EFFECT OF HIGH HYDROSTATIC PRESSURE ON THE FUNCTIONAL PROPERTIES OF SOY PROTEIN ISOLATE

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ABSTRACT

EFFECT OF HIGH HYDROSTATIC PRESSURE ON THE FUNCTIONAL PROPERTIES OF SOY PROTEIN ISOLATE

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Soy protein is a low-cost additive with high biological value, unique functional properties, and beneficial effects on health, and it is widely used in the food industry as an important ingredient. When the health and functional properties of soy protein are considered altogether, there are several benefits for consuming and using it in foods. High hydrostatic pressure (HHP) is a non-thermal novel processing technology that has been generally used to destroys vegetative cells, microorganisms and enzymes. Additionally, HHP treatment has an effect on protein structures, resulting changes in the functional properties of proteins. The aim of the study was to examine the effects of high hydrostatic pressure (HHP) treatment on functional properties of soy protein isolate (SPI). The experiments were carried out at different pressure parameters (300, 400 and 500 MPa) with a constant duration of 5 min at 25 ^{o}C and 40 ^{o}C. Also, the pH of the SPI samples was adjusted to pH 5 and pH 7 to evaluate the pH effect with HHP treatment on the functional properties of SPI prepared at 38% (w/v) concentration. Water holding capacity (WHC), solubility by Lowry method, emulsion activity, and viscosity of untreated and HHP-treated soy protein isolate were analyzed. Following that, characterization experiments including Fourier transform infrared (FTIR) spectroscopy and Nuclear Magnetic

Resonance (NMR) relaxometry were performed to determine the changes in secondary structure and hydration behavior of soy protein isolate (SPI), respectively. This study showed that HHP treatment significantly ($p<0.05$) decreased WHC compared to control, however there was no significant difference between WHC results of pH 5 and pH 7 ($p>0.05$). Moreover, HHP treatment significantly enhanced solubility of SPI at pH 7 compared to pH 5 and control, and also, at pH 5, the solubility of HHP-treated SPI at ambient temperature was significantly higher than that of SPI treated at 40 °C ($p<0.05$). Furthermore, although the emulsion activity results revealed no significant difference ($p>0.05$) between HHP-treated SPI at pH 7 and untreated SPI, providing the appropriate pressure increased emulsion activity at pH 5, but further increase in pressure resulted in reduced emulsion activity. Also, viscosity of SPI significantly reduced due to HHP treatment at pH 5 and pH 7 compared to control ($p<0.05$). In addition, FTIR results showed that HHP treatment caused remarkable changes in the secondary structure of SPI due to unfolding of protein. Moreover, HHP treatment had no influence on SPI hydration behavior at pH 7 ($p>0.05$), but T_2 values at pH 5 at 40 °C were significantly much higher than those obtained for control and other treated SPI. In conclusion, the results of this study showed that HHP application could be a valuable alternative to modify and improve the functional properties of soy protein, which has an important role in novel product development.

Keywords: Soy protein isolate (SPI), HHP, FTIR, NMR Relaxometry, functional properties

ÖZ

YÜKSEK HİDROSTATİK BASINCIN SOYA PROTEİN İZOLATININ FONKSİYONEL ÖZELLİKLERİ ÜZERİNE ETKİSİ

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Soya proteini, biyolojik değeri yüksek, benzersiz fonksiyonel özellikleri ve sağlığa yararlı etkileri olan düşük maliyetli bir katkı maddesidir ve gıda endüstrisinde önemli bir bileşen olarak yaygın olarak kullanılmaktadır. Soya proteininin sağlık ve fonksiyonel özellikleri bir arada düşünüldüğünde, onu tüketmenin ve gıdalarda kullanmanın çeşitli faydaları vardır. Termal olmayan yeni bir işleme teknolojisi olan yüksek hidrostatik basınç (YHB), genellikle vejetatif hücreleri, enzimleri, mikroorganizmaları yok etmek için kullanılmıştır. Ek olarak, YHB uygulamasının protein yapıları üzerinde etkisi vardır ve bu da proteinlerin fonksiyonel özelliklerinde değişikliklere neden olur. Bu çalışmanın amacı, yüksek hidrostatik basıncın soya proteini izolatının fonksiyonel özellikleri üzerindeki etkilerini araştırmaktır. Deneyler farklı basınç parametrelerinde (300, 400 ve 500 MPa) 25 ^{o}C ve 40 ^{o}C'de 5 dakika sabit süre ile gerçekleştirilmiştir. Ayrıca, %38 konsantrasyonda hazırlanan soya protein izolatının fonksiyonel özellikleri üzerindeki YHB ve pH etkisini değerlendirmek için soya protein izolat numunelerinin pH'ı 5 ve 7'ye ayarlanmıştır. Kontrol numunesi ve basınca maruz bırakılmış soya protein izolatlarının su tutma kapasitesi, Lowry yöntemiyle çözünürlük, emülsifikasyon aktivitesi ve viskozitesi analiz edilmiştir. Bunu takiben, soya proteini izolatının

ikincil yapısındaki değişiklikleri ve hidrasyon davranışındaki değişiklikleri belirlemek için sırasıyla Fourier transform infrared (FTIR) spektroskopisi ve Nükleer Manyetik Rezonans (NMR) Relaksometri ölçümünü içeren karakterizasyon deneyleri yapılmıştır. Bu çalışma, YHB uygulamasının su tutma kapasitesini kontrole kıyasla önemli ölçüde azalttığını ($p<0.05$) göstermiştir, ancak pH 5 ve pH 7'de su tutma kapasite sonuçları arasında önemli bir fark gözlemlenmemiştir ($p>0.05$). Ek olarak, YHB işlemi, pH 5 ve kontrol ile karşılaştırıldığında pH 7'de soya protein izolatının çözünürlüğünü önemli ölçüde arttırdığı ve ayrıca pH 5'te, YHB ile muamele edilmiş SPI'nın oda sıcaklığındaki çözünürlüğü, 40 °C'de muamele edilmiş SPI'den önemli ölçüde daha yüksek olduğu görülmüştür ($p<0.05$). Ek olarak, emülsiyon aktivitesi sonuçları, pH 7'de YHB ile muamele edilmiş SPI ile işlem görmemiş SPI arasında önemli bir fark ($p>0.05$) ortaya koymamasına rağmen, uygun basıncın sağlanması pH 5'te emülsiyon aktivitesini arttırmıştır, ancak basınçta daha fazla artış, emülsiyon aktivitesinin azalmasına neden olmuştur. Ayrıca, kontrole kıyasla pH 5 ve pH 7'de YHB işlemine bağlı olarak SPI'nın viskozitesi önemli ölçüde azalmıştır ($p<0.05$). Ek olarak, FTIR sonuçları, YHB uygulamasının, SPI'nın ikincil yapısında proteinin açılmasından kaynaklanan dikkate değer değişikliklere neden olduğunu göstermiştir. Ayrıca, YHB işleminin pH 7'de SPI'nın hidrasyon davranışı üzerinde hiçbir etkisinin olmadığı ($p>0.05$), fakat 40 °C'de pH 5'teki T_2 değerleri, kontrol ve diğer işlenmiş SPI için elde edilenlerden önemli ölçüde daha yüksek olduğu NMR ölçümü sonuçlarında görülmüştür. Sonuç olarak, bu çalışmanın sonuçları, yeni ürün geliştirmede önemli bir role sahip olan soya proteininin fonksiyonel özelliklerini değiştirmek ve geliştirmek için YHB uygulamasının değerli bir alternatif olabileceğini göstermiştir.

Anahtar Kelimeler: Soya protein izolatı, YHB, FTIR, NMR Relaksometresi, fonksiyonel özellikler

To the ones that always support me, especially my beloved family.

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ABBREVIATIONS

HHP	: High hydrostatic pressure
SPI	: Soy protein isolate
WHC	: Water holding capacity
FTIR	: Fourier transform infrared
NMR	: Nuclear magnetic resonance
CPMG	: Carr-Purcell-Meiboom-Gill
ANOVA	: Analysis of Variance
PEF	: Pulsed electric field
US	: Ultrasonication
RF	: Radiofrequency
EA	: Emulsion Activity
O/W	: oil-in-water
W/O	: water-in-oil
BSA	: Bovine serum albumin

CHAPTER 1

INTRODUCTION

1.1 Soy Protein

Soybean (*Glycine max L.*), an annual legume plant native to northeast China and other areas of Asia, is the world's major source of edible oil and vegetable protein. Soybeans have the greatest protein level of any food crop, as well as the second-highest fat content among all food legumes. On a dry weight basis, soybean seeds have a protein content of 36-38% and an oil content of 19%. Soybean seed composition is strongly influenced by environmental and genetic factors (Mojica et al., 2014).

Proteins have been increasingly more popular in the food industry in tandem with the growing world population. In addition, consumers' interest in healthy nutrition has increased over the years, prompting the food industry to shift in using plant proteins due to their nutritional properties. Soy protein has a high level of interest among the plant proteins, and this is owing to its excellent nutritional quality, availability, abundance, low cost, as well as its significant functional properties in food applications. Soy protein is a plant-based protein that is commonly utilized in the food industry. Its nutritional and industry-appropriate functional properties have contributed significantly to its popularity (Li et al., 2012). Soy protein products have been employed as healthy and functional dietary components in every food category supplied to consumers since the 1960s. Soybean protein, which has been the focus of much research, has played an increasingly important role in human nutrition in recent decades (Singh et al., 2008).

Storage proteins, which are primarily *globulins*, make up around 90% of the proteins in soybeans. Intracellular enzymes including amylase, urease, and lipoxygenase are among the remaining proteins, as are protein inhibitors, hemagglutinins, and membrane lipoproteins. **7S** (β-conglycine) and **11S** (glycine), which are the storage proteins, are the primary components of soy protein and dictate the functional properties of soy proteins. According to the literature, the relative proportions of 7S and 11S fractions differ substantially, and also conflicting study results can be attributed to the fact that these storage proteins display association-dissociation properties under different conditions. According to some estimations, glycine represents 60-70 % of the soybean globulins, and glycine is the protein, which precipitates at pH 4.5, which has a direct influence on the functional properties of soy proteins (Kinsella, 1979).

As a protein source, there are three types of soy protein products with content ranging from 50 to 90%. These include soy protein flours, concentrates, and isolates, from the lowest to the highest protein content. Soy protein isolates containing 90% or more protein are the most refined forms of soybean protein. In producing soy protein concentrates, oligosaccharides and other low molecular weight components are eliminated. Soy protein isolate, on the other hand, is prepared by removing water-insoluble polysaccharides in addition to the oligosaccharides and other low molecular weight components (Singh et al., 2008).

Soy based food consumption is expanding as a result of the stated benefits to human nutrition and health. It lowers plasma cholesterol, prevents cancer, diabetes, and obesity, and protects against intestinal and renal illnesses, especially when supplemented with other protein sources such as cereal grains, milk, and meat (Bressani, 1981; Hamilton & Carroll, 1976; Torún et al., 1981). Soy protein products' nutritional adequacy has been convincingly shown by their usage in infant formulas, where protein requirements are particularly crucial (Yetley & Park, 1995).

When the health and functional properties of soy protein are examined together, there are many positive effects of its consumption and usage in foods. Soy protein isolates and concentrates contain the same protein quality as meat, milk, and eggs, and are also very easy to digest compared to other protein commodities. In addition, soy proteins are cholesterol and lactose-free, making them appropriate to be used in cholesterol and lactose-free diets. Furthermore, there is an increasing demand for using soy products to obtain functional food, for example, in the production of bread, cereals, dairy products, and beverages, as well as in tablet and capsule supplements. It is also used to improve the texture of many foods such as meat, frozen desserts, cheese, peanut butter, by supporting moisture and flavor retention and helping emulsification (Singh et al., 2008).

1.1.1 Functional Properties of Soy Protein

Functional properties of proteins, in general, relate to any physicochemical property that impacts protein processing and behavior in food systems, as evaluated by the end product's quality. To predict the functional properties of soy protein, it is essential to understand the physicochemical states and interactions of the protein. The interaction of food components such as water, ions, proteins, and lipids, as well as environmental factors such as pressure, temperature, pH, and ionic strength, affect the functional properties of proteins, which contain information about the protein's internal physical properties such as composition, conformation, and structure.

Understanding the basis of functionality, such as the composition-structure connectivity of proteins is one of the most important aspects to obtain the functionality needed to modify proteins and predict potential applications. The physical behavior of a protein is governed by proteins' molecular size, amino acid composition, conformation, change distribution, intermolecular and intramolecular

bonding, and also environmental factors. In globular proteins, for example, the more polar charged amino acids are directed to the surface of the protein, the more solubility and hydration. Moreover, covalent and non-covalent bonding have significant impacts on protein functionality. The disulfide linkage, a covalent bond, has a significant influence on functional qualities of soy protein, such as gel formation. On the other hand, non-covalent bonds are hydrophobic interactions, hydrogen bonding, and electrostatic attraction and these bonds play a role in protein-protein and protein-solvent interactions that affect the functional properties of a protein. Aside from these, various factors such as the protein's genotype, manufacturing, and treatment conditions, harvesting, extraction method, isolation, technique, and storage can all influence the functional behavior of proteins.

Although the low cost and availability of soy proteins are appealing relative to other protein sources, the fundamental reason for their popularity is that they exhibit a wide range of functional qualities in various conditions. In the food industry, it has evolved into a product that meets important consumer demands such as texture (Al-Bakkush, 2008). At this point, it is essential to understand the impact of processing conditions on the functionality of plant proteins which have some restrictions compared to animal proteins. Because of the various functional properties of soy protein under various conditions, soy protein products will undoubtedly play a critical role in this new era of "restructured" food technology. As a result, protein modifications via different processing conditions become crucial in understanding and solving the problems about the functionality of plant proteins.

1.1.1.1 Water Holding Capacity (WHC)

Water-holding capacity (WHC), an important functional property of proteins, is the capability of proteins to prevent the release or ejection of water from their 3-D structures against gravity. It plays a critical role in food formulation in the food

industry, especially in foods such as baked dough and meat products, since if the WHC is too high or too low, it results in other ingredients being hydrated or sensitive to moisture during storage, respectively (Haque et al., 2016).

The determination of WHC (g water/g protein), which indicates the amount of water absorbed by the protein, is vital to understand the protein-water interaction (Zayas, 1997b). Studies have shown that WHC is affected by variables such as ionic strength, pH, temperature, time, and protein structure. The charge of protein molecules has a great influence on water-holding capacity, and it is usually lowest at the isoelectronic point where the net protein charge is zero and the protein-protein interaction predominates (Haque et al., 2016; Kneifel et al., 1991).

According to Yao et al. (1988), the ratio of glycinin and β-conglycinin, which are the major two proteins of soy protein, affects water holding capacity. The water holding capacity of SPI decreased with the increase of β-conglycinin to glycinin ratio. Furthermore, the study evaluated the role of sodium chloride on WHC, concluding that salt restricts the interaction of polar amino acids with water, lowering WHC. In another study, it was stated that the water holding capacity of SPI was directly related to its denaturation degree. Sidechains were more exposed to the surface by unfolding of proteins and WHC increased due to the increased interactions with water (Jovanovich et al., 2003).

1.1.1.2 Solubility

Solubility, the most feasible indicator of protein aggregation, is an important functional property of soy protein (Hu et al., 2013). Protein solubility is a determinant of water-protein interaction and is linked to other functional properties including emulsifying, gelling, and rheology (Boatright & Hettiarachchy, 1995; Huang et al., 2020). Moreover, protein-protein interaction is also associated with

solubility. Electrostatic and hydrophobic interactions between protein molecules have a decisive impact on the protein solubility. It is dominated by the delicate balance of repulsive and attractive intermolecular forces. When electrostatic repulsion is more than the hydrophobic interactions between proteins, they become more soluble; otherwise, the opposite takes place (Zayas, 1997a).

For applications in the food industry, understanding how the solubility of soy protein changes under the effect of diverse environmental conditions is critical. Since the solubility of soy protein is affected strongly from the physicochemical states of protein molecules, which are influenced by processes such as heating and drying during production and storage, solubility is one of the most defining characteristics of proteins. It is crucial to examine the variation of the solubility characteristic in relation to the parameters such as soy protein type (flour, concentrate, and isolate), protein concentration, ionic strength, pH, temperature, and/or pressure to have detailed knowledge about it (Lee et al., 2003; Manassero et al., 2015).

There is a very strong correlation between protein solubility and pH. Proteins become positively or negatively charged as they depart from the isoelectric point (pI), and this improves solubility. Soy protein has an isoelectric point of 4.5, at which the net charge is zero, resulting in decreasing solubility with the association of molecules (Rangavajhyala et al., 1997). According to a research, the change in solubility of SPI at pH 3 and pH 8 was investigated, and it was reported that the solubility of untreated SPI at pH 8 was higher than the solubility at pH 3 (Puppo et al., 2004). Moreover, the effect of temperature and pH on the solubility of soy protein isolate was examined in a study. The results revealed that solubility increased at pHs far from pI, and that temperature and pH improved the solubility synergistically (O'Flynn et al., 2021).

1.1.1.3 Emulsification

An emulsion is a system consisting of immiscible droplets dispersed in another liquid and stabilized by an interphasic component (Cherry, 1981). Emulsions generally consist of two phases: an internal and an external phase. The internal phase expression, also known as the discontinuous phase, refers to dispersed droplets. Furthermore, the external phase, also called the continuous phase, is the medium in which the droplets are dispersed (McWatters & Cherry, 1981). Additionally, oil-in-water (O/W) emulsions, in which oil droplets are suspended in the external water phase, and water-in-oil (W/O) emulsions, in which water droplets are suspended in the external oil phase, are the two most prevalent types of emulsions (Owusu-Apenten, 2004).

Proteins are a popular type of emulsifier since they commonly have surface-active characteristics which avoid droplet coalescence. They behave similarly to the surfactants by forming a film at the interface (McWatters & Cherry, 1981). Proteins with hydrophobic and hydrophilic groups are useful for emulsifying and are widely used in oil-in-water emulsions. The hydrophobic amino acids placed in the core of globular protein must migrate to the surface at the interface to support the emulsifying property of the proteins, hence partial denaturation of the proteins is required to coat the droplets. Following that, the proteins realign to place the surface hydrophobic amino acids in the internal phase and the hydrophilic amino acids in the continuous phase, forming a barrier that prevents coalescence (Nishinari et al., 2014). Also, Figure 1.1 illustrates the above-mentioned emulsifying mechanism of proteins in detail.

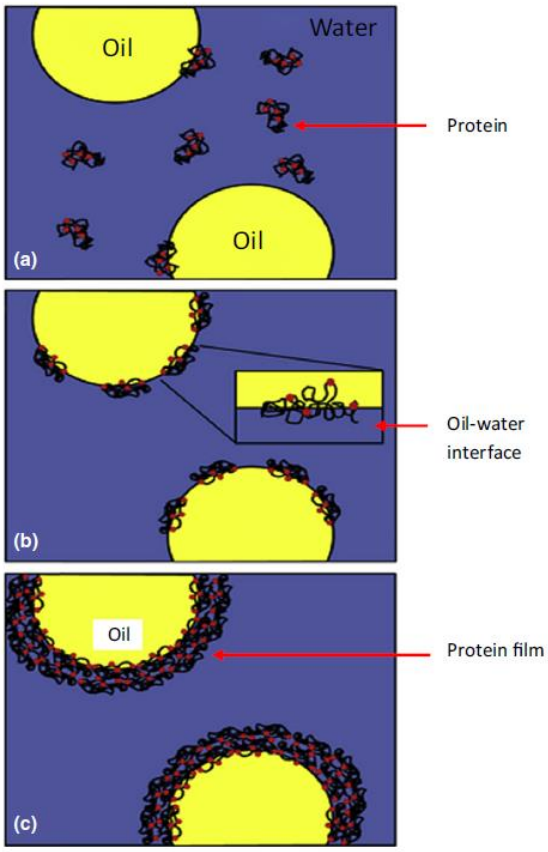

Figure 1.1 Protein behavior in an O/W emulsion. (a) movement of protein to the interface, (b) realign at the interface, (c) forming a viscoelastic film layer at the interface. Red dots illustrate hydrophobic amino acids (Haque et al., 2016).

Many studies have been conducted on the effects of various variables on the emulsifying characteristics of soy proteins. In a study, it was observed whether the emulsifying ability of soy protein isolates changed according to the different oil types in the emulsion at neutral pH and it was concluded that the oil type had an effect on the emulsifying ability of soy protein (Gu et al., 2009). The emulsification property of soy protein isolate at various pHs was studied by Qi et al. (1997) and they stated that the emulsification activity was the lowest at pH near to the pI point (pH 4-5) and increased as it moved away from the pI. They explained the reason for this situation

as soy protein had a more firm and stable behavior at pH near to the pI point at the interface and is not prone to film formation which is required for emulsification.

The emulsifying ability of soy protein is an important property widely used in the food industry for foods including sausages, bologna, soup, coffee whiteners, mayonnaise, salad dressings, and cakes batters (Kinsella, 1979).

1.1.1.4 Viscosity

The resistance to flow is known as viscosity, and it has a crucial role in food processing (Walnofer et al., 2005). Viscosity is a significant functional property of foods including beverages, soups, meats, and batters, as well as an important consideration for process line design in the food industry (Deak, 2004; Nazareth, 2009).

Viscosity, which is an important criterion for protein solutions, differs depending on the protein type and its physicochemical characteristics and conformations (Deak, 2004). Viscosity of proteins is completely controlled by protein molecules' molecular size, shape, charge, as well as solubility, and swelling capacity, and also greatly influenced by conditions such as concentration, processing temperature, ionicity, and pH (Hermansson, 1975; Kinsella, 1976).

According to the research by Wagner et al. (1992) on the rheological properties of SPI under various conditions, adding salt decreased the viscosity of proteins whereas raising temperature tended to increase it. In another study, Liu et al. (2017) reported that the viscosity inclined to increase as the concentration of SPI was increased and that this was associated with an enhancement in protein molecule entanglement. Moreover, in another study, it was demonstrated that the viscosity of soy protein may

be affected by conformational changes in proteins such as unfolding triggered by alkali and/or heat treatments (Nazareth, 2009).

1.2 Novel Processing Techniques

Recently, there has been an increase in consumer demand for fresh and lightly processed foods that retain their nutritional and organoleptic characteristics. The food industry is always looking for new and alternative food processing technologies and combinations of current methods to produce higher quality foods more efficiently and economically. These novel processing methods include ecologically friendly and sustainable food production techniques, such as minimal energy and water needs, without the drawbacks of traditional processing, by processing foods with the highest level of safety and quality. High hydrostatic pressure (HHP), pulsed electric field (PEF), ultrasonication (US), ultraviolet light, and cold plasma are among the most interesting novel processes. These are the examples of popular non-thermal food processing techniques that provide the greatest degree of food safety and quality, as well as the comprehension and management of complicated process-structure-function connections (Rastogi, 2013).

1.2.1 High Hydrostatic Pressure (HHP)

1.2.1.1 General View

There has been great interest in the application of high hydrostatic pressure (HHP), which is a non-thermal novel processing technology. In food processing, HHP has emerged as an alternative technique to the conventional heat treatment. It is used to eliminate pathogenic and spoilage bacteria and is also effectively used to improve the functional properties of the food (Alvarez et al., 2008). HHP treatment has minimal to no effect on the nutritional and organoleptic qualities of the food unlike traditional heat treatment, which degrades the nutritional content of foods such as

proteins, enzymes, and vitamins. So that, it is a better choice for heat-sensitive foods. In comparison to thermally processed foods, HHP treated foods have a superior texture, enhanced appearance, and fresher taste, as well as higher nutritional retention (Rastogi, 2013).

In 1883, Certes was the first to describe the consequences of high pressure on organisms. On the other hand, the impact of high hydrostatic pressures on foods was first demonstrated in 1899 by Bert Hite. The preservation of milk under 600 MPa high pressure was examined in Hite's study. Significant progress was made with the introduction of the first HHP treated product in Japan in 1992. High-pressure processing has been utilized efficiently in the food industry for the past 30 years and in the future, it may lead to a wide range of food products with longer shelf lives that offer a diversity of flavors and nutritional benefits to the consumers (Elamin et al., 2015). Currently, HHP treated fruit juices especially orange and apple juices, jellies, jams, dip sauces, seafood such as oysters, ready-to-eat meats, sauces, etc. are available on commercial food markets in the U.S., Japan, Canada, etc. (Rastogi, 2013).

HHP is suitable for a wide range of foods such as meat products, fruit and vegetable products, beverages, and dairy products. In theory, the product should contain enough water and be free of air voids for the treatment. Air gaps are undesirable because air is more compressible than water, resulting in a longer pressurization time (Aganovic et al., 2021).

HHP treatment is an emerging novel method that relies on the interplay of three physical variables: pressure, temperature, and time. The pressure level varies over a wide range depending on the application. For the treatment, the pressure and temperature levels range from 100 MPa to 1000 MPa and from 0 ◦C to 60 ◦C,

respectively, while the application time ranges from seconds to a few minutes. Depending on the purpose of the application and the type of product, the pressure, temperature, and pressurization time parameters of the process are decided. The simultaneous spread of pressure in all possible directions of the product is a significant advantage over thermal methods (Janowicz & Lenart, 2018).

HHP processing system is comprised of a pressure vessel, a pressure generator, a pressure and temperature controller and monitor, and a material handling system (Elamin et al., 2015). The product packed in a sterilized high-pressure container is loaded into a pressure vessel prefilled with pressure-transmitting fluid and exposed to hydrostatic pressure for specified periods until decompression. Water is the most preferred pressure-transmitting fluid when considering anti-corrosion properties, the viscosity of the fluid changes under pressure, compression temperature, and impacts on foods. (Aganovic et al., 2021).

HHP technology is based on two operating principles: Le Chatelier's Principle and Isostatic Principle. According to Le Chatelier's Principle, a chemical system in equilibrium undergoes a reaction change followed by a reduction in volume, which can be accelerated by pressure. The Isostatic Principle explains that the pressure is transmitted uniformly and instantaneously throughout the sample in all directions regardless of the size, shape, and composition of the food (Chawla et al., 2011). The fact that the food is not crushed during pressure is related to the uniform pressure transmission (Rastogi, 2013).

In food systems, the HHP process is being studied as a non-thermal technique for a variety of reasons, including the elimination of foodborne pathogens, the deactivation of undesired enzymes, and the enhancement of functional properties while maintaining nutritional qualities (Alpas et al., 2000; Bayindirli et al., 2006;

Dede et al., 2007; Okur et al., 2019). Moreover, as an important consequence of the HHP treatment, food biopolymers may undergo significant modifications such as gelatinizing starch, transiting lipid phase, and denaturing the protein (Yamamoto, 2017).

1.2.1.2 Effects of HHP treatment on Plant Proteins

High hydrostatic pressure (HHP) treatment has a significant role in modifying the functional properties of foods. Unlike heat treatments in foods, HHP application has no effect on small molecules like vitamins and amino acids. On the other hand, it causes modifications on large molecules such as the non-covalent bonds of proteins (Puppo et al., 2004).

The volume of a protein in a solution is mainly composed of the volume of its atoms, the volume of voids associated with poor atomic packing, and the volume change of water caused by hydration on the surface of the protein. The action of pressure on a chemical system in equilibrium pushes the equilibrium to the less voluminous state, which is known as Le Châtelier's principle. Proteins under high pressure favor a lower volume conformer within the scope of this principle (Kauzmann, 1959; Winter et al., 2007).

The application of pressure promotes compression and conformational changes in the protein structure. Pressure has a significant effect on non-covalent bonds, and since the compressibility of covalent bonds is usually negligible, pressure has no effect on the primary structure of proteins, but it has a direct effect on their secondary, tertiary, and quaternary structures (Queirós et al., 2018). In conclusion, the most major contributions of pressure on proteins are the collapse of voids caused by water penetration, which is linked with hydration and the weakening of hydrogen bonds. Intermolecular interactions occur because of these, and the tertiary structure

destabilizes, enabling the protein to unfold (Akasaka, 2006). As a result of HHP application, the structures of proteins are commonly modified compared to their native structures, resulting in changes in the functional properties of proteins (Queirós et al., 2018).

HHP processing combined with various pressure, temperature, and time parameters have been studied in the literature to observe the modifications in the functional properties of plant proteins. Plant proteins are demonstrated to be affected by high hydrostatic pressure application in terms of interactions, denaturation, and aggregation (Nikbakht Nasrabadi et al., 2021). Peyrano et al. (2016) studied the impacts of HHP treatment on the physicochemical and functional characteristics of cowpea protein isolates at 200, 400, and 600 MPa. It was observed that the solubility decreased dramatically up to 400 MPa, and at 600 MPa, it was near to the untreated condition. Furthermore, it was stated that the water holding capacity of cowpea protein raised in direct proportion to the pressure. Yin et al. (2008), on the other hand, found that high hydrostatic pressure application at 400 MPa pressure significantly enhanced the solubility of red kidney bean protein isolates. Aside from solubility, the researchers reported that while HHP treatment increases emulsifying activity up to 400 MPa, it declines at 600 MPa. Also, in a study carried out by Chao et al. (2018), it was observed that there was no change in the solubility of pea proteins as a result of the application of HHP at 200, 400, and 600 MPa for 5 minutes at various pH levels.

Although there is a growing interest in soy protein because of its nutritional and functional properties, there hasn't been much study on the effect of HHP application on soy protein's functional properties. In the study performed by Molina & Ledward (2003), soybean protein isolate was subjected to temperature-assisted HHP treatment at high pressure (300-700 MPa) for 15 min, and the gel formation and the structure of the gel formed were examined. As another example, Alvarez et al. (2008) also

focused on the gel formation feature of soy protein by examining the effects of concentration, pH, and additives in the HHP process applied at high pressure (up to 650 MPa) and different temperatures (20°C and 40°C) at varying durations (0.1-10 minutes). Studies have generally focused on the gel-forming ability of soy protein with HHP application, and other functional properties have remained in the background in the literature.

In addition to pressure, temperature, and duration, studies have also demonstrated that as a result of HHP application, the product is affected by conditions such as protein type, protein concentration, ion type, ion concentration, and pH (Alvarez et al., 2008; Manassero et al., 2015; Speroni & Añón, 2013).

1.3 Objective of the Study

The main objective of the study is to investigate the effects of high hydrostatic pressure (HHP) treatment on the functional properties of soy protein isolate (SPI). Furthermore, this study aims to evaluate the influence of the pressurization temperature by applying different temperatures during the HHP treatment. In addition, the pH effect on the functional properties of SPI by adjusting the pH prior to HHP treatment was studied. For this purpose, HHP treatment at different pressure (300, 400, and 500 MPa), temperature (25 and 40 °C), and pH (5 and 7) parameters were performed to investigate the effect on SPI functionality. To examine the modifications, water holding capacity, solubility by Lowry method, emulsion activity, and viscosity of untreated and HHP-treated soy protein isolate were statistically analyzed. Following that, Fourier transform infrared (FTIR) spectroscopy and Nuclear Magnetic Resonance (NMR) relaxometry analysis were performed to determine the changes in protein's secondary structure and hydration behavior of soy protein isolate (SPI), respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Soy protein isolate (SPI) was purchased from Pingdingshan Tianjing Plant Albumen Co., Ltd. (Henan, China) for this study. Total protein content of commercial soy protein isolate was measured by Kjeldahl method (%N × 6.25 for SPI) according to AOAC Official Method (AOAC, 2007). Total protein content of SPI was calculated as 88.28% (dry basis).

A number of chemicals were used in order to investigate the characteristics of both untreated and treated soy proteins. Sodium hydroxide (NaOH) and hydrochloric acid (HCl), Folin-Ciocalteau's phenol reagent, Bovine Serum Albumin (BSA), sodium potassium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$), Copper(II) sulfate pentahydrate ($CuSO_4.5H_2O$), sodium carbonate (Na_2CO_3), sulphuric acid (H_2SO_4), boric acid (H_3BO_3), phenolphthalein ($C_{20}H_{14}O$), and methyl red were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). In all analyses, distilled water was used. Also, Evin brand corn oil was bought from the local market (Ankara, Turkey) for use in determining emulsification characteristics.

2.2 Methods

2.2.1 Sample Preparation

Soy protein isolate powder was mixed with 1M HCl or 1M NaOH solutions at room temperature to adjust pH to 5 and 7. Protein samples with a concentration of 38 %

g/ml (w/v) were kneaded until homogenous dough-like sample was obtained. To assist the protein absorbing the solution, prepared samples were kept in a refrigerator (4 °C) for half a day before HHP treatment, and then HHP treatment was performed.

2.2.2 High Hydrostatic Pressure (HHP) Treatment

High Hydrostatic Pressure (HHP) treatments were carried out with 760.0118 type pressure equipment (SITEC-Sieber Engineering AG, Zurich, Switzerland) which was also shown in Figure 2.1. The device has a three main component that are pressure making unit, pressure vessel and pressure transfer medium. The vessel had a volume of 100 mL, a diameter of 24 mm, and a length of 153 mm. Also, there is a built-in heating-cooling system which was for keeping inner temperature constant (Huber Circulation Thermostat, Offenburg, Germany). The vessel was filled with distilled water, which acts as a pressure transfer medium. For the intended system, the rate of pressure increase was 340 MPa/min for 400 MPa and release was less than 5 seconds. For this reason, the pressure increases and release timings were not included in the pressurization time stated in this investigation.

Figure 2.1 High Hydrostatic Pressure Equipment

The prepared protein samples 38 % (w/v) with pH 5 and 7 were full-filled into 25 ml sterile polyethylene cryotubes (LP Italiana SPA) to perform HHP treatment. The treatment was performed at three different pressure (300, 400, and 500 MPa) and two different temperatures (25 and 40 °C) for 5 min. Pressurized samples were freeze-dried before being kept at -20 °C for further assays. Control samples, which were analyzed directly without any preparation, were not subjected to any temperature or pressure treatment.

2.2.3 Total Protein Content of Soy Protein Isolate by Kjeldahl Method

Kjeldahl method was performed to determine the total protein content of untreated soy protein isolate (AOAC, 2007). Crude protein content (%) of SPI was calculated by multiplying the total nitrogen content with the conversion factor that was 6.25 for soy protein isolate.

2.2.4 Water Holding Capacity (WHC)

Water holding capacity (WHC) was determined using the method described by Li et al. (2011) with some modifications. For the analysis, 5 % w/v protein solution prepared with distilled water was homogenized with the help of Ultra Turrax T-18 (IKA, Corp., Staufen, Germany) at 5,000 rpm for 5 minutes. Samples prepared for analysis were taken into pre-weighed centrifuge tubes and the final weights were recorded. Centrifugation was carried out at 4,000 rpm for 30 minutes. Following centrifugation, the supernatant was discarded, and the weight of the remaining part was recorded. The following equation was used to calculate the water holding capacity (Li et al., 2011).

WHC (g H_2O held by sample / g dry protein sample) = $\frac{(Wt - Wct - Ws - WSPI)}{WSPI}$

where

W_t = total weight
W_{ct} = weight of centrifuge tube
W_s = weight of supernatant liquid
W_{SPI} = weight of soy protein isolate

2.2.5 Protein Solubility by Lowry Method

The Lowry method was used to determine the solubility of the treated and untreated soy protein powders. 1 % (w/v) protein solutions were prepared and thoroughly mixed Ultra Turrax T-18 (IKA, Corp., Staufen, Germany) at 5,000 rpm for 5 minutes as a preliminary preparation. Afterwards, the solutions were centrifuged at 2,500 rpm for 15 minutes and the supernatant liquids were used for the solubility analysis.

The reagents required for Lowry method were listed in Table 2.1. After reagents A, reagent 1 and reagent 2 was prepared as indicated in Table 2.1 given below, Lowry ARC reagent was prepared using 100:1:1 volume ratio of reagent A, reagent 1 and

reagent 2, respectively. Following that, Folin-Phenol reagent was prepared by diluting 2N stock (commercial) with distilled water at a 1: 1 ratio.

Table 2.1 The reagents required for Lowry method

Reagent 1	2% CuSO4.5H2O
Reagent 2	2% Na-K Tartrate
Reagent A	2% (w/v) Na2CO3 dissolved in 0.1 N NaOH
Lowry ARC Reagent	Mixture of Reagent A:1:2 with a ratio of 100:1:1
Folin-Phenol Reagent	Diluted Folin-ciocalteu's Phenol Reagent with a ratio of 1:1

For the experiment, 0.5 ml of diluted supernatant was mixed with 2.5 ml of Lowry ARC reagent and the mixture was incubated at room temperature for 10 minutes. After that, 0.25 mL Folin-Phenol reagent was added and mixed well with a vortex-mixer (VM-10, Witeg Labortechnik GmbH, Germany) before incubating in a dark area at room temperature for 30 minutes. The absorbance values of the samples were measured with Optizen POP Nano-Bio UV spectrophotometer at 750 nm and the measurements were recorded.

Finally, a calibration curve was obtained from 1 g/L BSA (Bovine Serum Albumin) stock solution with serial dilutions from 0.5 to 0.03125 g/L. The calibration curve was generated by plotting absorbance vs. g/L BSA concentration and calibration curve with the equation was shown in Figure A.1. Solubility of treated and untreated soy protein samples was found by using the obtained absorbance values in the equation of the calibration curve.

2.2.6 Emulsion Activity

The emulsion activity of untreated and treated samples was measured using a modified version of the technique published by (Lee et al., 2006). For the experiment, firstly, 1% protein solution prepared using distilled water was mixed with Ultra Turrax T-18 (IKA, Corp., Staufen, Germany) at 5,000 rpm for 5 minutes. 1 ml of the protein solution was taken into a tube and 0.5 ml of corn oil was added to it. Afterwards, a silent crusher was used for 1.5 minutes to obtain an emulsion. The height of the prepared emulsion was measured and recorded. The emulsion was centrifuged at 10,000 rpm for 1 minute to observe the oil fraction which was separated at the top and the height of top layer was measured and recorded. Emulsion activity was calculated by using the formula given below (Lee et al., 2006).

$$\text{EA (\%)} = \frac{height\ of\ emulsion\ before\ centrifuge - height\ of\ top\ layer\ after\ centrifuge * 100}{height\ of\ emulsion\ before\ centrifuge}$$

2.2.7 Viscosity Determination

The viscosity of the samples was measured by using SV-10 Vibro Viscometer (A&D Company, JAPAN) to assess their flow characteristics. For the analysis, 5% protein solution containing soy protein isolate and distilled water was prepared, then mixed with Ultra Turrax T-18 (IKA, Corp., Staufen, Germany) at 6,000 rpm for 5 minutes. SV-10 Vibro Viscometer has the feature of measuring with the tuning-fork principle, which provides high measurement accuracy. The Sinewave Vibro Viscometer detects the driving electric current required to vibrate the two sensor plates at a constant frequency to measure viscosity. The experiment was carried out at room temperature and the viscosity values, whose unit was cP, were taken directly from the device.

2.2.8 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Fourier transform infrared (FTIR) spectroscopy is used to determine the effect of pressure application on the amino acid structure of soy protein. To characterize the structure of the dried samples, measurements was done by using IRSpirit Spectrometer with Attenuated Total Reflectance (ATR) attachment (Shimadzu Corporation, Kyoto, Japan) with a resolution of 4 cm^{-1} and 32 number of scans in the frequency range of 600-4000 cm^{-1}.

2.2.9 Nuclear Magnetic Resonance (NMR) Relaxometry

Nuclear magnetic resonance measurement was carried out to examine the hydration behavior of soy protein isolate. For the experiment, 0.2 g protein powder were mixed with 0.8 ml distilled water in 10 ml tubes. The experiment was performed by using a 0.5 T NMR spectrometer operating at a frequency of 20.34 MHz (Spin Track, Resonance Systems GmbH, Kirchheim/Teck, Germany) to measure spin-spin relaxation times (T_2) of untreated and treated samples prepared. T_2 data of the samples were determined by using CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence with 900 ms echo time, 600 echoes and 350 ms time of observation with 16 scan number. MATLAB (R2019b, The MathWorks Inc., Natick, MA, USA) was used to conduct the analysis.

2.2.10 Statistical Analysis

Statistical analysis was conducted in three replicates for each experiment, and results were analyzed using MINITAB (Version 16.1.1, Minitab Inc., Coventry, UK). Analysis of Variance (ANOVA) was carried out to ascertain the influence of factors on the functional properties by using the general linear model. Tukey's comparison test with a 95% confidence interval was used to determine significant differences.

Lowercase letters were used to indicate significant differences between HHP treatments at various pressure, temperature, and pH levels.

2.3 Experimental Design

Table 2.2 summarizing the factors, levels, and measured responses was provided to illustrate the entire experimental design.

Table 2.2 Experimental Design Parameters

Factors	Levels	Responses
Pressure Levels	300 MPa, 400 MPa, 500 MPa	1. Water Holding Capacity 2. Protein Solubility 3. Emulsion Activity (%) 4. Viscosity 5. Fourier Transform Infrared (FTIR) Spectroscopy 6. Nuclear Magnetic Resonance (NMR) Relaxometry
Temperature	25 ^{o}C, 40 ^{o}C	
Time	5 min	
pH	5 and 7	

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Water Holding Capacity (WHC)

There is a relation between water holding capacity (WHC) of proteins and product mouthfeel, and texture (Tao et al., 2019). For this reason, it is critical to identify the factors that affect the WHC of proteins. As a result of the experiments carried out within the scope of this study, the WHC of the untreated soy protein isolate (SPI) was determined as 9.32 g water /g protein. In the literature, the WHC of the commercial soy protein isolate varies in the range of 3.5 - 8.13 g water /g protein (Li et al., 2012; Tao et al., 2019; Wong & Kitts, 2003; Y. N. Zhang & Zhao, 2013). As also stated in the introduction section, WHC is affected by the ratio of the two main proteins of the soy protein, glycinin and β-conglycinin. In addition, depending on the cultivar and/or extraction procedure, the functional characteristics of SPI may differ considerably. As an expected result, the WHC of untreated SPI may change from one study to the next. Figure 3.1 illustrates the WHC of untreated soy protein isolate (control) and the WHC modifications of HHP-treated SPIs exposed to various pressure and temperature combinations with pH adjusted to pH 5 and pH 7 prior to HHP treatment.

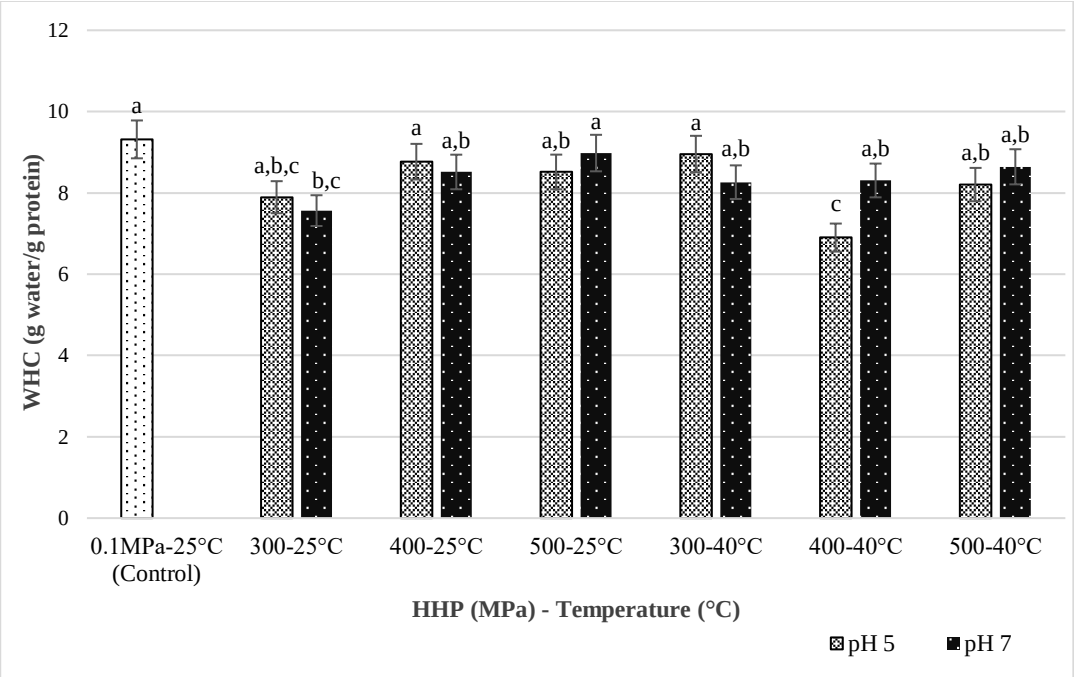

Figure 3.1 Water holding capacity (g water / g protein) results of both untreated and HHP-treated soy protein isolate (SPI) at pH 5 and pH 7. Different letters indicate significant difference between different HHP conditions

The statistical analysis revealed that the combination of pressure, temperature, and pH had a significant ($p<0.05$) impact on the WHC of SPI. When the pressure effect was analyzed alone, it was seen that the pressure was statistically significant ($p<0.05$) on the WHC results. While pressure reduced water holding capacity regardless of the level, this reduction was significant for 300 MPa and 400 MPa ($p<0.05$), except for 500 MPa, when compared to the control. The reduced capacity to bind water molecules caused by the change in protein native conformation with pressurization might explain why HHP-treated SPIs had lower WHC than the control. Temperature and pH combination were not significant on WHC especially at 500 MPa of pressurization ($p>0.05$), which was out of the downtrend and had WHC values close to control. On the other hand, 400 MPa, 40 °C HHP application at pH 5 ($p<0.05$) had the lowest WHC value (6.9 g water / g protein), a significant difference as can be

also seen in WHC results given Figure 3.1 detailed above. Reduction in availability of polar amino acids due to unfolding that can be caused by high pressure may explain the reduction in WHC for 400 MPa-40 °C treatment.

Water holding capacity should normally be lower at pHs around the isoelectric point of soy protein isolate (4.5) due to high protein-protein interaction on the surface at that point. Although pH 5 is predicted to result in lower WHC than pH 7 in a regular scenario, there was no significant difference between pH 5 and pH 7 ($p>0.05$). This could be interpreted as the pH impact being suppressed because of the structural and conformational changes in soy protein isolate induced by HHP treatment, and the HHP effect being more dominant than the pH effect. Although there was no significant difference between pHs, the lowest WHC value was for the process at 400 MPa 40 °C at pH 5, and this decrease may be due to the fact that SPIs with unfolding-induced loss in the suitability of polar amino acid groups were obtained with the effect of high pressure.

The effect of pressure-pH combinations on WHC was significant at 40°C. Specifically, the pH 5 adjusted SPIs which were treated at 400 MPa had a significantly lower WHC (6.9 g water / g protein) than the other HHP-treated SPIs and the control sample. Likewise, SPIs treated at 300 MPa at 25 °C showed a significant decrease in WHC (~7.73 g water / g protein) for both pH 5 and pH 7. Due to the pressurization at moderate levels (300 and 400 MPa), the native globular conformation of SPIs may change in a way that results a drop in WHC. So that, the unfolding of the polypeptide chains, which resulted in a loss in the suitability of the polar amino acid groups to which water binds, may have resulted in a reduction in WHC. On the other hand, the change in WHC of SPI adjusted to pH 7 was not significant and it could be because of having little influence on the structural characteristics of SPI at this pH for its water holding capacity. As a result, at pH 5, it can be said that at medium pressure levels, the protein structure was affected in a

way that decreased the availability of polar amino acids, resulting in a greater decrease in water holding capacity.

3.2 Protein Solubility by Lowry Method

Protein solubility under different environmental conditions is critical for the food industry to investigate its functional properties for possible food applications. Solubility of soy protein in solutions is insufficient and it is the one of the most important limitations of soy protein. In order to eliminate this limitation, the protein can be modified by applications such as high hydrostatic pressure. In this study, protein solubility was determined by the Lowry method and expressed as a percentage. The results obtained as a result of the analysis of untreated SPI and HHP-treated SPI with various pressure, temperature and pH parameters were shown in Figure 3.2.

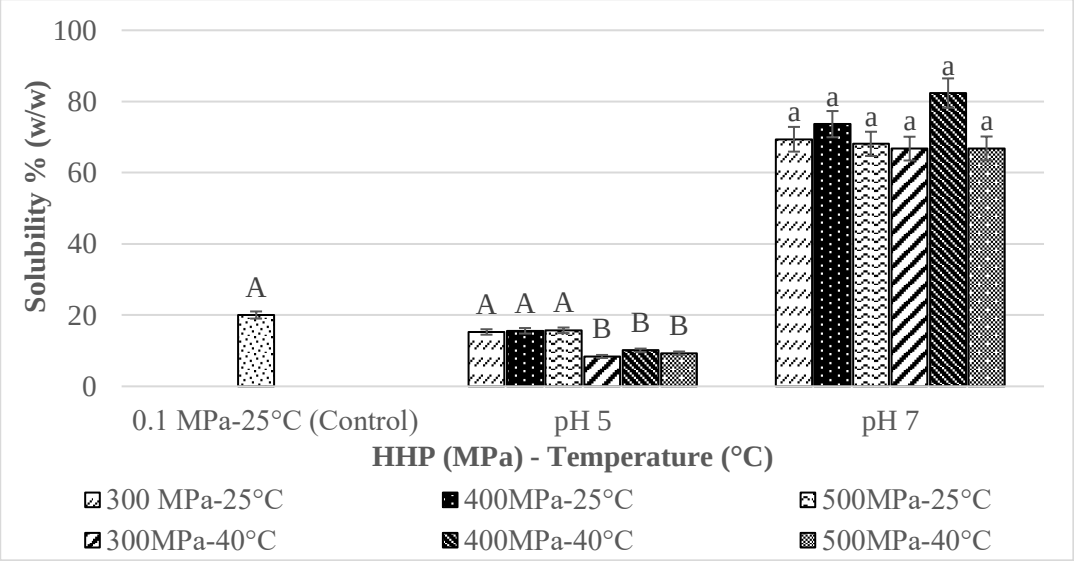

Figure 3.2 Protein solubility % (w/w) results of both untreated and HHP-treated soy protein isolate (SPI) at pH 5 and pH 7. Different capital letters indicate significant difference at pH 5 while small letters indicate significant difference at pH 7

As can be clearly seen from the Figure 3.2, since the solubility results at pH 5 and pH 7 were too much apart from each other, ANOVA analysis was performed and interpreted separately. Increasing the pH from 5 to 7 improved the solubility significantly ($p<0.05$). Proteins tend to aggregate at pH close to 4.5, which is the isoelectric point of soy protein isolate, as stated in the introduction part. Protein aggregation near the isoelectric point was assumed to be the cause of the low level of protein solubility at pH 5 compared to pH 7. The repulsion between charged molecules increased when the pH raised from 5 to 7, affecting the protein-water interaction in a positive way. As a result, an increase in protein solubility was directly associated with an increase in protein-water interaction. Similarly, in a finding reported by Li et al. (2011), the SPI solubility was compared at pH 6.8 and pH 3, and it was demonstrated that the solubility at pH 6.8 was higher than the solubility at pH 3. In addition, in another study, it was stated that the solubility was the lowest in the pH range of 3.5 and 5.5, and this was due to protein aggregation near pI point (Torrezan et al., 2007). Likewise, in the study conducted by Manassero et al., (2015), it was emphasized that changing pH from 5.9 to 6.4 resulted in a significant increase in protein solubility, and low solubility at pH near to the pI point was attributed to insoluble aggregates. The results of all these mentioned studies support that the solubility obtained at pH 7 is significantly ($p<0.05$) higher than the solubility at pH 5, which is close to the pI (4.5 for SPI) point.

In this study, the effect of high pressure alone on solubility was not significant ($p>0.05$) when pressurized samples were compared with control samples. This was most likely due to the fact that pH had a dominant effect in this experiment. On the other hand, when different pressure levels (300, 400, and 500 MPa) were statistically analyzed for all HHP-treated samples, there was a significant difference in protein solubility results ($p<0.05$). Although this significant difference was not seen at first glance since the lettering in Figure 3.2 is made over pHs, it can be clearly seen in

detailed ANOVA results depicted in Table B.2 (Appendix B.2). Among the HHP treatments applied at 300, 400 and 500 MPa, the solubility result obtained at 400 MPa is significantly ($p<0.05$) higher than the other pressure levels. For instance, the highest protein solubility (82.37 %) was observed for SPI solution adjusted to pH 7 and treated at 400 MPa, 40 °C. At pressure levels up to 400 MPa, unfolding the structure of soy protein isolate can promote the protein-water interaction, resulting in enhanced solubility. When the pressure was increased to 500 MPa, however, the solubility decreased significantly ($p<0.05$), and the loss in solubility may be correlated with the formation of insoluble high molecular weight aggregates. Factors promoting the formation of aggregates may be exposure of hydrophobic residues due to further increase of pressure. In this research, for example, untreated SPI and SPIs adjusted to pH 7 and subjected to 300, 400, and 500 MPa at 40 °C had solubility of 20.04 %, 66.77 %, 82.37 %, and 66.83 %, respectively. According to a study with similar results, the solubility of 1% (w/v) SPI solution exposed to HHP treatment at pH 6.8 increased significantly for 200-300 MPa pressurization and 5-15 min application time due to unfolding of soy protein isolate structure and decreased significantly with increasing the pressure level and prolonging the time (Li et al., 2011). Furthermore, in a study by Puppo et al (2004), the HHP application enhanced the solubility of SPI at 200 MPa, but no significant change was detected at 400 MPa and 600 MPa. Different results in the literature are highly dependent on the cultivars and SPI preparation method. Additionally, the concentration of the prepared SPI solution is critical to reveal the effect of pressure on functional properties since protein-protein and protein-solvent relationships are concentration dependent.

The statistical analysis revealed that, the effect of temperature alone and in combination with pressure on solubility was not significant for the overall results ($p>0.05$); but there was a significant effect of pressure and temperature together for pH 5 ($p<0.05$). The solubility of HHP-treated SPI at room temperature (~15.54 %) was significantly higher than those treated at 40 °C (~9.27 %). This was an expected result due to the effect of temperature on plant protein solubility. With the influence

of temperature, polypeptide chains unfolded, exposing the hydrophobic part which is at the center of the soy protein in its natural structure more and decreasing their solubility. However, at pH 7, an increase in temperature at 400 MPa with the synergistic effect of pressure and pH can be associated with increase in electrostatic interactions, which is a key factor in the protein-solvent interaction, and the highest solubility was obtained for the 400 MPa-40 °C treatment combination.

While the solubility of soy protein isolate was supported by increasing the pressure up to a certain level, further increasing the pressure caused a decrease in solubility. As a result of this research, it was shown that adjusting the pressure, temperature, and pH parameters could modify the solubility of soy protein isolate, with the best solubility (82.37 %) achieved at 400 MPa-40 °C for HHP-treated SPI adjusted to pH 7.

3.3 Emulsion Activity

The emulsifying ability of proteins, owing to their amphiphilic nature, is a frequently desired property in food systems. Emulsifying ability is strongly influenced by protein characteristics as well as processing parameters such as medium composition, pH, and temperature (Queirós et al., 2018). In this study, the effect of HHP treatment on emulsifying activity at various pressure, temperature and pH was investigated and the results were expressed as %.

The effects of the HHP treatment on the emulsion activity of SPI were shown in Figure 3.3. Obviously, statistical results demonstrated that pressure had a significant ($p<0.05$) influence on emulsion activity. Significantly higher results were obtained at pH 5 under 300 MPa compared to control and other treated samples. While the emulsion activity of untreated SPI was 59.34 %, the highest emulsion activity was recorded as 70.37 % for SPI treated at 300 MPa – 40 °C at pH 5 ($p<0.05$). Although

application of appropriate pressure improved emulsion activity, increasing pressure lowered emulsion activity, and in most cases, SPIs treated at 400 MPa and 500 MPa had emulsion activities quite similar to control ($p>0.05$). In addition, it should be noted that while the pressure effect can be observed clearly at pH 5, pressure did not have a significant effect on the emulsion activity at pH 7.

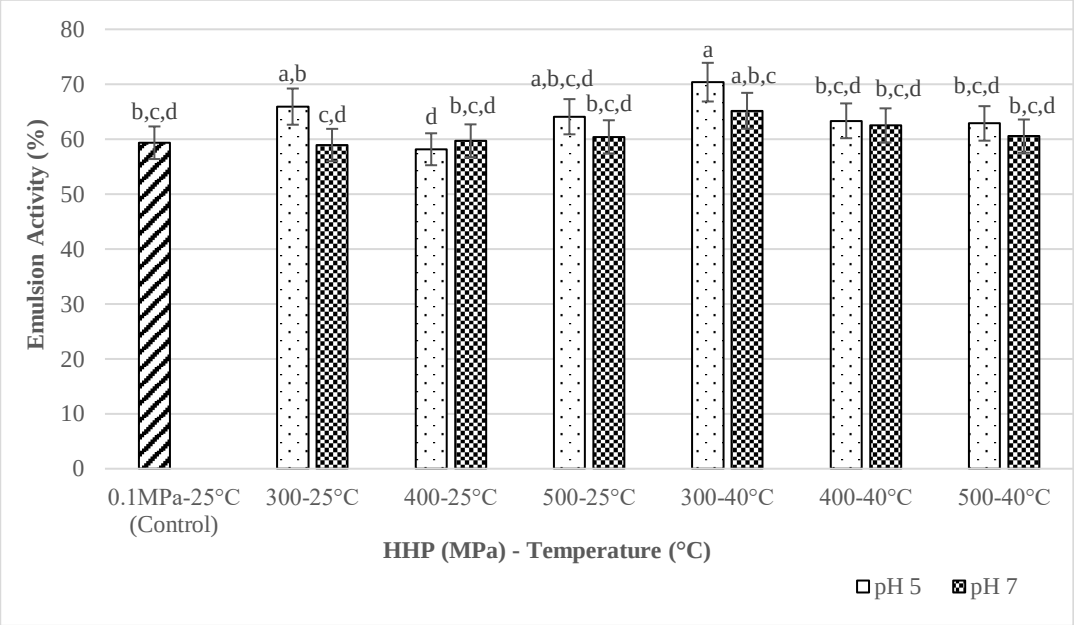

Figure 3.3 Emulsion activity (%) results of both untreated and HHP-treated soy protein isolate (SPI) at pH 5 and pH 7. Different letters indicate significant difference between different HHP conditions

To interpret the pressure effect, the findings showed that moderate protein unfolding with HHP treatment may enhance emulsifying ability of SPI, but HHP treatment at high pressure levels may hinder its emulsifying properties because of decrease in molecular flexibility of SPI due to high molecular weight aggregates. In a study, when the emulsion activity results of HHP treated red kidney protein isolates at 200, 400 and 600 MPa were examined, it was stated that the emulsion activity index of 200 and 400 MPa increased and there was a significant decrease at 600 MPa

compared to the control (Yin et al., 2008). In another research, the change in emulsion activity index for SPI subjected to HHP application for 15 min. at 200-500 MPa was evaluated, and pressure treatment had favorable effects on emulsion activity up to 300 MPa, but further increase in pressure caused a reduction in emulsion activity which was still greater than control (Li et al., 2011). On the other hand, as Molina et al. (2001) stated in their study, the 7S and 11S fractions, which are the main proteins of SPI, could be affected by HHP treatment (200 – 600 MPa; 20 °C) in a different way. According to their study, SPI showed the highest emulsion activity index at 400 MPa, and at this pressure level, the aggregation of 11S reduced surface hydrophobicity, while denaturation of 7S increased surface activity. Since the ratio of 11S and 7S fractions may vary depending on cultivar, it should be considered that the emulsion activity values obtained by HHP may also alter.

Furthermore, there was no significant difference ($p>0.05$) between the emulsion activity results obtained at pH 7, and the results were also similar when compared to the control. The reason for this may be that the application of HHP had little influence on the structural characteristics of polypeptides within SPI at pH 7 for its emulsion properties.

In addition, when all the results were considered in general, it was seen that the temperature increase improved the emulsion activity significantly ($p<0.05$). For example, at pH 5, emulsion activities of SPIs treated at 300 MPa – 25 °C and 300 MPa – 40 °C were found as 65.92 % and 70.38 %, respectively. This change in emulsion activity may be caused by an increase in temperature, leading to a change in the direction that supported protein unfolding. Also, in a study, it was stated that heating of rice protein isolates up to moderate time interval increased the emulsion activity of protein isolates from kidney beans due to change in conformational structure of protein (Tang & Ma, 2009b).

3.4 Measurement of Viscosity

The resistance to flow, or viscosity, is a critical feature in food processing in the food industry. Food processing, processing design, new product development, and consumer-desired qualities such as mouthfeel and physical appearance all rely heavily on viscosity determination (Kinsella, 1976; Walnofer et al., 2005). Soy protein is suitable for use in beverages with its low viscosity feature. Soy protein's low viscosity, along with its nutritious characteristics, makes it a reasonable choice for use in creamers, milk replacers, and infant formulas (Singh et al., 2008). This study examined how pressure, temperature, and pH factors affect the rheological characteristics of a modified soy protein isolate, and the viscosity results were shown in Table 3.1. As a result of the analysis, the viscosity of 5% commercial soy protein isolate solution was determined as 10.3 cP.

Table 3.1 Viscosity (cP) results of HHP-treated soy protein isolate (SPI) at pH 5 and pH 7

	Pressure (MPa) – Temperature (°C)	Viscosity (cP)
Control	0.1 MPa-25°C	10.33 ± 0.26^{a}
	300 MPa-25°C	$4.09 \pm 0.08^{d,e}$
	400 MPa-25°C	$4.14 \pm 0.02^{d,e}$
pH 5	500 MPa-25°C	3.55 ± 0.11^{e}
	300 MPa-40°C	2.56 ± 0.02^{f}
	400 MPa-40°C	3.50 ± 0.39^{e}
	500 MPa-40°C	2.69 ± 0.02^{f}
	300 MPa-25°C	$4.67 \pm 0.25^{b,c,d}$
	400 MPa-25°C	$4.97 \pm 0.16^{b,c}$
pH 7	500 MPa-25°C	7.49 ± 0.67^{a}
	300 MPa-40°C	5.37 ± 0.43^{b}
	400 MPa-40°C	$4.72 \pm 0.11^{b,c,d}$
	500 MPa-40°C	$4.56 \pm 0.20^{c,d}$

Different letters indicate significant difference between different HHP conditions

According to statistical analysis, pressure, temperature, and pH both individually and in combination had a significant ($p<0.05$) effect on the viscosity of SPI. HHP-treated SPI had significantly lower viscosity ($p<0.05$) compared to control (10.3 ± 0.36 cP). For example, viscosity of SPI treated at 300 MPa - 25 °C at pH 7 was found as 4.67 cP. Similarly, Li et al. (2011) found that HHP treatment (300 MPa, 15 min) reduced viscosity remarkably at pH 6.8. Besides that, in a study, the viscosity of cowpea

protein isolate was analyzed at various pressure levels (200-600 MPa) at pH 8 and pH 10 and it was concluded that the pressure significantly reduced the viscosity. At that point, modified protein structure due to HHP application, as well as the disintegration of aggregates held by weak bonds, might explain the HHP-induced reduction in viscosity (Peyrano et al., 2016). On the other hand, the increase in viscosity at 500 MPa-25°C for pH 7 could be explained by network formation of aggregated protein due to HHP treatment.

The results show that the viscosity at pH 5 was significantly ($p<0.05$) lower than at pH 7. Among all results including the control, the lowest viscosity was recorded at pH 5 as 2.56 cP for SPI processed at 300MPa – 40 °C. Also, the highest viscosity was found at pH 7 as 7.49 cP for SPI treated at 500 MPa – 25 °C compared to HHP-treated SPI. Similarly, in the study of Kinsella, (1979), it was reported that an increase in pH from 5 to 10.5 had an increasing effect on soy protein viscosity.

Many studies have shown a correlation between viscosity and WHC (Arrese et al., 2002; Hermansson, 1975; Remondetto et al., 2001). When the results in this study were analyzed, it was concluded that there was a moderate positive correlation between the viscosity of SPI and WHC. By taking advantage of the parallel results, it can be interpreted that the reason for this decrease in viscosity and WHC was due to the unfolding that occurs in the of SPI under the applied treatment conditions. Finally, as a result of viscosity analysis, it was concluded that of SPI can be modified with different combinations of variables, and this knowledge is invaluable for soy protein isolate, which is used in a wide variety of food applications in the food industry.

3.5 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR spectroscopy analysis is an effective technique for getting detailed information regarding changes in protein structure, particularly secondary structure. Also, it is known that HHP treatment induces significant modifications on protein structures. For this purpose, FTIR spectra were used to examine structural changes in soy protein isolates subjected to HHP treatment with various parameters. In this study, the new peaks and peak band area were used to examine changes in protein structure. FTIR spectra of HHP-treated SPI in the frequency range of 600-4000 cm^{-1} was shown on Figure 3.4 and Figure 3.6, together with control sample spectra. The results for pH 5 and pH 7 were depicted separately for clarity. In addition, to make the differences more readable and understandable, the changes of peak position and relative area were also indicated on Figures 3.5 and 3.7.

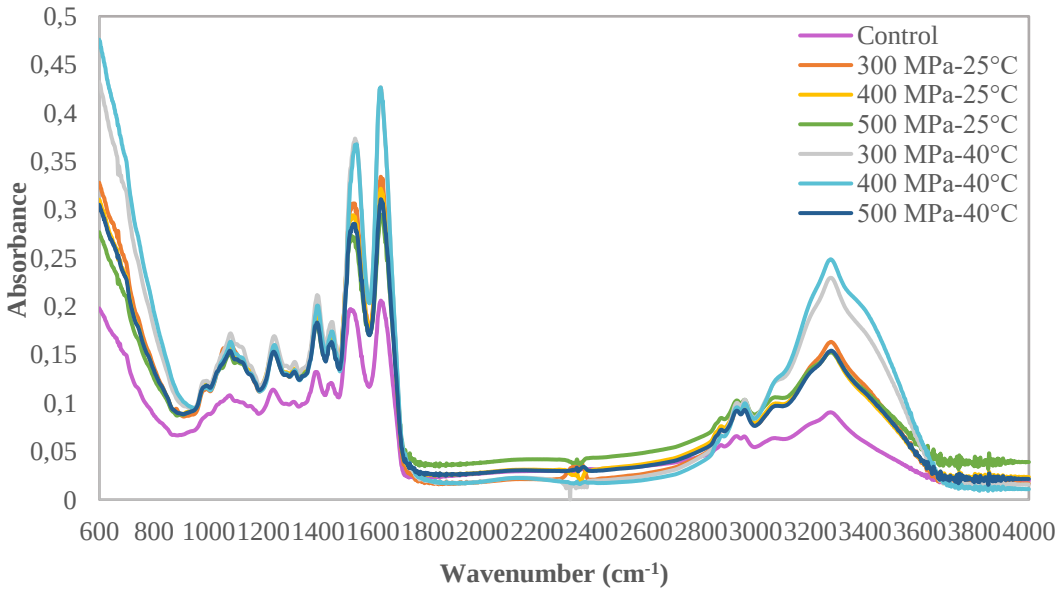

Figure 3.4 FTIR spectra of both untreated and HHP-treated soy protein isolate (SPI) at pH 5

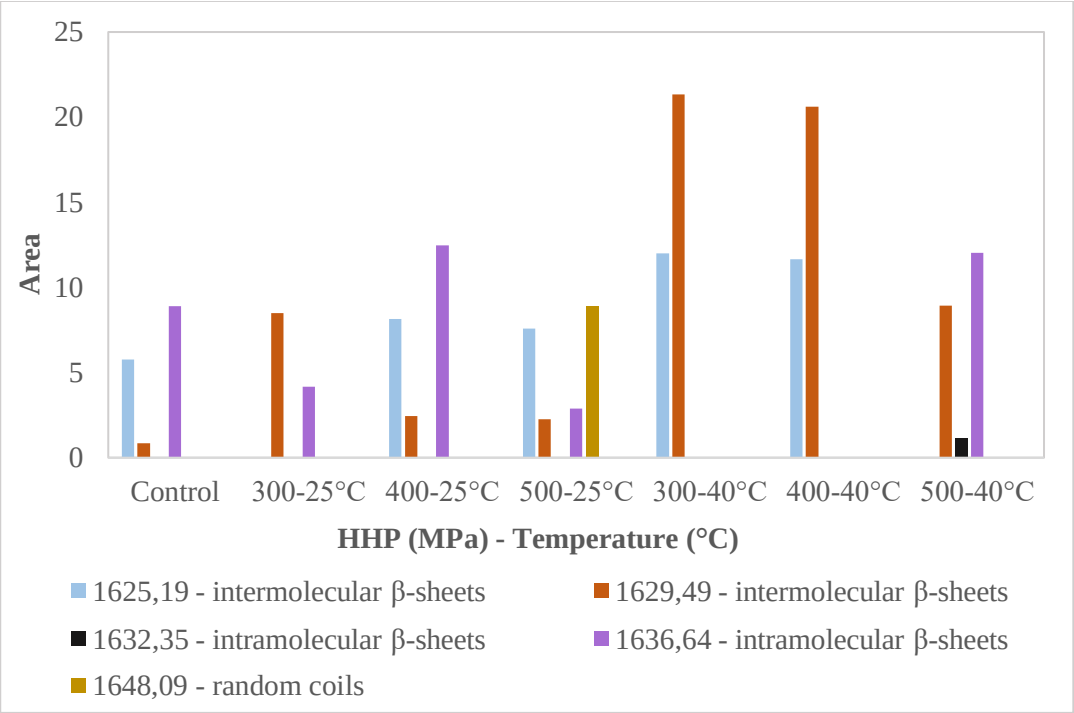

Figure 3.5 Relative area of peaks located in amide I band for both control and HHP-treated soy protein isolate (SPI) at pH 5

The FTIR analysis can provide information on the components of the secondary structures of the untreated and HHP-treated SPI. The amide I band, which is in 1600-1700 cm^{-1} spectrum band, is mostly used for the evaluation of secondary structures of proteins. In proteins, the amide I band is linked with the C=O stretching of peptide bonds. Because amide I region offers useful information regarding protein conformation and unfolding, it is appropriate for studying these changes. The corresponding relationships between the components of the secondary structure of the proteins and their peak positions in the amide I band are 1610–1630 & 1685–1695 cm^{-1}, 1630–1640 & 1670–1684 cm^{-1}, 1640–1648 cm^{-1}, 1648–1659 cm^{-1}, and 1660–1668 cm^{-1} for intermolecular β-sheets, intramolecular β-sheets, random coils, α-helices, and β-turns, respectively (Cui et al., 2019; Zhang et al., 2022). Secondary structure of commercial soy protein isolate is quite similar to that of 7S or 11S

globulins which they commonly contain very high levels of β-sheets and low amounts of α-helices (Tang, 2009a).

As can be clearly seen in Figures 3.4, 3.5, 3.6, and 3.7, the differences in the amide I region of the HHP treatment can be easily observed in this study. Significant changes were recorded in the secondary structure of SPI by HHP application at various pressure, temperature, and pH levels. While there were 1625.19, 1629.49 and 1636.64 cm^{-1} peak bands for the control sample, there was a shift to the 1632.35, 1648.09, 1653.81 and 1675.27 cm^{-1} peak bands and the intensity of these peaks varied for different treatment conditions.

The most obvious sign of modification in the protein structure was the decrease or even disappearance of the intramolecular β-sheet peak band (1636.64 cm^{-1}) under most of the conditions. In addition, when viewed in general, it was seen that the intensity of peak band for intermolecular β-sheets at 1629.49 cm^{-1} band increased with HHP treatment for most of the cases. The reason for the shift from the intramolecular β-sheets to the intermolecular β-sheets may be indicative of changes in hydrogen bonding or change in secondary structure of protein due to partial unfolding (Choi & Ma, 2005).

The disappearance of the 1636.64 cm^{-1} peak band and a remarkable increase in the 1629.49 cm^{-1} peak bands were observed for SPI treated at 300 MPa-40 °C-pH 5 and 400 MPa-40 °C-pH 5. The loss in intramolecular β-sheet and enhancement in intermolecular β-sheet may be considered as an improvement in intermolecular hydrogen bond strength in the β-sheet structure as a result of partial unfolding. Similarly, in a study, changes in spectral bands for autoclaved glycinin were examined by FTIR and this change in intramolecular and intermolecular β-sheet structure was associated with protein unfolding (Long et al., 2015). Furthermore,

decrease in intramolecular β-sheets and formation of random coils were associated with protein unfolding. SPI treated at 500 MPa – 25 °C at pH 5 was the most evident example of this situation with the highest intensity of random coils band (1648.09 cm^{-1}). On the other hand, for SPI treated at 500 MPa-40 °C, the intramolecular β-sheet band was formed back and even the 1632.35 cm^{-1} peak band was detected. This may be due to the refolding of the protein as a result of further increasing the pressure. Also, Wang et al., (2011) showed in a study with FTIR results that HHP application caused rearrangement in β-conglycinin structure with the unfolding. As clearly seen from the results, HHP application causes different changes on the secondary structure of SPI depending on the pressure level.

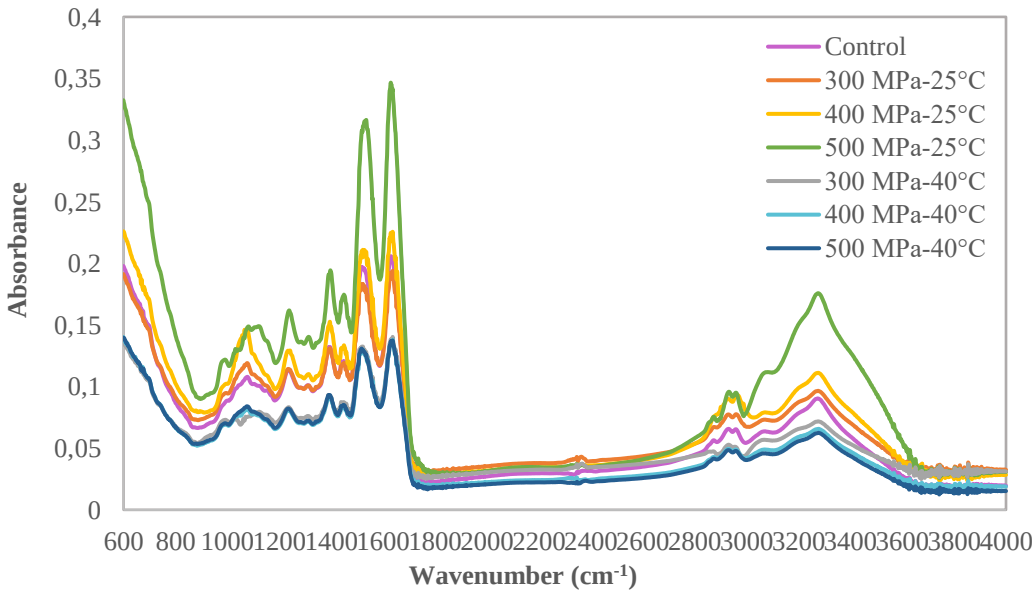

Figure 3.6 FTIR spectra of both untreated and HHP-treated soy protein isolate (SPI) at pH 7

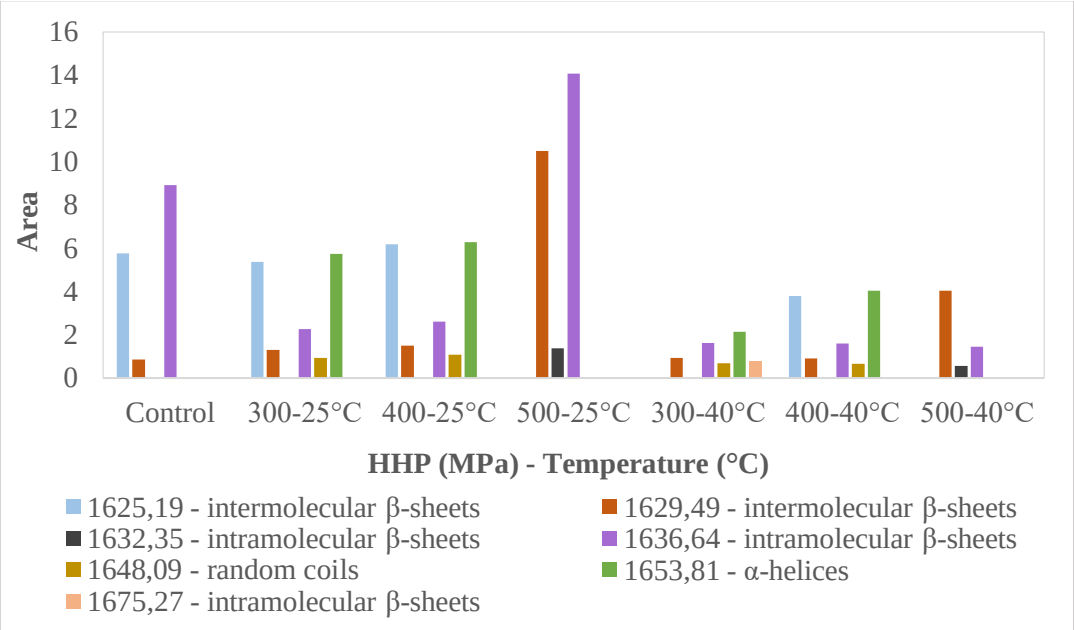

Figure 3.7 Relative area of peaks located in amide I band for both control and HHP-treated soy protein isolate (SPI) at pH 7

Also, at the results obtained for pH 7, α-helix band formations were detected, unlike pH 5, as seen in Figure 3.7. The formation of α-helix structure (formation of 1653.81 cm^{-1} peak bands) and the decrease of intramolecular hydrogen bonding of β-sheet in HHP-treated samples other than 500 MPa were explained by partial protein unfolding with an increase in soluble aggregates. Contrary to the general result, SPI at pH 7 with the further pressure increase (500 MPa) tended to form aggregate as a result of the hydrophobic groups embedded in the globular structure of native SPI coming to the surface due to the unfolding. This was also consistent with the solubility results.

3.6 Hydration Behavior by Nuclear Magnetic Resonance (NMR) Relaxometry

NMR relaxometry is a helpful method for observing the state and distribution of water in food systems as well as understanding the interaction between water and macromolecules, especially proteins. Transverse relaxation (T_2) time is a reliable indication of mobile protons in the samples and provides structural information on protein hydration (Chen et al., 2010; Dekkers et al., 2016; Hinrichs et al., 2003). T_2 values were recorded at the end of NMR relaxometry experiment in order to investigate the hydration behavior of SPI. T_2 results of control and HHP-treated SPI were illustrated in Figure 3.8.

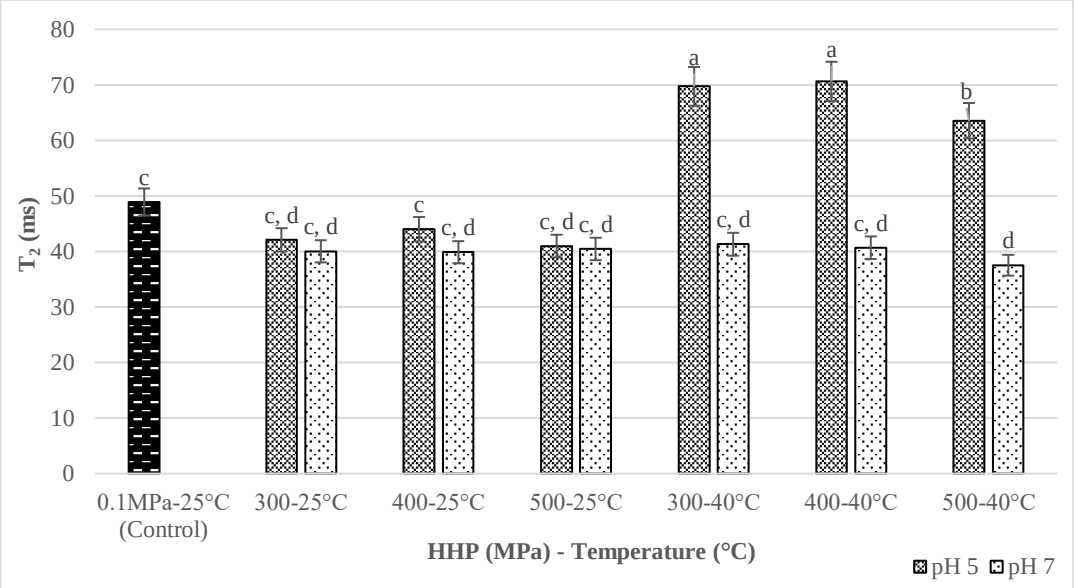

Figure 3.8 T_2 (ms) results of both untreated and HHP-treated soy protein isolate (SPI) at pH 5 and pH 7. Different letters indicate significant difference between different HHP conditions

The larger reduction in T_2 value meant that the more water molecules were integrated and bound to the protein structure effectively (Yildiz et al., 2018). For this reason, NMR measurement was performed to examine the hydration behavior of HHP-treated soy protein isolate (SPI) and T_2 values were noted. As seen in Figure 3.8, HHP-treated SPI had different hydration behaviors for different process parameters. When all parameters of the HHP application were evaluated jointly, the influence of SPI on hydration behavior was not statistically significant ($p>0.05$), but there was a significant difference ($p<0.05$), when parameters analyzed individually or in binary combinations.

At pH 7, there was no significant effect of pressure and temperature ($p>0.05$) changes on the SPI hydration behavior. On the other hand, the T_2 results measured at pH 5 at 40 °C were significantly ($p<0.05$) higher than the T_2 values at 25 °C. Furthermore, when these values were compared to the control (48.91 ms) and pH 5, a significant difference ($p<0.05$) was reported. The longest T_2 results were obtained at pH 5 for SPI treated at 300 MPa – 40 °C (69.74 ms) and 400 MPa – 40 °C (70.65 ms). Long T_2 relaxation time demonstrated that the amount of free water was high, indicating that SPI was less hydrated under these treatment conditions. To explain the scenario here with another perspective, as Roche et al., (2017) explained in their paper, the penetration of water molecules into the protein core may have been supported by the conformational changes in the protein structure caused by pressure and temperature. As a result, water molecules penetrating inner structure of soy protein isolate due to the impact of weakened hydrogen bonds caused by pressure, increased the water mobility of the system, resulting in longer transverse relaxation (T_2) time.

When the T_2 results at 40 °C and pH 5 were compared, raising the pressure to 500 MPa resulted in a significant reduction in T_2, which was still greater than the control. This reduction in T_2 relaxation time might be explained by the fact that more water was closely bound to the protein structure due to HHP application. Furthermore,

although the results at pH 7 were non-significant when compared to the control, this was not the case for 500 MPa – 40 °C. A significant decrease in T_2 values was recorded for the soy protein isolate subjected to these processing conditions. This reduction in T_2 relaxation time at 500 MPa – 40 °C for both pH 5 and pH 7 may be proof that SPI was modified under high pressure and that more water could be bound with the effect of pressure. Similarly, in the research of Zhao et al., (2018), there was an increase in T_2 relaxation time up to 400 MPa and decreased with further pressure increase up to 550 MPa for HHP treated sweet potato protein at pH 3, and it was reported that HHP treatment lowered water mobility and enhanced hydration. In addition, Zhao et al., (2018) stated that T_2 relation time increased for pH 6, but it was not significant.

Lastly, in this research, a negative Pearson correlation ($r = -0.661$, $p < 0.05$) was found between the T_2 values obtained by NMR relaxometry and solubility results obtained by the Lowry method. The increase in solubility might be attributed to improved water-solvent interaction, meanwhile the decrease in T_2 values may be associated to the development of soluble protein aggregates as a result of HHP-induced protein folding.

CHAPTER 4

CONCLUSIONS & RECOMMENDATIONS

In this study, the effect of HHP application on the functional properties of soy protein isolate was investigated. The effects of pressure, temperature, and pH on the functional characteristics of the soy protein isolate were easily detectable when the results were analyzed.

According to water holding capacity (WHC) of SPI results, WHC significantly ($p<0.05$) reduced by HHP treatment compared to control. The lowest WHC value in the study was found for SPI treated at 400 MPa – 40 °C – pH 5. Also, there was no significant difference between WHC results of pH 5 and pH 7 ($p>0.05$).

In addition, solubility results show that HHP application caused significant changes on solubility of soy protein isolate. The solubility results of HHP-treated SPI at pH 7 were significantly ($p<0.05$) higher than at pH 5 and control. Also, although the pressure alone did not have a significant effect, when all factors are considered, SPI treated at pH 7 at 400 MPa and 40 °C had the maximum solubility.

Moreover, the emulsion activity results illustrated that there was no significant difference ($p>0.05$) between HHP-treated SPI at pH 7 and untreated SPI. However, there was a complex scenario at pH 5. At this pH, applying the right pressure raised emulsion activity, but further increase in pressure lowered emulsion activity.

Furthermore, HHP treatment reduced viscosity of SPI for both pH 5 and pH 7 significantly compared to control ($p<0.05$). Also, the results indicated that at pH 5, the viscosity was significantly lower ($p<0.05$) than at pH 7.

In addition, the secondary structure of the SPI was examined with FTIR spectroscopy analysis and it was concluded that the HHP application caused great changes in the secondary structure of the SPI due to HHP induced protein unfolding.

NMR relaxometry analysis was conducted to obtain information about the hydration behavior of the soy protein isolate. It was concluded that the effect of HHP application on the hydration behavior of SPI at pH 7 was not significant ($p>0.05$). In addition, T_2 results at pH 5 at 40 °C were significantly greater than control and other treated SPI.

Based on this research, it is the most comprehensive result obtained that the HHP treatment modifies SPI and gives various functional properties as a result of this modification. It was observed that solubility and hydration behavior improved in HHP-treated SPIs adjusted to pH 7. Solubility is one of the most important limitations of SPI, and this limitation can be avoided thanks to HHP-induced modification. By modifying SPI, which is utilized in many areas in food institutions, it is possible to generate new alternative approaches and apply it in novel food applications by taking advantage of the HHP process. In addition, since it is possible to change the emulsifying ability in HHP-treated SPIs, according to the results of this study, HHP-treated SPIs at pH 5 may be used in novel product development.

In conclusion, this study highlighted that modification of the soy protein isolate is possible with the help of HHP treatment. SPI is affected by the application of high hydrostatic pressure in terms of interactions, denaturation, and aggregation. Therefore, the HHP treatment may be a feasible option for obtaining soy protein isolates with a wide range of functional properties.

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APPENDICES

A. Calibration Curve

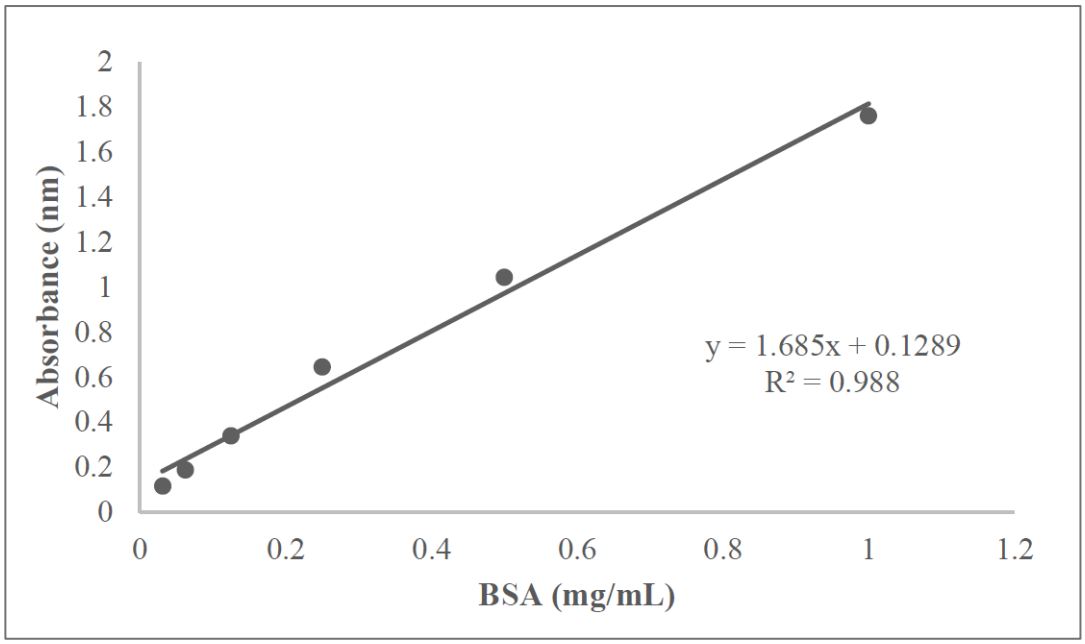

Figure A.1. Calibration curve for Lowry Method prepared by Bovine Serum Albumin (BSA)

Absorbance (at 750 nm) = 1.685 * (mg BSA/ml) + 0.1289 where $R^2 = 0.988$

B. Statistical Analyses

Table B.1 ANOVA and Tukey's Comparison Test with 95% confidence level for Water Holding Capacity (WHC)

General Linear Model: WHC versus Pressure; Temperature; pH

```
Factor       Type   Levels  Values
Pressure     fixed       3  300,0; 400,0; 500,0
Temperature  fixed       2  25; 40
pH           fixed       2  5; 7


Analysis of Variance for WHC, using Adjusted SS for Tests

Source                   DF   Seq SS  Adj SS  Adj MS      F      P
Pressure                  2   1,5592  1,5592  0,7796   5,63  0,010
Temperature               1   0,2314  0,2314  0,2314   1,67  0,208
pH                        1   0,2666  0,2666  0,2666   1,93  0,178
Pressure*Temperature      2   5,6442  5,6442  2,8221  20,38  0,000
Pressure*pH               2   2,1219  2,1219  1,0610   7,66  0,003
Temperature*pH            1   0,4017  0,4017  0,4017   2,90  0,101
Pressure*Temperature*pH   2   1,7594  1,7594  0,8797   6,35  0,006
Error                    24   3,3232  3,3232  0,1385
Total                    35  15,3076


S = 0,372110   R-Sq = 78,29%   R-Sq(adj) = 68,34%


Unusual Observations for WHC

Obs      WHC      Fit   SE Fit  Residual  St Resid
 20  7,52529  8,20446  0,21484  -0,67917     -2,24 R
 21  8,82595  8,20446  0,21484   0,62149      2,05 R
 23  6,70609  7,56448  0,21484  -0,85839     -2,83 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95,0% Confidence

Pressure   N  Mean  Grouping
  0,1      3   9,3  A
500,0     12   8,6  A B
300,0     12   8,2    B
400,0     12   8,1    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

Temperature   N  Mean  Grouping
```

25	18	8,4	A
40	18	8,2	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

pH	N	Mean	Grouping
7	18	8,4	A
5	18	8,2	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	Temperature	N	Mean	Grouping
500,0	25	6	8,7	A
400,0	25	6	8,6	A
300,0	40	6	8,6	A
500,0	40	6	8,4	A
300,0	25	6	7,7	B
400,0	40	6	7,6	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature (pH 5)	N	Mean	Grouping
30040	3	9,0	A
40025	3	8,8	A
50025	3	8,5	A
50040	3	8,2	A
30025	3	7,9	A B
40040	3	6,9	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature (pH 7)	N	Mean	Grouping
50025	3	9,0	A
50040	3	8,6	A
40025	3	8,5	A B
40040	3	8,3	A B
30040	3	8,3	A B
30025	3	7,6	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (25°C)	N	Mean	Grouping
5007	3	9,0	A

4005	3	8,8	A B
5005	3	8,5	A B C
4007	3	8,5	A B C
3005	3	7,9	B C
3007	3	7,6	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (40°C)	N	Mean	Grouping
3005	3	9,0	A
5007	3	8,6	A
4007	3	8,3	A
3007	3	8,3	A
5005	3	8,2	A
4005	3	6,9	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (300MPa)	N	Mean	Grouping
405	3	9,0	A
407	3	8,3	A B
255	3	7,9	A B
257	3	7,6	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (400MPa)	N	Mean	Grouping
255	3	8,8	A
257	3	8,5	A
407	3	8,3	A
405	3	6,9	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (500MPa)	N	Mean	Grouping
257	3	9,0	A
407	3	8,6	A
255	3	8,5	A
405	3	8,2	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	Temperature	pH	N	Mean	Grouping
500,0	25	7	3	9,0	A
300,0	40	5	3	9,0	A
400,0	25	5	3	8,8	A
500,0	40	7	3	8,6	A B
500,0	25	5	3	8,5	A B
400,0	25	7	3	8,5	A B
400,0	40	7	3	8,3	A B
300,0	40	7	3	8,3	A B
500,0	40	5	3	8,2	A B
300,0	25	5	3	7,9	A B C
300,0	25	7	3	7,6	B C
400,0	40	5	3	6,9	C

Means that do not share a letter are significantly different.

Table B.2 ANOVA and Tukey's Comparison Test with 95% confidence level for protein solubility by Lowry Method

General Linear Model: Solubility versus Pressure; Temperature; pH

General Linear Model: Solubility versus Pressure-Temperature (pH 5)

Factor	Type	Levels	Values
Pressure-Temperature (pH 5)	fixed	6	30025; 30040; 40025; 40040; 50025; 50040

Analysis of Variance for Solubility, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure-Temperature (pH 5)	5	181,485	181,485	36,297	74,34	0,000
Error	12	5,859	5,859	0,488		
Total	17	187,344				

S = 0,698746 R-Sq = 96,87% R-Sq(adj) = 95,57%

Unusual Observations for Solubility

Obs	Solubility	Fit	SE Fit	Residual	St Resid
10	6,6730	8,3949	0,4034	-1,7219	-3,02 R
11	9,7116	8,3949	0,4034	1,3167	2,31 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature (pH 5)	N	Mean	Grouping
50025	3	15,7	A
40025	3	15,6	A

30025	3	15,3	A
40040	3	10,1	B
50040	3	9,3	B
30040	3	8,4	B

Means that do not share a letter are significantly different.

General Linear Model: Solubility versus Pressure-Temperature (pH 7)

Factor	Type	Levels	Values
Pressure-Temperature (pH 7)	fixed	6	30025; 30040; 40025; 40040; 50025; 50040

Analysis of Variance for Solubility, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure-Temperature (pH 7)	5	546,94	546,94	109,39	3,00	0,055
Error	12	437,54	437,54	36,46		
Total	17	984,48				

S = 6,03834 R-Sq = 55,56% R-Sq(adj) = 37,04%

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature (pH 7)	N	Mean	Grouping
40040	3	82,4	A
40025	3	73,6	A
30025	3	69,4	A
50025	3	68,1	A
50040	3	66,8	A
30040	3	66,8	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	N	Mean	Grouping
400,0	12	45,4	A
500,0	12	40,0	A
300,0	12	40,0	A
0,1	3	20,0	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	N	Mean	Grouping
400,0	12	45,4	A
500,0	12	40,0	B
300,0	12	40,0	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature	N	Mean	Grouping
25	18	43,0	A
40	18	40,6	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

pH	N	Mean	Grouping
7	18	71,2	A
5	18	12,4	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (25°C)	N	Mean	Grouping
4007	3	73,6	A
3007	3	69,4	A
5007	3	68,1	A
5005	3	15,7	B
4005	3	15,6	B
3005	3	15,3	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (40°C)	N	Mean	Grouping
4007	3	82,4	A
5007	3	66,8	B
3007	3	66,8	B
4005	3	10,1	C
5005	3	9,3	C
3005	3	8,4	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (300MPa)	N	Mean	Grouping
257	3	69,4	A
407	3	66,8	A
255	3	15,3	B
405	3	8,4	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH

```
(400MPa)        N  Mean  Grouping
407             3  82,4  A
257             3  73,6  A
255             3  15,6    B
405             3  10,1    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH
(500MPa)        N  Mean  Grouping
257             3  68,1  A
407             3  66,8  A
255             3  15,7    B
405             3   9,3      C

Means that do not share a letter are significantly different.
```

Table B.3 ANOVA and Tukey's Comparison Test with 95% confidence level for Emulsion Activity

General Linear Model: Emulsion Activity versus Pressure; Temperature; pH

```
Factor       Type   Levels  Values
Pressure     fixed       3  300,0; 400,0; 500,0
Temperature  fixed       2  25; 40
pH           fixed       2  5; 7


Analysis of Variance for Emulsion Activity, using Adjusted SS for Tests

Source                   DF   Seq SS   Adj SS  Adj MS      F      P
Pressure                  2  113,189  113,189  56,595  11,91  0,000
Temperature               1   77,118   77,118  77,118  16,23  0,000
pH                        1   76,475   76,475  76,475  16,10  0,001
Pressure*Temperature      2   57,010   57,010  28,505   6,00  0,008
Pressure*pH               2   62,332   62,332  31,166   6,56  0,005
Temperature*pH            1    0,136    0,136   0,136   0,03  0,867
Pressure*Temperature*pH   2    7,964    7,964   3,982   0,84  0,445
Error                    24  114,025  114,025   4,751
Total                    35  508,250


S = 2,17969   R-Sq = 77,57%   R-Sq(adj) = 67,28%


Unusual Observations for Emulsion Activity

     Emulsion
Obs  Activity      Fit  SE Fit  Residual  St Resid
 13   74,2700  70,3767  1,2584    3,8933      2,19 R
```

```
 14   66,0000  70,3767  1,2584   -4,3767     -2,46 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95,0% Confidence

Pressure   N  Mean  Grouping
300,0     12  65,1  A
500,0     12  62,0  A B
400,0     12  60,9    B
  0,1      3  59,3    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

Temperature   N  Mean  Grouping
40           18  64,1  A
25           18  61,2    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

pH   N  Mean  Grouping
5   18  64,1  A
7   18  61,2    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature
(pH 5)               N  Mean  Grouping
30040                3  70,4  A
30025                3  65,9  A B
50025                3  64,1    B
40040                3  63,3    B C
50040                3  62,9    B C
40025                3  58,2      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature
(pH 7)               N  Mean  Grouping
30040                3  65,2  A
40040                3  62,5  A B
50040                3  60,6  A B
50025                3  60,4  A B
40025                3  59,7  A B
30025                3  58,9    B

Means that do not share a letter are significantly different.
```

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (25°C)	N	Mean	Grouping
3005	3	65,9	A
5005	3	64,1	A B
5007	3	60,4	B C
4007	3	59,7	B C
3007	3	58,9	B C
4005	3	58,2	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (40°C)	N	Mean	Grouping
3005	3	70,4	A
3007	3	65,2	A B
4005	3	63,3	B
5005	3	62,9	B
4007	3	62,5	B
5007	3	60,6	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (300MPa)	N	Mean	Grouping
405	3	70,4	A
255	3	65,9	A
407	3	65,2	A
257	3	58,9	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (400MPa)	N	Mean	Grouping
405	3	63,3	A
407	3	62,5	A
257	3	59,7	A
255	3	58,2	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (500MPa)	N	Mean	Grouping
255	3	64,1	A
405	3	62,9	A
407	3	60,6	A
257	3	60,4	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	Temperature	pH	N	Mean	Grouping
300,0	40	5	3	70,4	A
300,0	25	5	3	65,9	A B
300,0	40	7	3	65,2	A B C
500,0	25	5	3	64,1	A B C D
400,0	40	5	3	63,3	B C D
500,0	40	5	3	62,9	B C D
400,0	40	7	3	62,5	B C D
500,0	40	7	3	60,6	B C D
500,0	25	7	3	60,4	B C D
400,0	25	7	3	59,7	B C D
300,0	25	7	3	58,9	C D
400,0	25	5	3	58,2	D

Means that do not share a letter are significantly different.

Table B.4 ANOVA and Tukey's Comparison Test with 95% confidence level for Viscosity

General Linear Model: Viscosity versus Pressure; Temperature; pH

Factor	Type	Levels	Values
Pressure	fixed	3	300,0; 400,0; 500,0
Temperature	fixed	2	25; 40
pH	fixed	2	5; 7

Analysis of Variance for Viscosity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	0,9639	0,9639	0,4820	7,79	0,002
Temperature	1	7,5717	7,5717	7,5717	122,45	0,000
pH	1	31,7157	31,7157	31,7157	512,90	0,000
Pressure*Temperature	2	4,3184	4,3184	2,1592	34,92	0,000
Pressure*pH	2	5,4727	5,4727	2,7364	44,25	0,000
Temperature*pH	1	0,0812	0,0812	0,0812	1,31	0,263
Pressure*Temperature*pH	2	7,0311	7,0311	3,5156	56,85	0,000
Error	24	1,4841	1,4841	0,0618		
Total	35	58,6388				

S = 0,248669 R-Sq = 97,47% R-Sq(adj) = 96,31%

Unusual Observations for Viscosity

Obs	Viscosity	Fit	SE Fit	Residual	St Resid
28	8,1500	7,4933	0,1436	0,6567	3,23 R
29	7,0100	7,4933	0,1436	-0,4833	-2,38 R

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 31     5,8300  5,3867  0,1436    0,4433      2,18 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95,0% Confidence

Pressure   N  Mean  Grouping
  0,1      3  10,3  A
500,0     12   4,6    B
400,0     12   4,3    B
300,0     12   4,2    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

Temperature   N  Mean  Grouping
25           18   4,8  A
40           18   3,9    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

pH   N  Mean  Grouping
7   18   5,3  A
5   18   3,4    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

PressureTemperature
(pH 5)               N  Mean  Grouping
40025                3   4,1  A
30025                3   4,1  A
50025                3   3,6    B
40040                3   3,5    B
50040                3   2,7      C
30040                3   2,6      C

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95,0% Confidence

PressureTemperature
(pH 7)               N  Mean  Grouping
50025                3   7,5  A
30040                3   5,4    B
40025                3   5,0    B
40040                3   4,7    B
30025                3   4,7    B
50040                3   4,6    B

Means that do not share a letter are significantly different.
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Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (25°C)	N	Mean	Grouping
5007	3	7,5	A
4007	3	5,0	B
3007	3	4,7	B C
4005	3	4,1	C D
3005	3	4,1	C D
5005	3	3,6	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (40°C)	N	Mean	Grouping
3007	3	5,4	A
4007	3	4,7	B
5007	3	4,6	B
4005	3	3,5	C
5005	3	2,7	D
3005	3	2,6	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (300MPa)	N	Mean	Grouping
407	3	5,4	A
257	3	4,7	B
255	3	4,1	B
405	3	2,6	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (400MPa)	N	Mean	Grouping
257	3	5,0	A
407	3	4,7	A
255	3	4,1	B
405	3	3,5	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (500MPa)	N	Mean	Grouping
257	3	7,5	A
407	3	4,6	B
255	3	3,6	C
405	3	2,7	D

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	Temperature	pH	N	Mean	Grouping
500,0	25	7	3	7,5	A
300,0	40	7	3	5,4	B
400,0	25	7	3	5,0	B C
400,0	40	7	3	4,7	B C D
300,0	25	7	3	4,7	B C D
500,0	40	7	3	4,6	C D
400,0	25	5	3	4,1	D E
300,0	25	5	3	4,1	D E
500,0	25	5	3	3,6	E
400,0	40	5	3	3,5	E
500,0	40	5	3	2,7	F
300,0	40	5	3	2,6	F

Means that do not share a letter are significantly different.

Table B.5 ANOVA and Tukey's Comparison Test with 95% confidence level for T_2 data by NMR Relaxometry

General Linear Model: T2 versus Pressure; Temperature; pH

Factor	Type	Levels	Values
Pressure	fixed	3	300,0; 400,0; 500,0
Temperature	fixed	2	25; 40
pH	fixed	2	5; 7

Analysis of Variance for T2, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	69,77	69,77	34,89	11,09	0,000
Temperature	1	1445,37	1445,37	1445,37	459,37	0,000
pH	1	2078,39	2078,39	2078,39	660,56	0,000
Pressure*Temperature	2	37,11	37,11	18,56	5,90	0,008
Pressure*pH	2	21,45	21,45	10,72	3,41	0,050
Temperature*pH	1	1510,26	1510,26	1510,26	479,99	0,000
Pressure*Temperature*pH	2	0,28	0,28	0,14	0,04	0,956
Error	24	75,51	75,51	3,15		
Total	35	5238,14				

S = 1,77381 R-Sq = 98,56% R-Sq(adj) = 97,90%

Unusual Observations for T2

Obs	T2	Fit	SE Fit	Residual	St Resid
13	73,3160	69,7413	1,0241	3,5747	2,47 R
28	43,3720	40,4513	1,0241	2,9207	2,02 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	N	Mean	Grouping
0,1	3	48,9	A
400,0	12	48,8	A
300,0	12	48,3	A
500,0	12	45,6	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature	N	Mean	Grouping
40	18	53,9	A
25	18	41,2	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

pH	N	Mean	Grouping
5	18	55,2	A
7	18	40,0	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature (pH 5)	N	Mean	Grouping
40040	3	70,6	A
30040	3	69,7	A
50040	3	63,6	B
40025	3	44,0	C
30025	3	42,1	C
50025	3	41,0	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-Temperature (pH 7)	N	Mean	Grouping
30040	3	41,3	A
40040	3	40,7	A
50025	3	40,5	A
30025	3	40,0	A
40025	3	39,9	A
50040	3	37,5	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (25°C)	N	Mean	Grouping
4005	3	44,0	A
3005	3	42,1	A
5005	3	41,0	A
5007	3	40,5	A
3007	3	40,0	A
4007	3	39,9	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure-pH (40°C)	N	Mean	Grouping
4005	3	70,6	A
3005	3	69,7	A
5005	3	63,6	B
3007	3	41,3	C
4007	3	40,7	C
5007	3	37,5	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (300MPa)	N	Mean	Grouping
405	3	69,7	A
255	3	42,1	B
407	3	41,3	B
257	3	40,0	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (400MPa)	N	Mean	Grouping
405	3	70,6	A
255	3	44,0	B
407	3	40,7	B C
257	3	39,9	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Temperature-pH (500MPa)	N	Mean	Grouping
405	3	63,6	A
255	3	41,0	B

257	3	40,5	B
407	3	37,5	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95,0% Confidence

Pressure	Temperature	pH	N	Mean	Grouping
400,0	40	5	3	70,6	A
300,0	40	5	3	69,7	A
500,0	40	5	3	63,6	B
400,0	25	5	3	44,0	C
300,0	25	5	3	42,1	C D
300,0	40	7	3	41,3	C D
500,0	25	5	3	41,0	C D
400,0	40	7	3	40,7	C D
500,0	25	7	3	40,5	C D
300,0	25	7	3	40,0	C D
400,0	25	7	3	39,9	C D
500,0	40	7	3	37,5	D

Means that do not share a letter are significantly different