Figure 18, 19). The vacuumed sealed pouches were loaded into the cylindrical cavity of the vessel. The experiments were carried out according to a Box Behnken design of experiments as described in Section 2.6. All experiments were conducted at room temperature (22 ºC □ 25 ºC). The pressurization rate was around 110 MPa/min and the depressurization took about 10 seconds. Water was used as the pressure transmitting fluid.

The pressurization was accompanied by an increase in temperature about 3 ºC/100 MPa. The temperature dropped to the original temperature as the vessel was depressurized.

Figure 18: 1.5 L High Pressure Processing vessel at Washington State University, Pullman, WA.

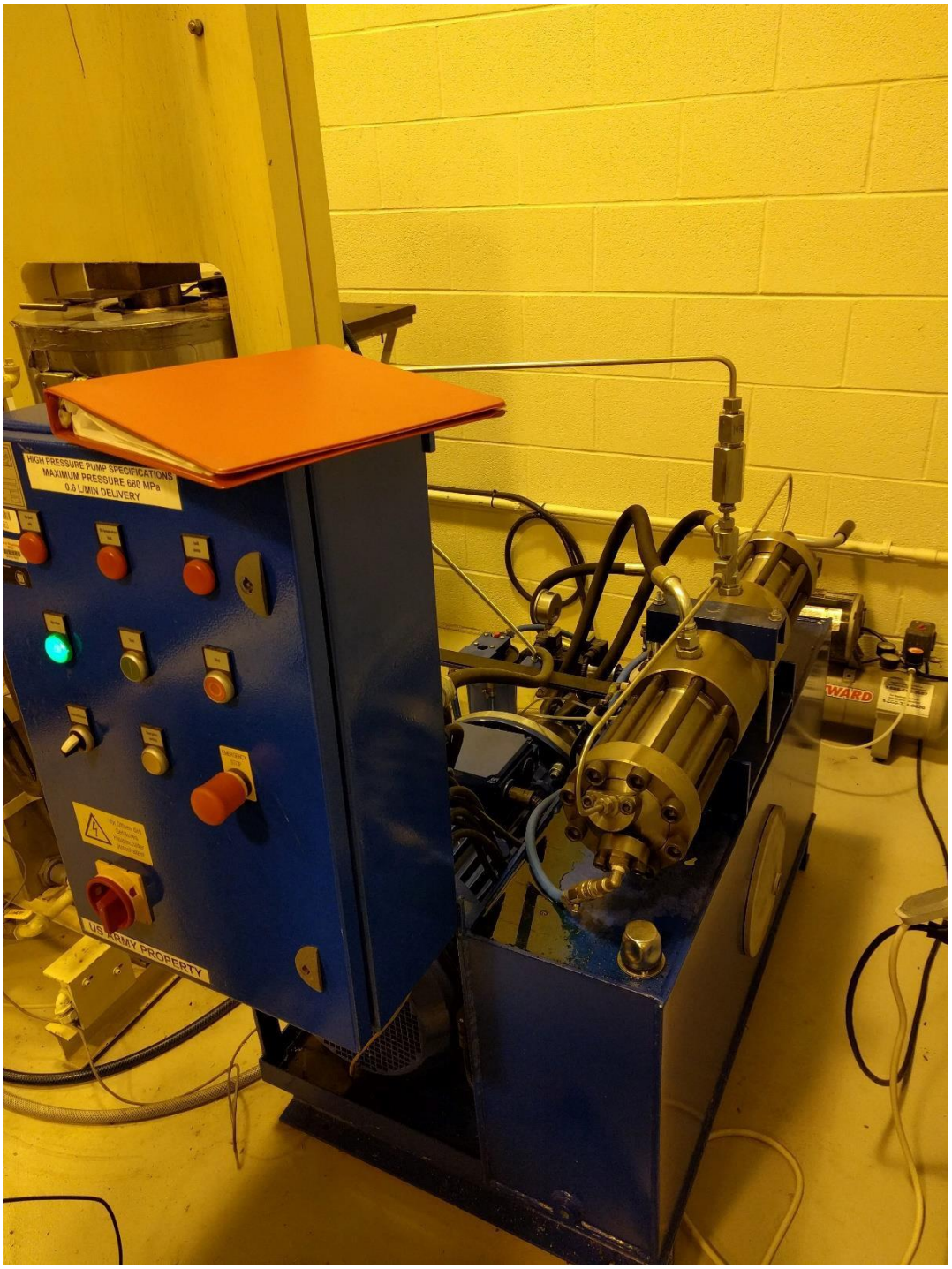



Figure 19: HPP pump at Washington State University, Pullman, WA

The second part of the study, that is, the experiments for isolating the effect of pressure on the properties of YLPC, was carried out at University of California, Davis using a 2 liter Avure Isostatic Food Press (Avure Technologies Inc, Franklin, TN, USA) (Figure 20).

Figure 20: 2 Liter HPP unit at University of California, Davis.

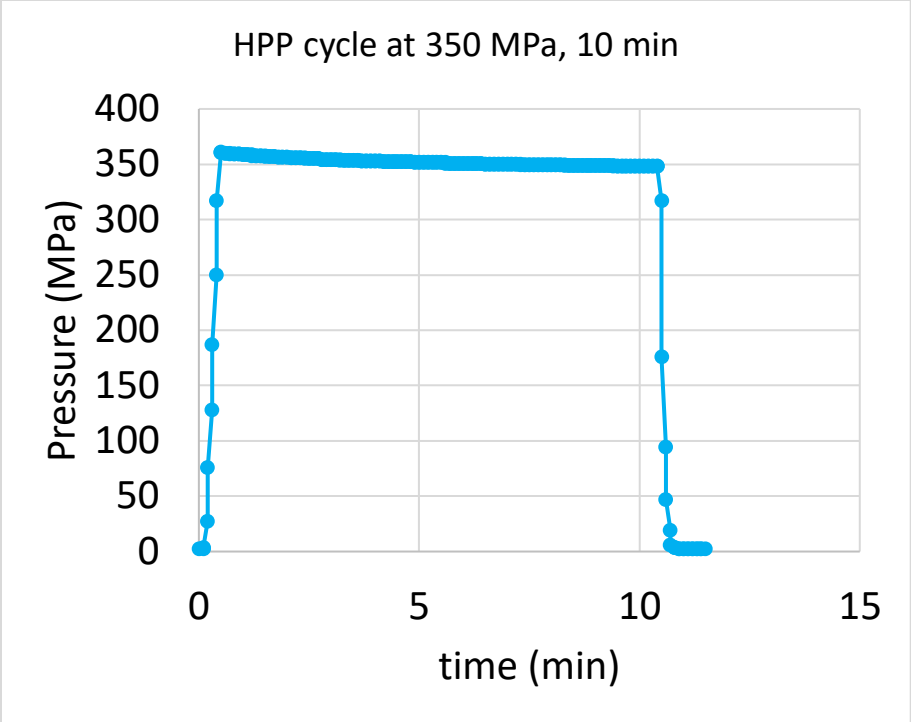



Figure 21: High Pressure Processing cycle performed as a part of the BBD experiments at Washington State University, Pullman, WA.

## 2.4 Post processing of samples

The pressure treated samples were frozen for 24 h before freeze drying for 12h. The freeze dried, pressure treated protein concentrate powder was then vacuum sealed in polyethylene pouches (Figure 22) and stored under refrigeration for further analysis.

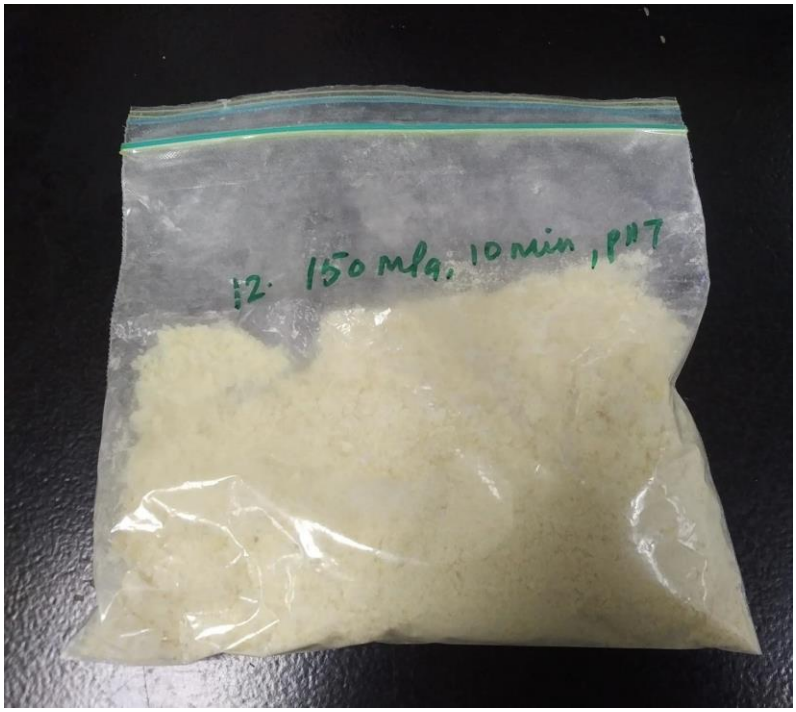



Figure 22: Freeze dried, High Pressure Processed YLPC powder

## 2.5 Analysis:

### 2.5.1 Emulsification ability and stability

#### 2.5.1.2 Sample preparation:

The freeze dried protein powder was dispersed in the citrate phosphate buffer of appropriate pH (3 or 7) at a concentration of 50 mg/ml by dispersing the protein concentrate powder in the buffer at 500 rpm for 1 h. Emulsions were prepared using 5

ml of 50 mg/ml of the pressure treated protein concentrate powder in citrate phosphate buffer and 1 ml of vegetable oil. A Polytron PT 1600e (company) homogenizer (Figure 23) was used to form the emulsion (Figure 24) at 20000 rpm for 1 minute, followed by a 5 second pause, followed again by 20000 rpm for 1 min.


Figure 23: Polytron PT1600E homogenizer, Department of Food Science, Rutgers University.

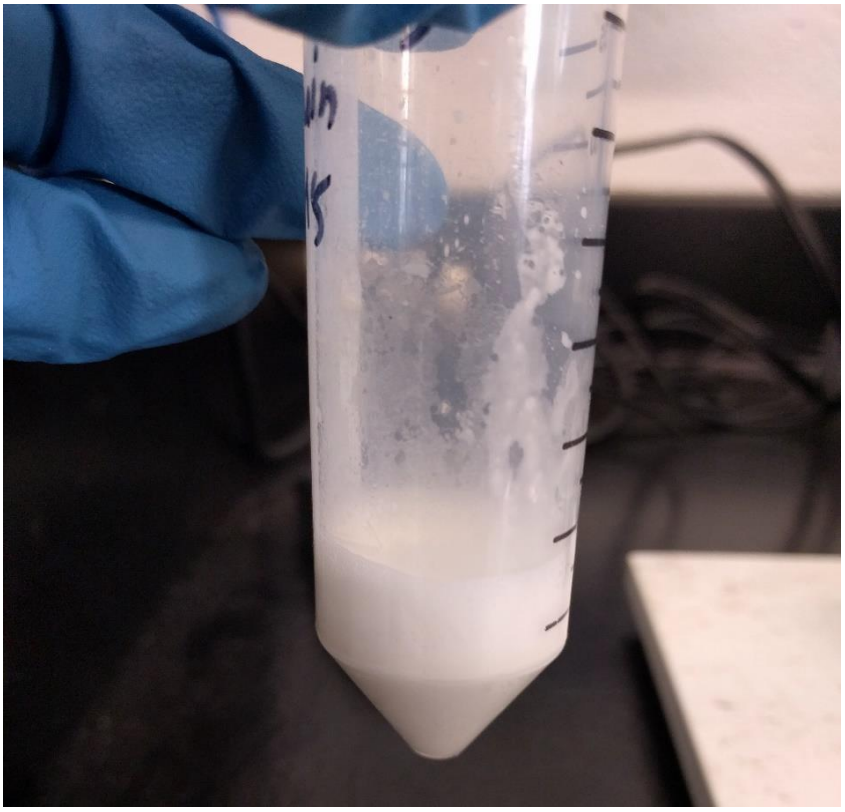


Figure 24: Oil in water emulsion stabilized using high pressure treated YLPC.

2.5.1.3 Droplet size Analysis

The emulsion droplet size, represented by the volume mean diameter ($D_{4,3}$) was measured as the emulsification ability (EA) immediately after forming the emulsion using Malvern Mastersizer 3000 (Malvern Instruments Ltd, Malvern, UK) (Figure 25) with a particle size range of 0.1 µm to 1000 µm. For emulsion stability (ES), the emulsion droplet size was measured after 120 min of standing at room temperature. The ratio of the initial to final droplet size ($D_{4,3}$) was calculated as the emulsion stability of the sample.


Figure 25: Mastersizer 3000 (Malvern Instruments, Malvern, UK) at Department of Material Science and Engineering at Rutgers University, New Brunswick.

Thus, Emulsification Ability (EA) is based on the size distribution of the oil droplets in the emulsion immediately after the emulsion is prepared i.e., at time 0 min. There are two values which give information about the oil droplet size distribution in an emulsion system- $D_{90}$ and $D_{4,3}$.

$D_{90}$ – This value represents the diameter which is greater than 90% of the droplets in the emulsion.

$D_{4,3}$ – This value represents the average diameter of all the droplets in the emulsion. This is different from D50 (or the median), which is the value which represents the diameter, above and below which lie equal number of oil droplets.

Emulsification stability (ES%) = ($EA/D_{4,3}$ 120 min after emulsion is prepared)*100

### 2.5.2 Foaming ability and foam stability

The freeze-dried protein powder was dispersed in the citrate phosphate buffer of appropriate pH (3 or 7) at a concentration of 50 mg/ml. 5 ml of this solution was subjected to homogenization using the Polytron PT 1600E for 1 min at 15000 rpm. The increased volume after homogenization was measured as the foaming ability of the sample. For foam stability, the homogenized sample was allowed to stand at room temperature for 30 min in a 250 ml graduated cylinder before measuring the final volume (Figure 26). The ratio of the final to initial volume was calculated as the foam stability of the sample.

Figure 26: Foam stabilized using high pressure treated YLPC.

Foaming ability = [(Increased final volume-initial volume without foam)/initial volume]*100

Foam stability= (Volume of foam after 30 min/initial foam volume)*100

### 2.5.3 Protein solubility

The freeze-dried protein concentrate powder was dispersed in citrate phosphate buffers (pH 3 or 7) at a concentration of 1% (w/v). The solution was stirred on a Thermolyne Nuova magnetic stirrer at 500 rpm for 1 h. This was followed by centrifugation at 10000 g for 20 min in a Thermo scientific Sorvall Legend X1R centrifuge . The protein content of the supernatant was determined using a BCA (Bisinchonicic acid) kit from Sigma Aldrich (Figure 27). The protein solubility was calculated as:

Protein solubility = protein content of supernatant/ total protein content * 100

Figure 27: BCA protein assay kit (Sigma Aldrich)

### 2.5.4 Fat binding capacity

0.6 g of YLPC powder was weighed in a centrifuge tube. 10 mL of vegetable oil was added to the tube and the weight was noted. The YLPC was thoroughly dispersed in the oil using a vortex mixer for 30 s, and allowed to stand for 30 min. The YLPC-oil mixture was then centrifuged at 3000 g for 10 min. The supernatant oil was discarded, and the centrifuge tube was inverted and allowed to stand for 30 minutes. Then the contents of the centrifuge tube were weighed.

Fat binding capacity = (weight of centrifuge tube, oil and YLPC after discarding supernatant-weight of YLPC and centrifuge tube before adding oil) / 0.6

2.5.5 Surface zeta potential

2.5.5.1 Sample preparation:

0.1 % (w/v) solution of freeze dried, pressure treated protein were prepared in citrate phosphate buffers (pH 3 or 7). The weighed amount of protein was dissolved in citrate-phosphate buffer for 1 h at 500 rpm, followed by centrifugation at 10000g for 20 min. The supernatant solutions were injected into the folded capillary cell (Figure 28) using a syringe.

Figure 28: Folded Capillary cell used to measure zeta potential.

2.5.5.2 Zeta potential measurement

The surface zeta potential was measured using the Malvern Zetasizer 2000 (Malvern Instruments Ltd, Malvern, UK) (Figure 29).

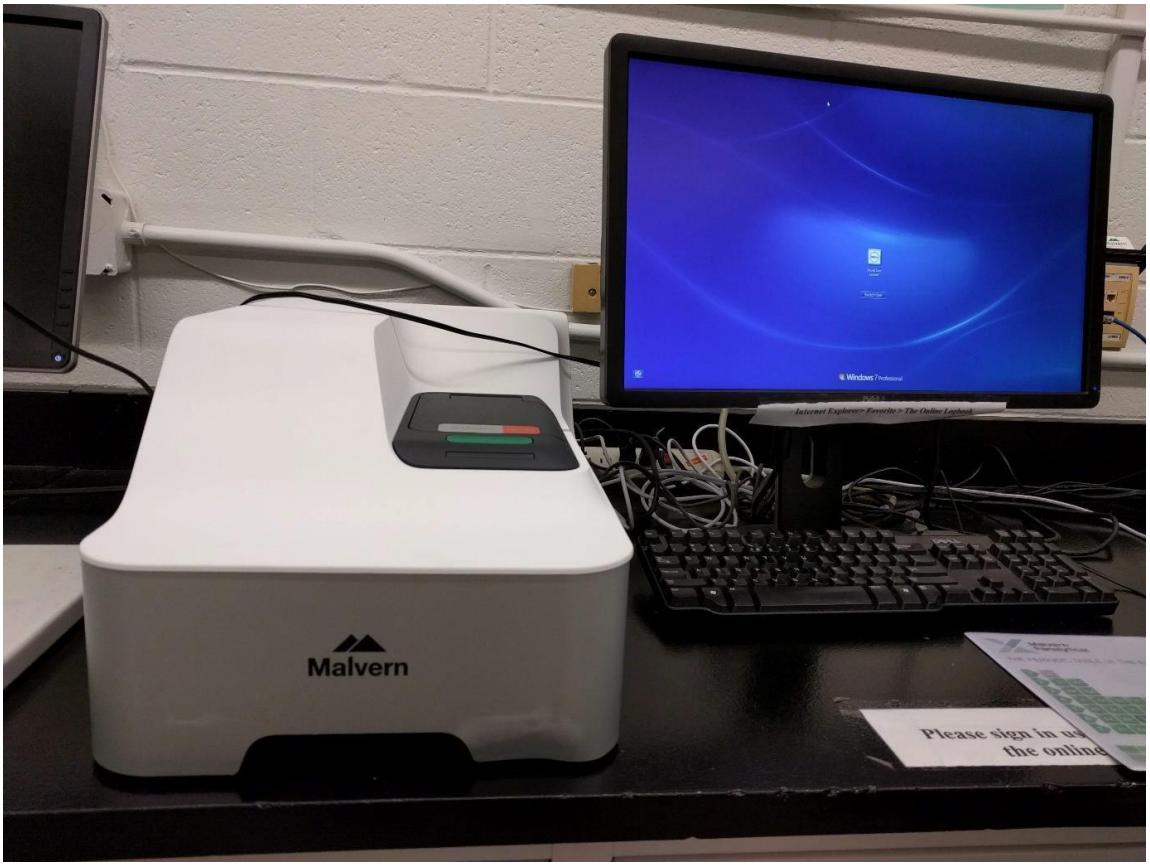



Figure 29: Zetasizer 2000 (Malvern Instruments, Malvern, UK).

2.5.6 Surface hydrophobicity

1.5% (w/v) solution of freeze dried, pressure treated protein were prepared in citrate phosphate buffers (pH 3 or 7). A solution of 0.008 M ANS was prepared as stock. To prepare a sample for analysis, 4 ml of the buffer and 20 µL of ANS stock were added to

increasing amounts of protein solutions (10, 20, 30, 40, 50 mL). The solutions were vortexed for 30 s. Fluorescence intensity (FI) at 470 nm was measured using a fluorescence spectrofluorometer RF 5301 PC with 390 nm as the excitation wavelength with a 4 nm constant slit for excitation and emission (Figure 30). FI was also measured for the protein in buffer without ANS. The FI for each sample was calculated by subtracting this value from the FI of the sample with ANS. The surface hydrophobicity was determined as the initial slope of the FI versus protein concentration plot.


Figure 30: Fluorescence spectrofluorometer.

## 2.6 Experimental design

Response surface methodology (RSM) is a statistical method which can be used to optimize a response or a dependent variable, according to certain independent variables which influence it. This helps in designing a set of experiments which can evaluate the effect of the independent variables on the response and fit it to a mathematical model. The model equation for a non-linear quadratic RSM is

$$y=\alpha0+ \alpha1x1+ \alpha2x2+ \alpha3x3+\alpha11x12+ \alpha22x22+ \alpha33x32+ \alpha12x1x2 + \alpha13x1x3 + \alpha23x2x3. \quad .. (3)$$ (Ignoring the higher order interactions)

where the y is the dependent variable or the response, which is dependent on the three independent variables x1, x2, and x3 and their interactions. Here, α0 is the model constant, α1, α2, and α3 are the linear coefficients, α12, α23, and α13 are interaction or cross product coefficients and α11, α22, and α33 are quadratic coefficients. In a Box Behnken design of experiments, this model is a quadratic model in which the experimental design requires three levels of each independent variable. The conditions are defined by the midpoints of the edges of a cuboid, which is the process space. The condition at the centre of this cuboid is performed in triplicate (1+2). The number of runs (N) in a RSM design is determine by the equation:

N=2*number of independent factors*(number of independent factors-1) + number of centre points.

Thus, a total of 15 experimental runs are performed according to BBD. This allows for a significant saving of time and resources as it negates the need to perform a full factorial

design (33), that is, 81 experimental runs. Figure 31 represents the BBD in the present study. Each axis represents an independent variable.

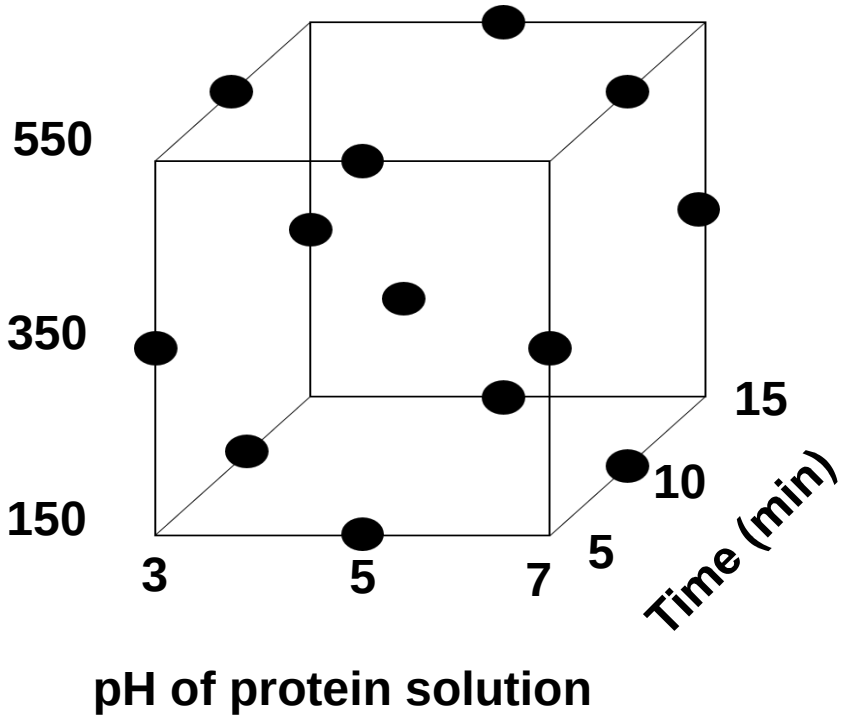



Figure 31: Box-Behnken design of experiments performed

The 15 experiments were performed in random order and a total of six responses were recorded for each run. These included the emulsification ability, foaming ability, solubility, fat binding capacity, surface hydrophobicity and surface zeta potential. The three levels of three independent factors used for the present study are shown in Table 2.

| | **Pressure (MPa)** | **Time (min)** | **pH** |
|---|---|---|---|
| Lower level | 150 | 5 | 3 |
| Mid level | 350 | 10 | 5 |
| Upper level | 550 | 15 | 7 |

Table 2: Three levels of independent factors used for designing the BBD

The values of the independent variables were expressed as coded values for convenience. +1 represents the upper value of the variable, 0 represents the midpoint value and -1 represents the lower value of the variable. Uncoded values are the actual numerical values of the three levels of the independent variable. Thus, Table 3 represents the coded and uncoded values of the three independent variables in this study.

| **Independent variable** | **Coded value** | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| Pressure (MPa) | 150 | 350 | 550 |
| Time (min) | 5 | 10 | 15 |
| pH | 3 | 5 | 7 |

Table 3: Nomenclature for coded and uncoded values of 3 independent variables used in the BBD.

The 15 experiments performed are given in Table 4

| Run # | Coded values | | | Uncoded values | | |
|---|---|---|---|---|---|---|
| | X1 | X2 | X3 | Pressure(MPa) | Time(min) | pH |
| 1 | -1 | -1 | 0 | 150 | 5 | 5 |
| 2 | 1 | -1 | 0 | 550 | 5 | 5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | -1 | 1 | 0 | 150 | 15 | 5 |
| 4 | 1 | 1 | 0 | 550 | 15 | 5 |
| 5 | -1 | 0 | -1 | 150 | 10 | 3 |
| 6 | 1 | 0 | -1 | 550 | 10 | 3 |
| 7 | -1 | 0 | 1 | 150 | 10 | 7 |
| 8 | 1 | 0 | 1 | 550 | 10 | 7 |
| 9 | 0 | -1 | -1 | 150 | 5 | 3 |
| 10 | 0 | 1 | -1 | 350 | 15 | 3 |
| 11 | 0 | -1 | 1 | 350 | 5 | 7 |
| 12 | 0 | 1 | 1 | 350 | 15 | 7 |
| 13 | 0 | 0 | 0 | 350 | 10 | 5 |
| 14 | 0 | 0 | 0 | 350 | 10 | 5 |
| 15 | 0 | 0 | 0 | 350 | 10 | 5 |

Table 4: Coded and uncoded variables used in the BBD.

Varying the pressure

In order to isolate the effect of pressure, the hold time and pH of the protein solution were kept constant. The pressure was varied from 50 MPa to 550 MPa, and the freeze dried samples were analysed similar to the BBD samples. The experiments in Table 5 were carried out.

| **Pressure (MPa)** | **Hold time (min)** | **pH of protein solution** |
|---|---|---|
| 50 | 10 | 7 |
| 100 | 10 | 7 |

| | | |
|---|---|---|
| 150 | 10 | 7 |
| 350 | 10 | 7 |
| 550 | 10 | 7 |

Table 5: Experiments carried out to check the effect of pressure alone on the physicochemical and functional properties of YLPC.

## 2.7 Statistical analysis:

The statistical analysis of the data from for the Box-Behnken Design was done using Minitab® software. The statistical analysis for the pressure experiments carried out keeping the time of treatment and pH constant were analysed using One Way Analysis of Variance (ANOVA) using Microsoft Excel 2013 and Tukey Test using Minitab software. A significance level ($p<0.05$) was used to test if the differences between the means were significantly different.

# 3. RESULTS AND DISCUSSION

## 3.1 Analysis of controls

The controls used were unprocessed Yellow Lentil Protein Concentrate and Sodium Tripolyphosphate, a commercially used food grade surfactant analysed for the surface hydrophobicity, surface zeta potential, emulsifying ability, emulsion stability, fat binding capacity, foaming ability and foam stability.

Table 6 shows that the emulsifying ability and emulsion stability of sodium tri-polyphosphate was significantly better than that of the unprocessed YLPC in a pH 3 system. This can be because of the high water solubility of sodium tri-polyphosphate. Sodium tri-polyphosphate being a slightly alkaline phosphate, increases the pH of the system. This leads to electrostatic repulsion between or within protein chains, which creates more space for water binding (Glorieux et al., 2017). However, the emulsifying ability of unprocessed YLPC in a pH 7 system is better than that of sodium tri-polyphosphate. But the emulsions stabilized by sodium tri-polyphosphate were more stable than those stabilized by unprocessed YLPC. When molecules of sodium tri- polyphosphate cover oil droplets and the since these molecules are charged, like charges on different oil droplets repel each other and keep the oil droplets from coalescing.. Sodium tri-polyphosphate does not form a foam on applying shear to 5% (w/v) solutions using a homogenizer. This indicates the ability of YLPC to act as an additive capable of performing more than one function in a food system.

| Property | Unprocessed YLPC | Sodium tri-polyphosphate |
|---|---|---|
| Emulsifying ability in a pH 3 system | 15.93 µm | 0.76 µm |
| Emulsifying ability in a pH 7 system | 7.23 µm | 11.31 µm |
| Emulsion stability in a pH 3 system | 82.77% | 87.07% |
| Emulsion stability in a pH 7 system | 83.21% | 91.81% |
| Foaming ability in a pH 3 system | 7.5 mL increased foam volume | No foam formed |
| Foaming ability in a pH 7 system | 7.5 mL increased foam volume | No foam formed |
| Foam stability in a pH 3 system | 100% after 30 min standing | No foam formed |
| Foam stability in a pH 7 system | 33.33% after 30 min standing | No foam formed |
| Fat binding capacity | 88.88% | |

Table 6: Analysis of controls for functional properties

## 3.2 Summary of results of BBD experiments

The Table 7 gives a summary of responses, as obtained through the analysis of the treated samples, to each parameter (Pressure-P, time-t, pH of the protein solution), and their interactions effects. A predictive equation can be obtained based on the coefficients of each of these parameters, after dropping the insignificant terms, based on the p-value. Here, the responses are:

Emulsification ability (EA) at pH 3 (EA-3), EA at pH 7 (EA-7), Emulsion stability (ES) at pH 3 (ES-3), ES at pH 7 (ES-7), protein solubility (S) at pH 3 (S-3), protein Solubility at pH 7 (S-7), Foaming ability at pH 3 (FA-3), Foaming ability at pH 7 (FA-7), Foam

stability at pH 3 (FS-3), Foam stability at pH 7 (FS-7), protein surface hydrophobicity at pH 3 (H-3), protein surface hydrophobicity (H), Fat binding capacity (FBC), surface zeta potential at pH 3 (Z-3) and surface zeta potential at pH 7 (Z-7).

| **Response** | **P** | **T** | **pH** | **P.t** | **P.pH** | **t.pH** | **$P^2$** | **$t^2$** | **$pH^2$** |
|---|---|---|---|---|---|---|---|---|---|
| EA-3 ($d_{4,3}$) | $3.87^b$ | $3.01^b$ | 1.62 | -1.8 | 0.89 | -3.7 | $-4.23^b$ | $-8.41^a$ | $3.53^a$ |
| EA-7 ($d_{4,3}$) | 1.898 | -0.745 | $-2.07^b$ | $-2.573^b$ | 0.517 | -0.197 | 1.137 | 1.712 | 0.458 |
| ES-3 ($d_{4,3}$) | 3.86 | 0.88 | 3.74 | -2.39 | 0.48 | $-10.42^b$ | $12.38^b$ | 2.78 | 7.72 |
| ES-7($d_{4,3}$) | 5.77 | -5.11 | $17.22^a$ | -1.51 | -4.32 | -2.99 | -7.74 | -15.09 | -5.1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S-3 | -3.63 | -1.37 | 0.65 | 0.57 | -0.45 | 2.49 | -0.02 | -0.28 | -0.66 |
| S-7 | -10.5[a] | -1.25 | 4.325[a] | 2.45[b] | -4.5[a] | -0.6 | 6.325[a] | -0.425 | -1.525 |
| FA-3 | -0.313 | -0.313 | -0.25 | -0.25 | 0.625 | 0.375 | -0.458 | 0.042 | 0.417 |
| FA-7 | 0 | 0.25 | -0.5 | 0 | 0.5 | 0 | 0.5 | 1 | -1 |
| FS-3 | 2.57 | -2.26 | 5.02 | -1.25 | -6.38 | -0.89 | -3.76 | 3.9 | -5.78 |
| FBC | 33.33[a] | -2.09 | -27.08[a] | 12.5 | -12.5 | -8.33 | -13.89 | -1.39 | -34.72[a] |
| H | 1159[b] | 404 | -1374[a] | 133 | -27 | -33 | -614 | 215 | 120 |

Table 7: Results of BBD experiments

a- Very significant ($p<0.01$) b- Significant ($0.01<p<0.05$)

No symbol- not significant

## 3.3 Effect of processing parameters

### 3.3.1 Physicochemical properties

#### 3.3.1.1 Protein surface hydrophobicity (PSH)

From the Table 7, it can be seen that protein surface hydrophobicity in a pH 7 system varied significantly with the Pressure (P) applied and the pH of the protein solution at the time of treatment. The predictive equation can thus be written as:

PSH at pH 7 = 6492 + 1159 P – 1374 pH…(R2 = 0.87)…..(4)

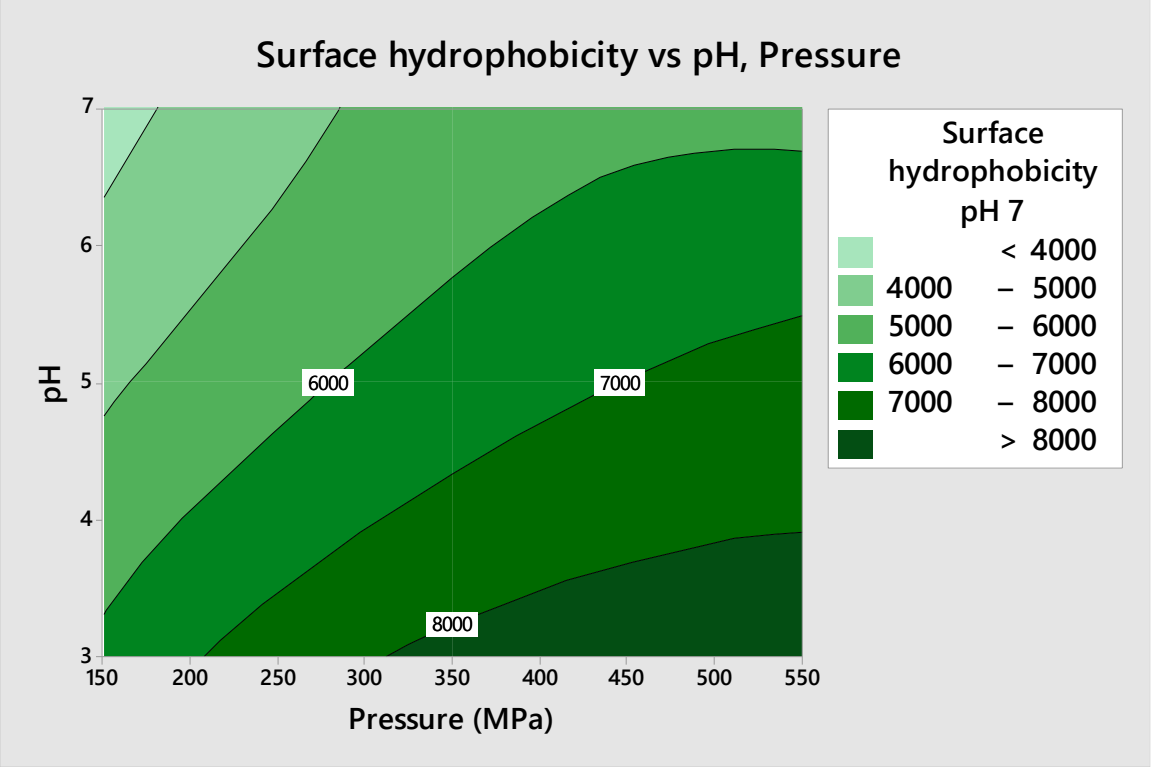



Figure 32: Effect of pressure and pH on protein surface hydrophobicity (PSH) in a pH 7 system.

Figure 32 shows a contour plot of PSH varying with Pressure and pH of the protein solution. Higher pressures (350 MPa - 550 MPa) caused the hydrophobicity to be higher. This may be because pressure causes the native structure of the protein to gradually unfold, exposing hydrophobic sites which are mainly non-polar amino acid residues, previously buried in the interior of the structure. The positive pressure coefficient indicates that an increase in the pressure applied caused the surface hydrophobicity to increase. Higher surface hydrophobicity might lead to increased binding of non-polar molecules (fats). The highest hydrophobicity index observed was about 8000 for samples treated at 350 MPa - 550 MPa. The negative pH coefficient indicates that lower the pH of the YLPC solution undergoing pressure treatment, higher was the observed surface hydrophobicity. Near the isoelectric pH of the protein (=5), the protein is more resistant to the effects of pressure since the protein molecules tend to aggregate before pressurization (He et al., 2014). At pH 7, there could be a shielding effect of negative charges on the protein molecule at play during pressurization, which cause the surface hydrophobicity to be lower (Li et al., 2018). This can be seen in the contour plot at pH 3.

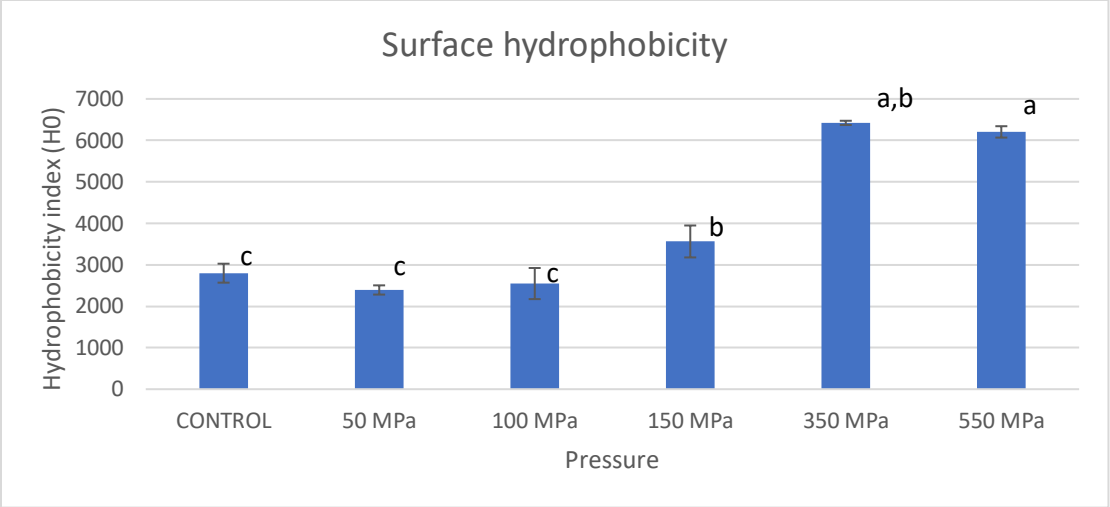



Figure 33: Protein surface hydrophobicity as a function of pressure compared to unprocessed control. (Error bars indicate standard deviation; data that do not share the same letter are significantly different from each other (one way ANOVA ($p<0.05$) and Tukey test))

Figure 33 indicates that the surface hydrophobicity did change with an increase in the pressure from 50 MPa to 550 MPa. There was a significant increase in the hydrophobicity from 150 MPa to 350 MPa from around Ho = 3500±385 to Ho = 6500±542, possibly due to gradual unfolding of the native structure under high pressure. However, there was no further increase in surface hydrophobicity if the pressure is increased beyond 350 MPa. The increase in the number of hydrophobic groups on the surface of the protein molecule might lead to an increase in hydrophobic interactions between these groups, and the hydrophobic groups will no longer be available to bind to the ANS dye. This could explain why there is no further increase in surface hydrophobicity beyond 350 MPa.

3.3.1.2 Surface zeta potential

None of the varied independent factors affected the surface zeta potential significantly for both pH systems. A high zeta potential (either positive or negative) is essential for emulsion/foam stability. It is also indicative of increased electrostatic interactions. The unchanged zeta potential might be because the protein molecule assuming a specific conformation at the pH values tested. The values tested might be too close to the isoelectric point of the YLPC, at which the protein has no net charge Aggregate formation due to increased hydrophobicity could also explain why the surface zeta potential did not change significantly. The protein could also assume a conformation in which the charged functional groups buried in the interior of the protein prior to HPP would be exposed to the solvent. Therefore, the net charge would be the same as before because of simultaneous exposure of hydrophobic and charged groups.

### 3.1.1 Functional properties of YLPC

#### 3.3.3.1 Protein solubility

From the table, we can see that in a pH 7 system, protein solubility depended significantly on the magnitude of pressure (P), the pH of the YLPC solutions (pH), the interaction of the pressure and time of treatment (Pt), the interaction of pressure and pH (P*pH) and the square of magnitude of pressure (P2).

PS at pH 7 = 29.4+4.325 pH + 2.45 Pt – 4.5 P*pH + 6.325 P2 (R2=0.9)….(5)

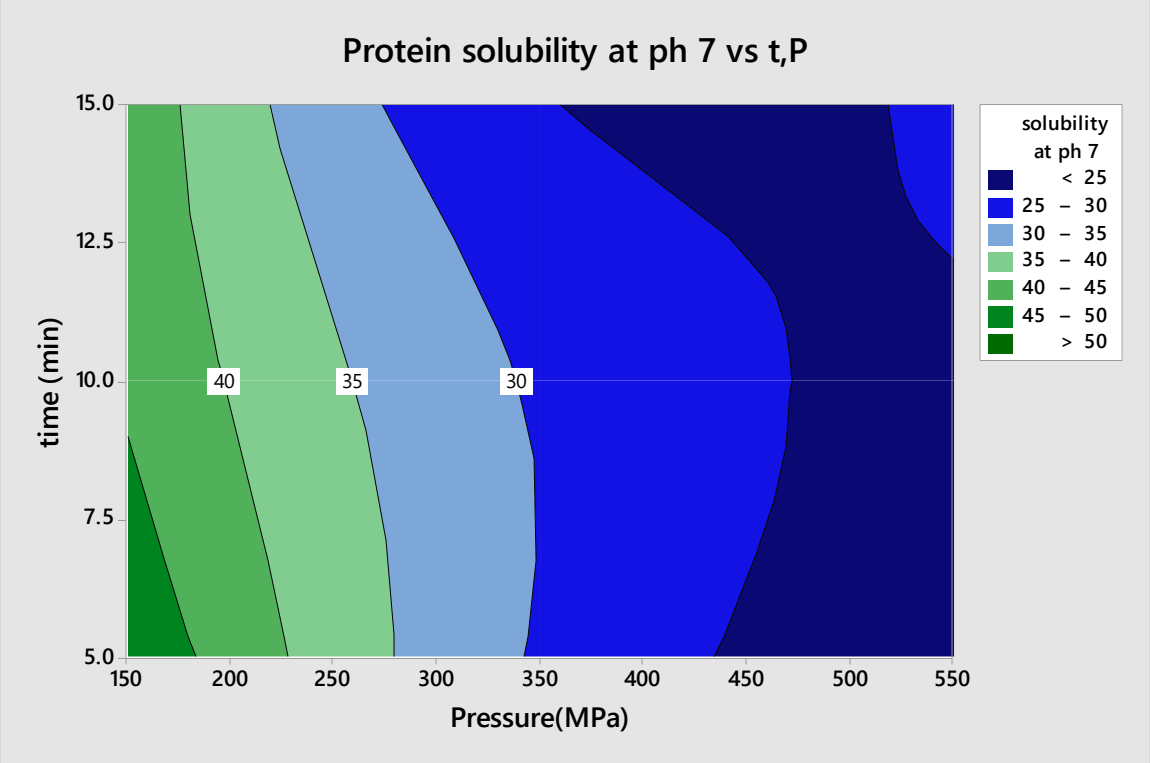



Figure 34: Effect of pressure-time interaction on the protein solubility in a pH 7 system

From the above contour plot in figure 34, it is seen that higher protein solubility, around 40%, was displayed by the protein samples subjected to milder HPP treatments, compared to the samples subjected to higher pressures like 550 MPa, which is around 30% or lower.

A possible explanation of this behaviour is that protein solubility in water is dependent on the surface charge of the protein, and the surface hydrophobicity. Since the surface hydrophobicity of YLPC increased with an increase in the pressure at around 350 MPa - 550 MPa, the ability of the protein to interact with water reduced. At lower pressures, it is possible that certain charged groups were exposed due to random uncoiling of the protein molecule which caused the protein to bind a slightly higher amount of water compared to the untreated YLPC.

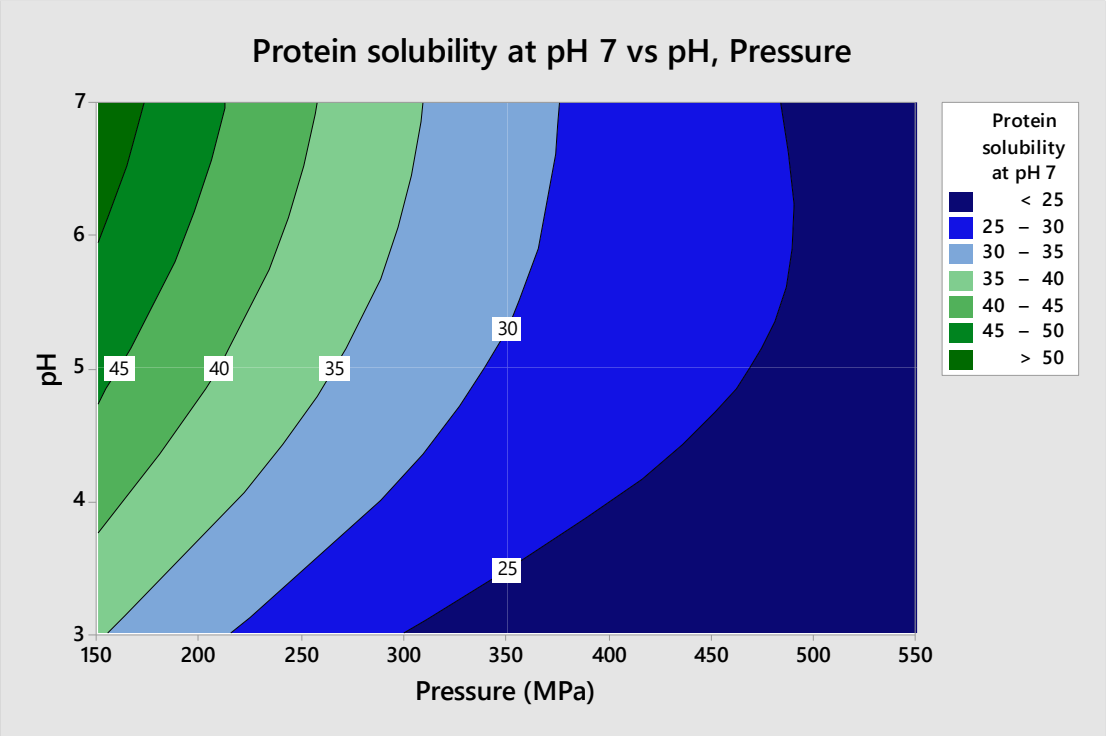



Figure 35: Effect of pressure-pH interaction on the protein solubility in a pH 7 system

In Figure 35, the contour plot of protein solubility in a pH 7 system varying with Pressure and pH of the protein solution, it can be seen that a combination of lower pressures and neutral protein solutions favour higher protein solubility. Increased solubility can be correlated with higher surface charge, which will allow for interaction with water

molecules. As we have seen earlier, the surface hydrophobicity was seen to decrease for protein solutions with neutral pH at the time of pressurization, possibly due to the shielding effects of negative protein charges to pressure. This could explain an increased interaction of water molecules with the protein, leading to higher solubility for these conditions.

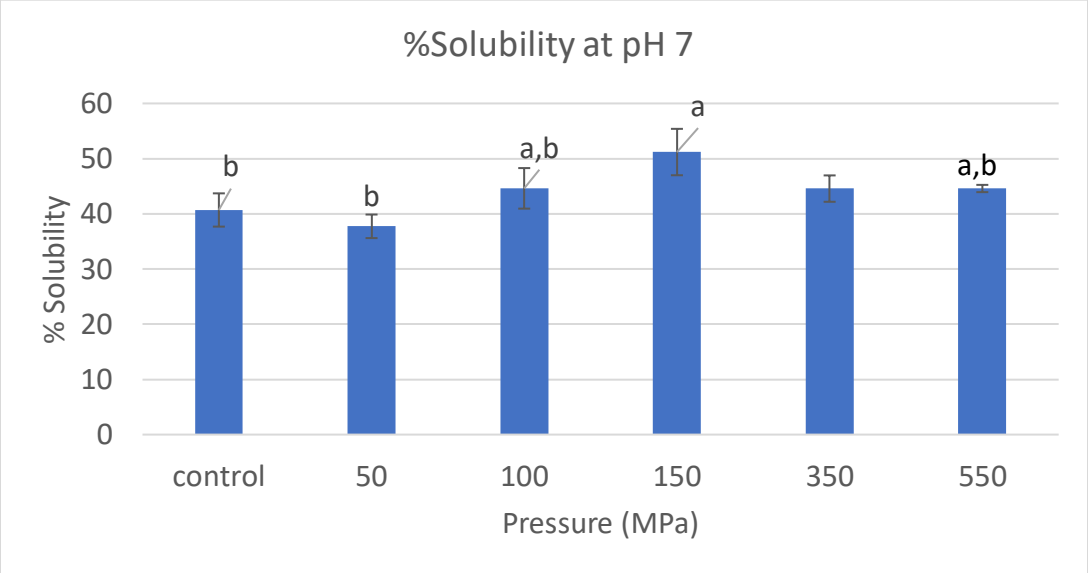



Figure 36: Protein solubility in a pH 7 system as a function of pressure compared to unprocessed control. (Error bars indicate standard deviation; data that do not share the same letter are significantly different from each other (one way ANOVA ($p<0.05$) and Tukey test)).

The graph in Figure 36 shows the % solubility of YLPC in a pH 7 system, when pressurized at increasing pressures, keeping the time of pressurization and pH of the YLPC solution constant. The protein solubility of the untreated YLPC is around 40%. The protein solubility increased slightly to around 45% for samples treated at 100MPa and further increases to 50% until about 150 MPa, but did not increase any further for samples which

underwent pressure treatment at higher pressures. Pressure unfolding could expose charged groups such as sulfhydryl groups (Yin et al., 2008), which could interact with water molecules, contributing to the increase in protein solubility. The protein solubility decreased to around 45% for samples treated at higher pressures. The formation of aggregates due to hydrophobic interactions between the exposed hydrophobic groups at higher pressures could explain the decreased surface hydrophobicity. Since the protein-protein interactions dominate, there was no interaction between protein molecules and water. The surface zeta potential remaining constant could be a reason for the increase in protein solubility not being very steep.

pH 3:

The solubility in a pH 3 system is independent of all the parameters tested in this study. This could be due to the protein assuming a particular conformation. The amino acids with side chains containing functional groups that are uncharged at pH 3 could be the reason for no improvement in solubility after HPP. (He et al., 2006).

3.3.2.1 Emulsification ability (EA):

pH 3:

Emulsifying ability is the size of the oil droplet when the emulsion is formed. According to the BBD experiments performed, in a pH 3 system, the EA depended significantly on the squares of Pressure (P2), time (t2) and pH (pH2). Thus, ignoring the insignificant terms, the predictive equation obtained from the BBD data for EA in a pH 3 system is:

EA (pH 3) = 30.45 + 2.87 pH – 6.2 P*P – 7.87 t*t – 4 pH*pH (R2 = 0.82)…..(6)

The positive coefficient of the pressure term indicates that an increase in the pressure treatment leads to an increase in the droplet size of the emulsion formed using the treated YLPC. The negative co-efficient of the time term indicates that increase in the treatment time lead to a decrease in the droplet size of the emulsion formed using the treated YLPC. This might indicate that samples treated with lower pressures (150 MPa) in combination with intermediate to high treatment times (10 min - 15 min) might form better emulsions in terms of initial droplet size.

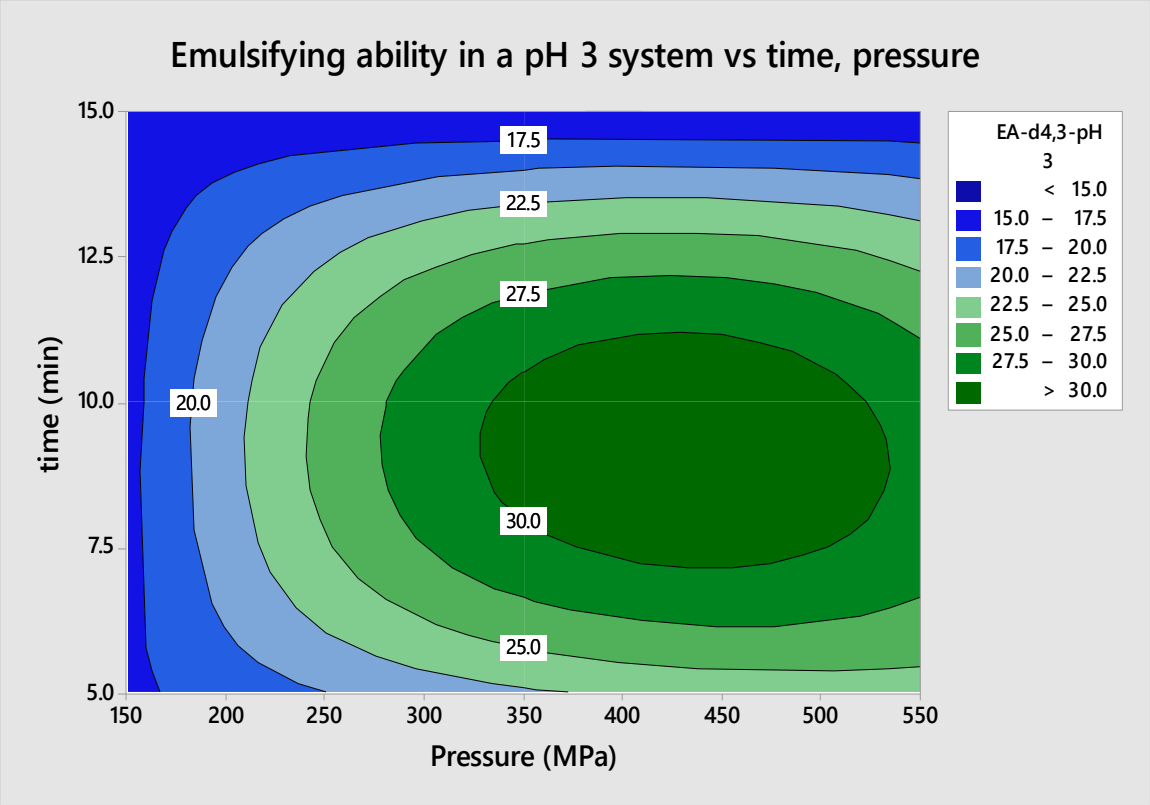



Figure 37: Effect of Pressure-Time interaction on emulsifying ability in a pH 3 system

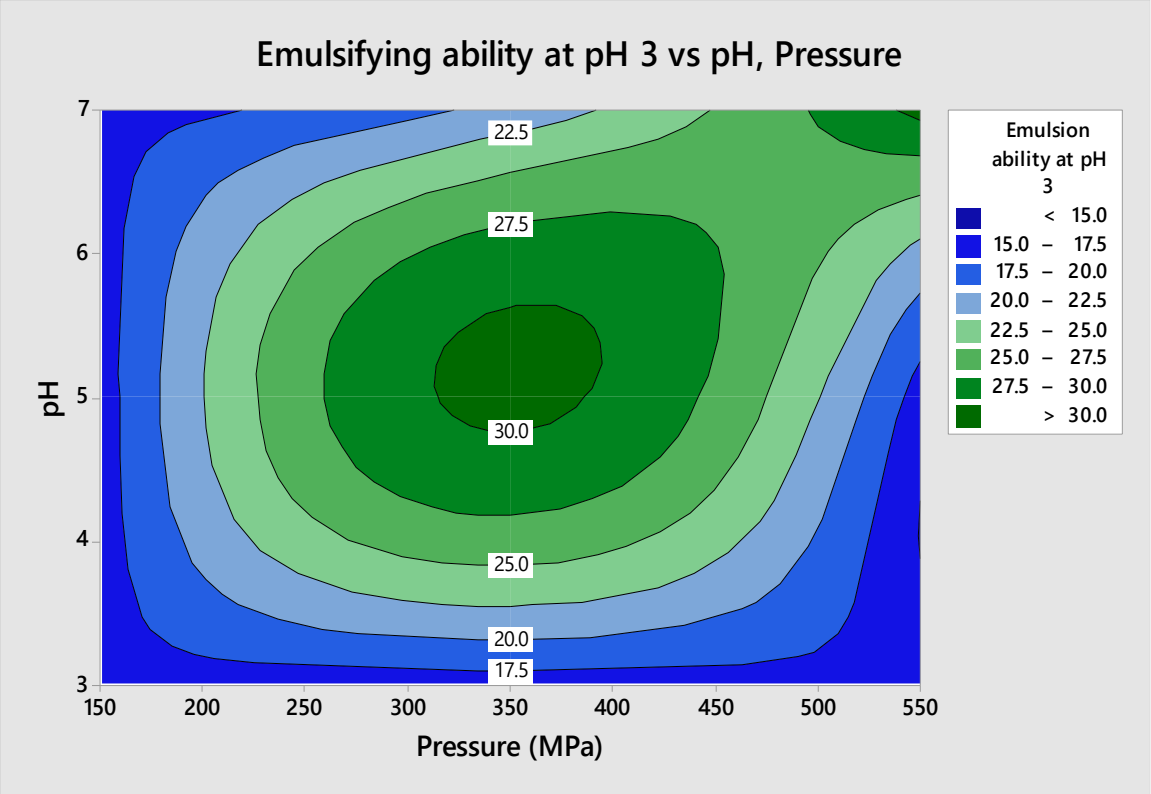



Figure 38: Effect of Pressure-pH interaction on the emulsifying ability in a pH 3 system

Lower oil droplet size indicated better emulsifying ability. Thus, oil droplet sizes of 30 µm indicate poor emulsifying ability, which was exhibited by samples processed at higher pressures and intermediate treatment times. The contour plot in figure 37 indicates that lower pressures and milder treatments on YLPC are more effective for the YLPC to form emulsions with smaller droplet size of dispersed phase. However, the lowest pressure in the BBD is 150 MPa. Therefore, it was necessary to see if pressures lower than 150 MPa are more effective in terms of emulsifying ability improvement in a pH 3 system.

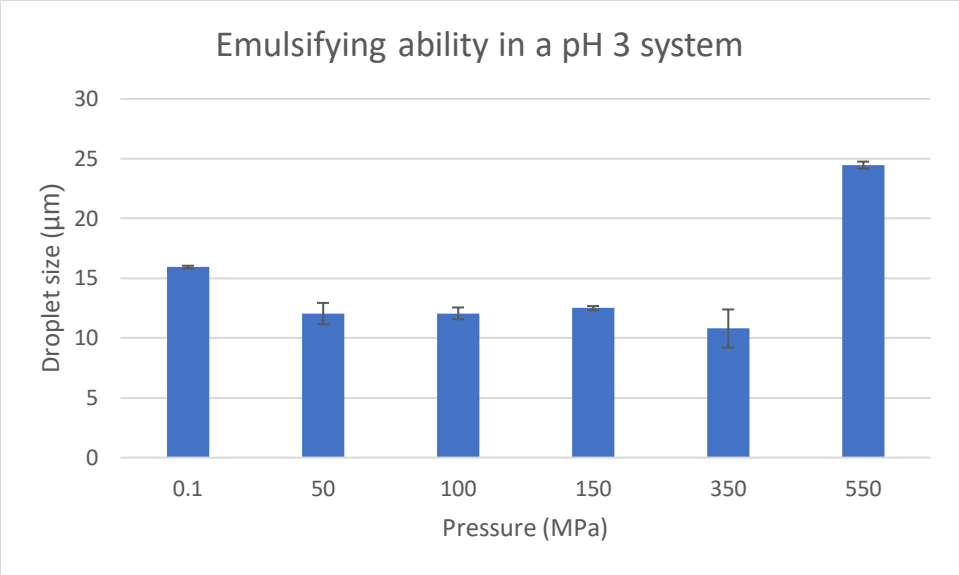



Figure 39: Emulsifying ability in a pH 3 system as a function of pressure compared to unprocessed control. (Error bars indicate standard deviation; data that do not share the same letter are significantly different from each other (one way ANOVA ($p<0.05$) and Tukey test))

The graph in Fig. 39 represents the droplet size after the formation of the emulsion for YLPC treated at increasing pressures, keeping the time of treatment constant at 10 minutes and the pH of the YLPC solution constant at 7. From the above graph, it can be seen that the EA improved slightly as the pressure increases, until 350 MPa, but decreased significantly as the pressure was increased to 550 MPa. The droplet size of the emulsion stabilized by the control, that is, the unprocessed YLPC is around 15 µm. The droplet size dropped slightly to around 12 µm for samples pressurized at lower pressures and increases to close to 25 µm for the emulsion stabilized by the YLPC sample pressurized at 550 MPa. Thus, increase in the intensity of pressure treatment negatively impacts the emulsifying ability of YLPC at pH 3. This might be because even though the protein surface hydrophobicity increased at higher pressures, the surface zeta potential did not significantly

vary. Thus, the protein might be able to bind a higher amount of fat, but not an increased amount of water. For an emulsion to be stable, the protein should be able to bind both oil and water effectively in order to prevent coalescence of oil droplets. Emulsifying ability is strongly related to solubility of the emulsifier in the food system. As seen in the previous section on the effect of high pressure on solubility, it can be seen that the solubility is not affected and remains largely the same as that of unprocessed YLPC under pressure. An increasing surface hydrophobicity was not accompanied by an increased surface charge and high solubility. Thus, the emulsifying ability of the processed YLPC does not increase significantly as the pressure increases. Also, the pH of the YLPC solution during the HPP treatment does not have a significant effect on its emulsifying ability.

pH 7:

According to the BBD experiments, the emulsifying ability in a pH 7 system depended significantly on the magnitude of pressure (P), the pH of the YLPC solution during the HPP treatment (pH) and the interaction of pressure and time (Pt). Thus, dropping the insignificant terms from the equation, the predictive equation for the emulsifying ability of YLPC in a pH 7 system is:

EA at pH 7 = 9.41+1.898 P-2.075 pH-2.573 Pt (R2 = 0.87),…….(7)

The positive co-efficient of pressure in the equation indicates that an increase in the magnitude of pressure corresponds to an increase in the droplet size of the dispersed phase of the emulsion. This means that YLPC treated under high pressures will form emulsions with larger dispersed phase droplets, that is, it will decrease the emulsifying ability of the

YLPC. Similarly, the negative co-efficient of the pH term indicates that a decrease in pH corresponds to bigger sized dispersed phase droplets.

Thus, higher values of pH of the YLPC solution during HPP treatment will increase the emulsifying ability of the YLPC.

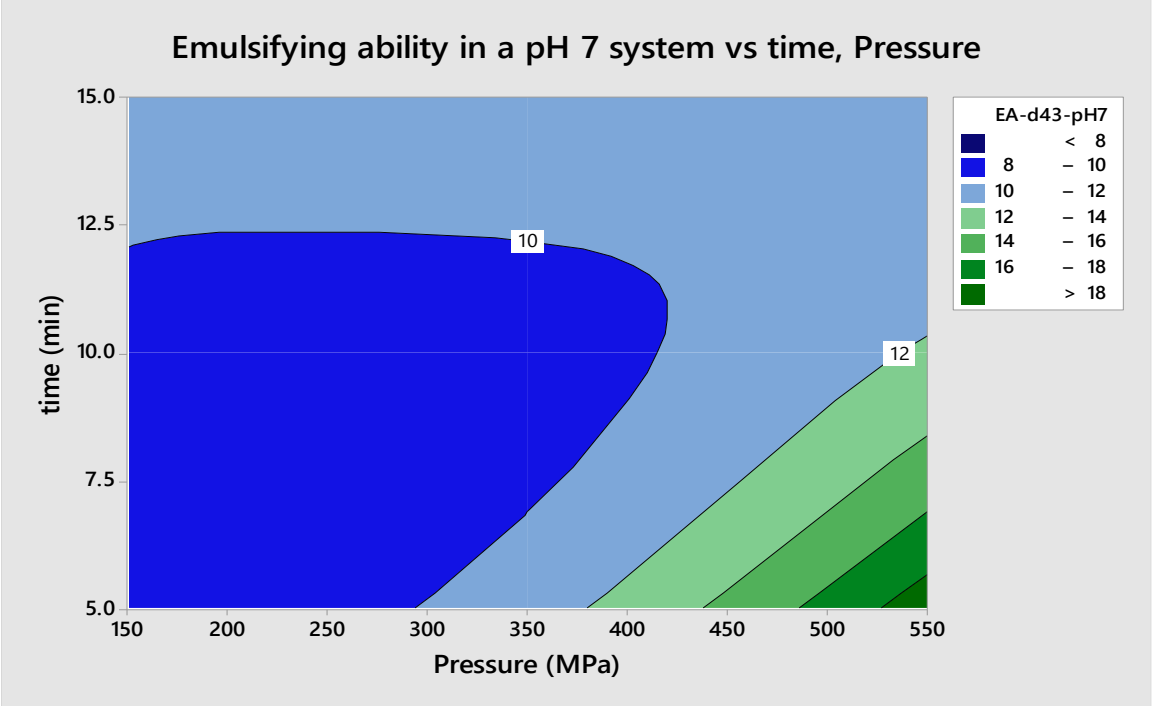



Figure 40: Effect of Pressure-Time interaction on the emulsifying ability in a pH 7 system

Figure 40 shows the contour plot generated based on the BBD data shows the variation of emulsifying ability of the YLPC in a pH 7 system as a function of the pressure and time of treatment. Lower droplet size of the dispersed phase and better emulsifying ability is observed at lower pressure and time combinations- the droplet size of dispersed phase in emulsions stabilized at pressures greater than 400 MPa slightly increases from 10 µm to 12 µm. The protein solubility, as seen in earlier sections, is higher for samples treated at

lower pressures (150 MPa). This could explain the better quality of emulsions at lower pressure-time combinations.

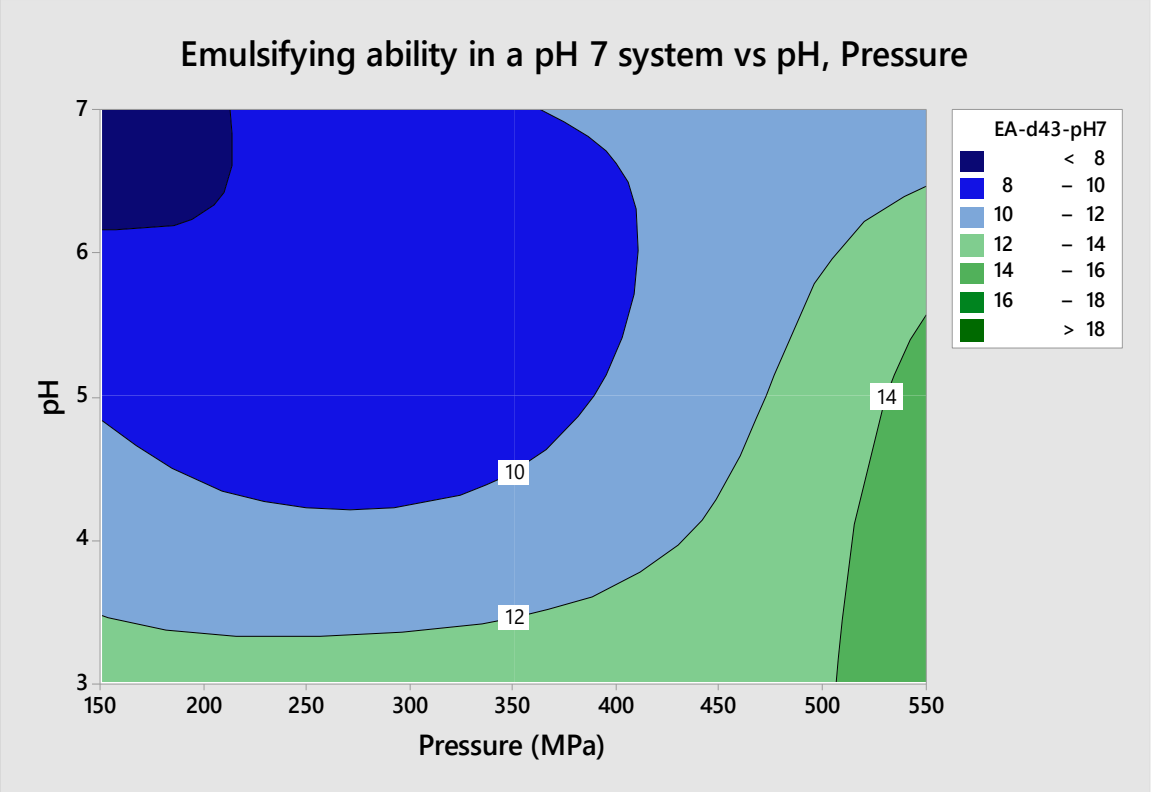



Figure 41: Effect of Pressure-pH interaction on the emulsifying ability in a pH 7 system

Figure 41 shows the contour plot above shows the variation of emulsifying ability of YLPC as a function of pH and pressure. It can be seen that lower pressures (150 MPa) and neutral protein solutions contributed to better emulsifying ability at pH

The lower pressure and milder treatment contributing to better emulsifying ability is similar to the pH 3 system. Since the BBD data does not consider the emulsifying ability of the unprocessed control, the following graph is plotted keeping the treatment time (10 min) and pH of the YLPC constant, and increasing the magnitude of pressure treatment from 50 MPa to 550 MPa.

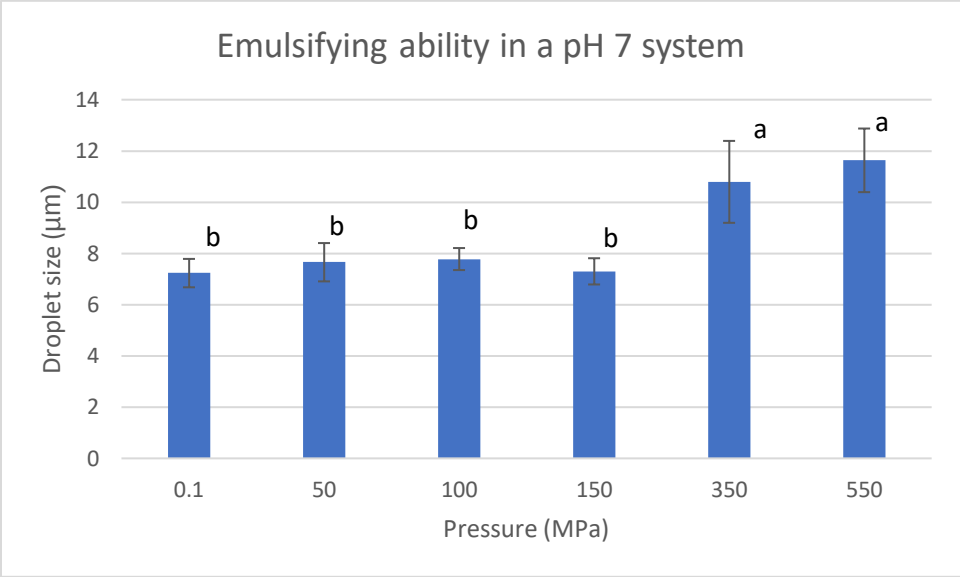



Figure 42: Emulsifying ability as a function of pressure in a pH 7 system compared to unprocessed control. (Error bars indicate standard deviation; data that do not share the same letter are significantly different from each other (one way ANOVA ($p<0.05$) and Tukey test))

3.3.2.2 Emulsion stability: pH 3:

According to the BBD data, the emulsion stability in a pH 3 system depended significantly on the interaction of time and pH (t*pH) and the square of the pressure term (P2). Dropping the insignificant terms, the predictive equation for the emulsion stability at pH 3 is:

ES at pH 3 = 66.67 – 10.42 t*pH + 12.38 P2 (R2=0.82)……(8)

The equation (8) indicates that higher pressures impart higher stability to the emulsions formed by the pressurized YLPC

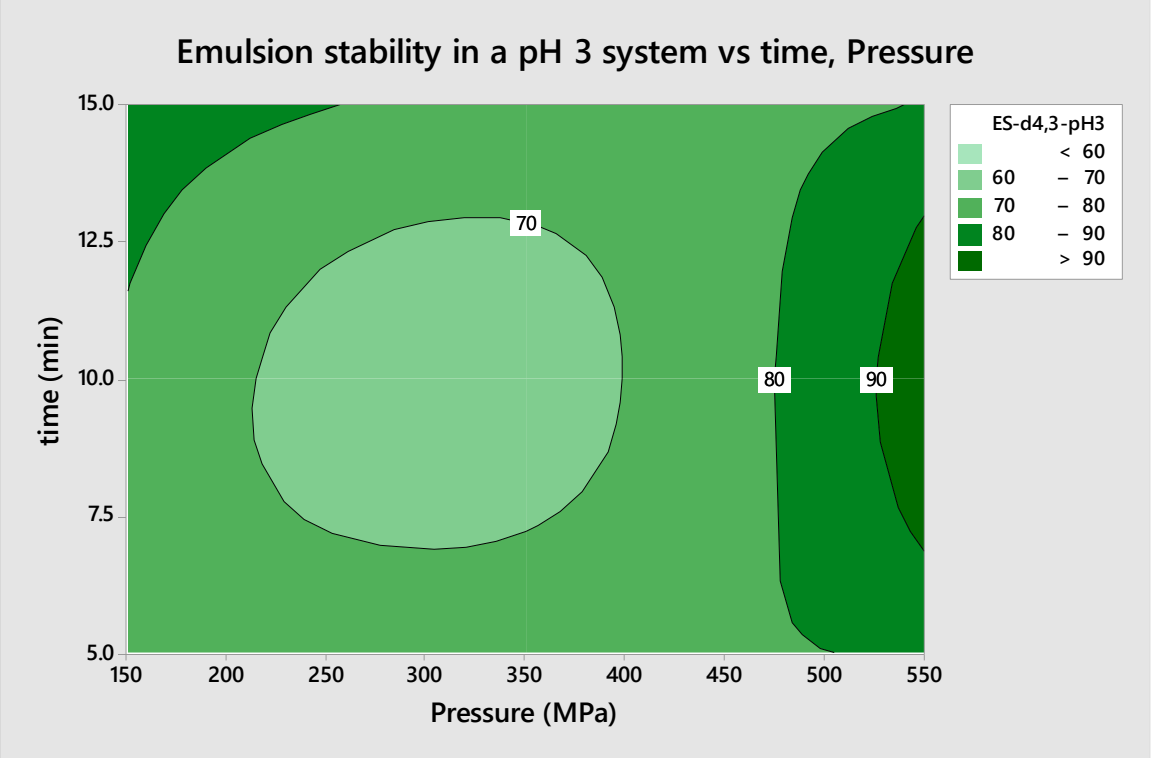



Figure 44: Effect of pressure-time interaction on the emulsion stability in a pH 3 system

The contour plot in figure 43 shows the variation of emulsion stability in a pH 3 system as a function of the time of pressure treatment and the magnitude of pressure. It can be seen that the highest emulsion stability, around 90% is observed for emulsions stabilized by YLPC samples treated at higher pressures and intermediate time combinations. It was observed that the droplet sizes observed in a pH 3 system for the protein solutions treated

at higher pressures such as 550 MPa was the largest. Bigger dispersed phase droplets (as observed in the EA at pH 3) are more stable as compared to smaller droplets, which could explain why the emulsions formed using protein samples treated at higher pressures are more stable.

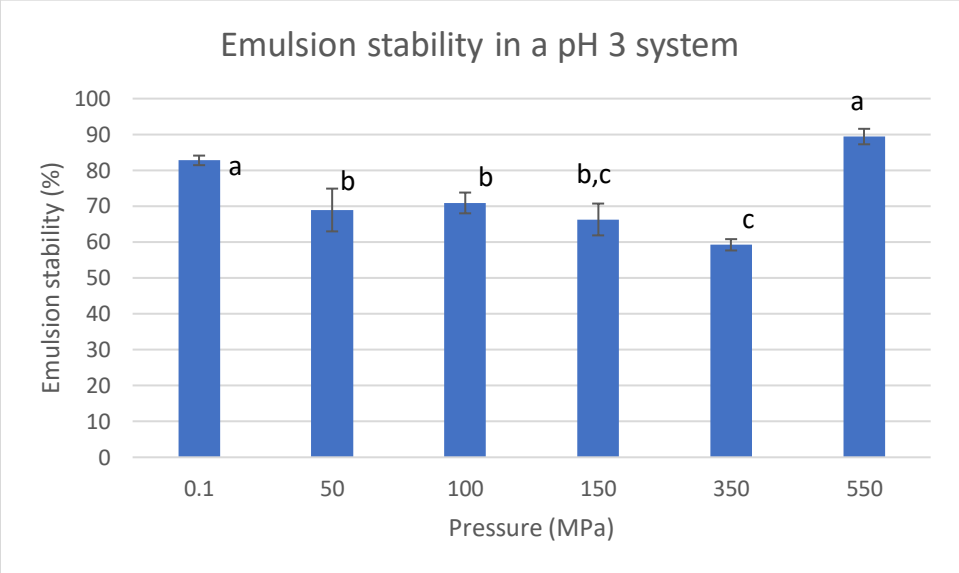



Figure 45: Emulsion stability in a pH 3 system as a function of pressure compared to the unprocessed control. (Error bars indicate standard deviation; data that do not share the same letter are significantly different from each other (one way ANOVA ($p<0.05$) and Tukey test))

Figure 45 represents the emulsion stability of YLPC solutions treated at increasing magnitudes of pressure keeping the treatment time and pH of the solution constant for all (10 min, pH 7). It confirms the BBD results that emulsion stability increased with an increase in the magnitude of pressure. It was the highest at 550 MPa, which was also the same pressure at which the dispersed phase droplets were the biggest in size. Thus the

increased stability may be because of the inherent stability of bigger droplets compared to smaller sized ones.

pH 7:

The BBD data indicates that the emulsion stability in a pH 7 system depends on the pH of the YLPC solutions that undergo the HPP treatment. Ignoring all the other insignificant terms, the predictive equation for the emulsion stability in a pH 7 system is:

ES at pH 7 = 91+17.22 pH (R2 = 0.85)……..(9)

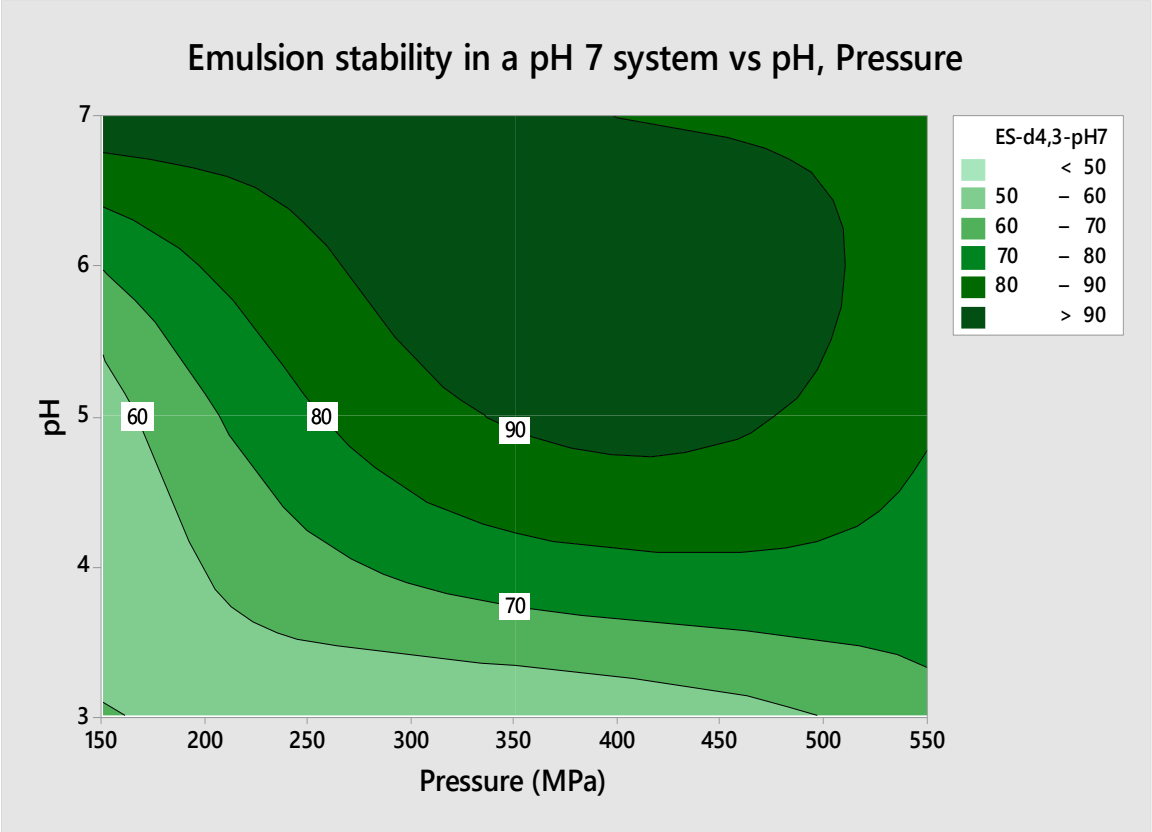



Figure 46: Effect of pressure-pH interaction on the emulsion stability in a pH 7 system

The contour plot in figure 46 shows the variation of emulsion stability in a pH 7 system as a function of pressure and pH of the YLPC solutions.

Higher emulsion stability is observed for samples that underwent pressure treatment in solutions near a neutral pH. Pressure did not significantly affect the emulsion stability in a pH 7 system, as indicated by the BBD data analysis. Higher emulsion stability at or near neutral pH could be because of the right balance of lower surface hydrophobicity and increased protein solubility. Since the protein molecules have a net negative charge at pH 7, they will repel each other on surrounding the oil droplets, thus keeping the emulsion intact.

3.3.2.4 Fat binding capacity:

According to the BBD data, the fat binding capacity of the YLPC significantly depends on the magnitude of pressure applied (P), the pH of the YLPC solution (pH) and the square of the pH (pH2). Dropping the insignificant terms, the predictive equation for the fat binding capacity of YLPC is:

FBC = 127.77 + 33.33 P – 27.08 pH – 34.72 pH2  (R2 = 0.9)……..(10)

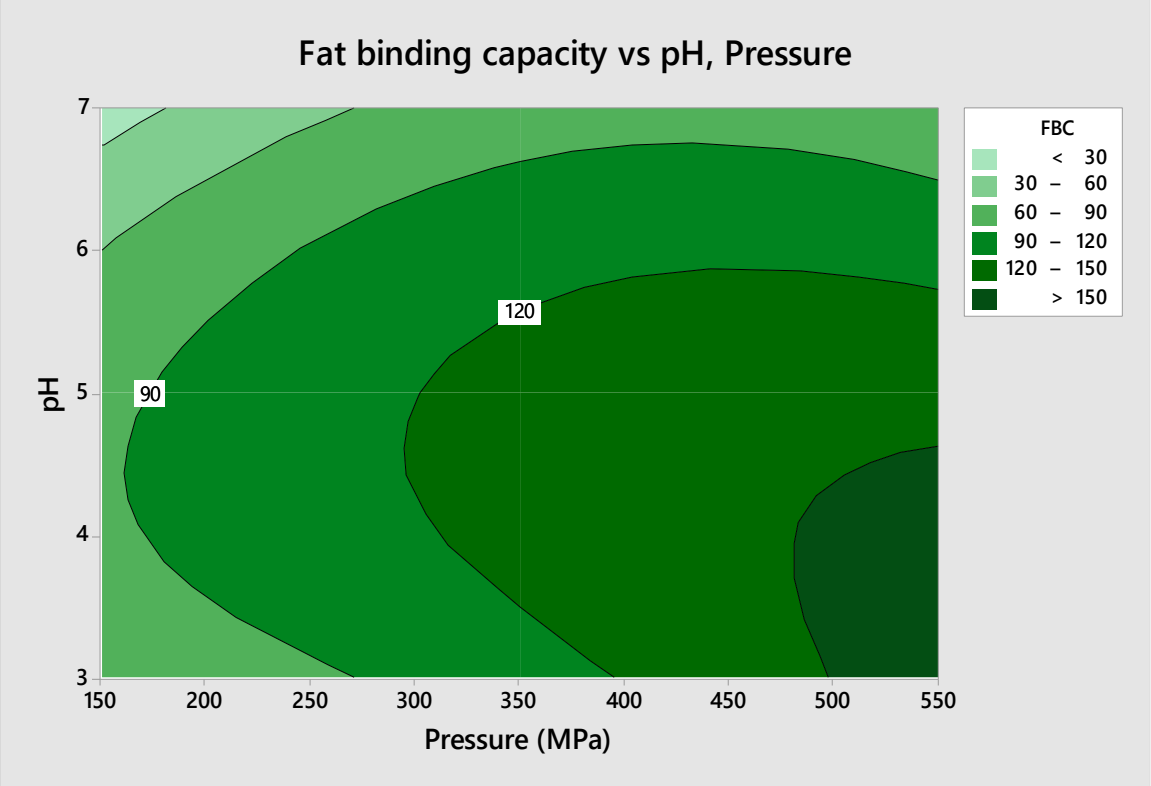



Figure 47: Effect of pressure-pH interaction on the fat binding capacity of YLPC

The contour plot above in Fig. 46 shows the variation of the fat binding capacity with the pressure applied and the pH of the YLPC solution. We can see that the fat binding

capacity increased with an increase in the pressure applied and is the highest for the YLPC solutions that had a lower pH value (3-4). It increased to around 120% or higher for samples treated at 550 MPa. This increase in the ability to bind fat may be because of an increase in the surface hydrophobicity for samples treated at higher pressures.

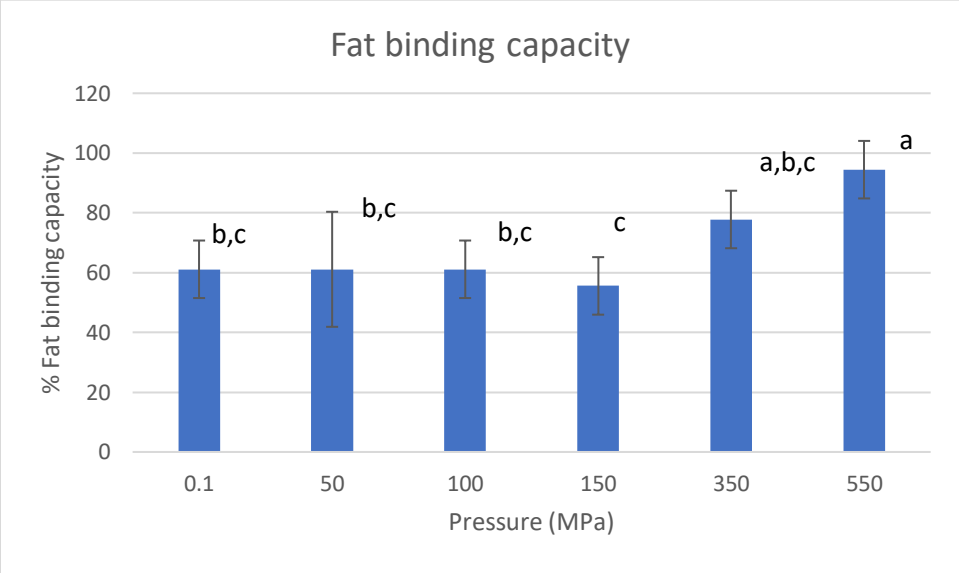



Figure 48: Fat binding capacity of YLPC as a function of pressure compared to unprocessed control. (Error bars indicate standard deviation; data that do not share the same letter are significantly different from each other (one way ANOVA ($p<0.05$) and Tukey test))

The bar chart in Fig. 48 represents the fat binding capacity of treated YLPC keeping the treatment time and the pH of YLPC solutions constant. The fat binding capacity of the untreated YLPC is around 60%. It can be seen that the fat binding capacity increases for higher pressures (550 MPa) to about 94%, which is slightly higher than the fat binding capacity of the control (unprocessed YLPC). This gradual increase could be because of the increased surface hydrophobicity of the YLPC. Non- polar amino acid residues, exposed to the surroundings due to gradual unfolding of the protein molecule can thus bind

significantly more fat than the less unfolded protein molecules subjected to lower pressures.

3.3.2.5 Foaming ability and foam stability:

The foaming ability and foam stability of YLPC did not vary significantly for any of the conditions studied for either of the pH systems studied. Even though the hydrophobicity increased with an increase in the pressure, this did not significantly affect the foaming ability of the YLPC. This could be because a good foam requires the hydrophilic groups oriented towards the water and the hydrophobic groups towards the air bubbles. Since the surface charge (zeta potential) did not change for both the pH systems, the YLPC could not bind water as effectively and give rise to a stable foam.

# 4. CONCLUSIONS

The surface hydrophobicity of Yellow Lentil Protein Concentrate (YLPC) increased after high pressure processing. The pressure treated YLPC was able to bind more fat as compared to untreated YLPC. The fat binding capacity of YLPC treated at 550 MPa was 90%, and that of the untreated control was 60%.

Experiments conducted using the Box Behnken Design (BBD) of experiments showed that Pressure (MPa), hold time (min), the pH of the YLPC solutions undergoing pressure treatment and their interaction effects significantly affected the functional and physicochemical properties of YLPC in two different pH systems, namely pH = 3 and pH = 7.

The surface zeta potential of YLPC was not significantly affected by the high pressure processing parameters studied.

In the pH 7 system, the solubility of YLPC subjected to lower pressure (150 MPa) increased slightly from 40% for the untreated control to 50%. However the solubility decreased to 45% when the YLPC was subjected to higher pressure (350 MPa).

A similar trend was observed for the emulsifying ability in both the pH systems. The emulsifying ability of YLPC deteriorated at higher pressure (350-550 MPa) in both systems.

The foam ability and foam stability of YLPC was not significantly affected by the high pressure processing parameters studied.

Thus, functional properties that require a high surface hydrophobicity as well as a high surface zeta potential did not improve after high pressure processing of YLPC, possibly because only the surface hydrophobicity increased significantly after pressure treatment.

Thus, high pressure processing of YLPC can improve its functional properties at mild processing conditions.

# 5. FUTURE WORK

In this study, the physicochemical and functional properties of pressure treated Yellow Lentil Protein Concentrate were studied. The effect of pressure was isolated to check its effect on the properties of YLPC. The effect of pH and hold time could be isolated for their effect on the properties of YLPC. The concentration of YLPC solutions was kept constant at 10% (w/v) during this study. This could be varied to see if it affects the way the protein unfolds under pressure at different concentrations.

A study of the structural changes due to high pressure could give a more detailed insight into the changes taking place in the protein structure under pressure. Gel electrophoresis, scanning electron microscopy of the pressure treated protein could give an idea about aggregate formation and unfolding after high pressure processing.

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