Universidade de Aveiro Departamento de Química

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**Rui Pedro Figueiredo Neto Queirós**

# Manipulação de interações entre proteínas com tratamentos enzimáticos e alta pressão para alterar as propriedades dos alimentos

# Manipulating protein-protein interactions through high pressure and enzymatic combined treatments to tailor food properties

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência e Tecnologia Alimentar e Nutrição, realizada sob a orientação científica do Professor Doutor José António Teixeira Lopes da Silva, Professor Auxiliar do Departamento de Química da Universidade de Aveiro e do Professor Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho à Andreia, aos meus pais e irmã.

**o júri**

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**palavras-chave** processamento por alta pressão, transglutaminase, inactivação enzimática, proteina de ervilha, proteina de soja, funcionalidade proteica.

**resumo**

Verifica-se um crescente aumento da procura por proteínas para satisfazer as necessidades nutricionais da população a nível global, em particular de proteínas vegetais devido a preocupações nutricionais e ambientais. As proteínas de origem vegetal aparecem assim como uma alternativa vantajosa às proteínas de origem animal, no entanto, as suas propriedades tecno-funcionais precisam ser melhor conhecidas e otimizadas. O processamento de alta pressão (AP) é uma tecnologia não térmica que permite modificar a estrutura das proteínas, permitindo alterar várias das suas propriedades. Enzimas, como a transglutaminase microbiana (MTG), também podem modificar as propriedades tecno-funcionais das proteínas, no entanto, muitas proteínas globulares mostram baixa suscetibilidade à ação desta enzima. A AP, capaz de alterar a conformação de proteínas, pode ser uma ferramenta útil para aumentar a acessibilidade das proteínas à ação da MTG. No entanto, as condições de processamento precisam ser adequadamente otimizadas para evitar a diminuição da atividade enzimática quando sujeita a pressão.

A inativação da MTG sob diferentes condições de pressão (200 – 600 MPa; 20 – 40 °C; 10 – 30 min) foi avaliada em diferentes valores de pH. Pelo menos 20% da MTG foi inativada quando foram usadas baixas pressões (< 300 MPa) a pH 4 e 5, enquanto foi necessária uma pressão acima de 400 MPa para obter uma inativação semelhante a pH 6 ou 7. A inativação por pressão da MTG seguiu uma cinética de primeira ordem em todas as condições testadas. As constantes cinéticas de inativação diminuíram com o aumento da pressão a uma temperatura constante a pH 4, com um volume de ativação positivo, enquanto o contrário foi verificado para os demais valores de pH. Tanto a energia de ativação quanto o volume de ativação foram dependentes do pH. No geral, a MTG pode ser considerada relativamente resistente à pressão, particularmente próximo do seu pH óptimo.

Foi avaliada a influência da pressão (200 – 600 MPa; 5 – 15 min), aplicada individualmente ou em combinação com MTG (até 30 $U \cdot g^{-1}$), sobre propriedades selecionadas de proteínas de ervilha e soja com concentrações entre 1 e 9% (m/v). Para uma concentração de proteína de 1 % (m/v), a AP aumentou a solubilidade da proteína de ambos os isolados quando aplicada individualmente. Da mesma forma, a hidrofobicidade de superfície também aumentou com a AP nas proteínas de ambas as fontes, aumentando, em geral, com o aumento da pressão e do tempo. Pelo contrário, o conteúdo de grupos sulfidrilo livres diminuiu com a pressão nas proteínas de ambas as fontes. O efeito da AP nas propriedades emulsificantes das proteínas, considerando quer a fração total de proteína no isolado, quer a fração solúvel, foi dependente do pH e das condições de AP (pressão, tempo). A AP parece ter efeitos mínimos na tensão superficial de ambas as proteínas e a ausência geral de efeitos negativos na atividade emulsificante resulta dos efeitos de agregação de proteínas induzidas pela AP. Por outro lado, os tratamentos individuais de MTG não produziram efeitos sobre as propriedades estudadas. Para as demais concentrações de proteínas utilizadas, a AP aumentou a solubilidade de dispersões de baixa concentração, diminuindo-a nas mais altas. Independentemente da concentração, a AP diminuiu o conteúdo de grupos sulfidrilo livres para as proteínas de ervilha, no entanto, teve o efeito contrário para as proteínas de soja. Comparativamente à solubilidade, a hidrofobicidade de superfície aumentou para concentrações baixas de proteína e o contrário foi verificado para concentrações altas. A MTG diminuiu a solubilidade e aumentou o conteúdo de grupos sulfidrilo livres de ambas as as proteinas. A enzima diminuiu a hidrofobicidade de superfície de ambas as proteínas quando estas se encontravam em concentração relativamente elevada. Quando combinados, AP e MTG parecem ter efeitos antagonisticos na solubilidade e no conteúdo de grupos sulfidrilo livres e efeitos sinergisticos na viscosidade. Os resultados obtidos indicam que tratamentos simultâneos de AP e MTG podem ser usados para modificar a estrutura das proteínas e consequentemente adaptar suas propriedades tecno-funcionais.

**keywords** high pressure processing, transglutaminase, enzyme inactivation, pea protein, soy protein, protein functionality.

**abstract**

The demand for proteins is rising and alternatives to animal-based proteins are necessary, either for nutritional or environmental reasons. Plant-based proteins appear as an alternative, however, their techno-functional properties need improvement. High-pressure processing (HPP) is a non-thermal technology that allows modifying proteins' structure hence allowing to change several of their properties. Enzymes, such as microbial transglutaminase (MTG), can also modify the techno-functional properties of proteins, however, many globular proteins show low susceptibility to the action of this enzyme. HPP, being able to change protein conformation, may be a useful tool to increase the accessibility of proteins to the action of MTG. Nevertheless, HPP conditions need to be carefully optimized to avoid the expected decrease in enzymatic activity when subjected to pressure.

Pressure inactivation of MTG under different HPP conditions (200 – 600 MPa; 20 – 40 °C; 10 – 30 min) was evaluated at different pH values. At least 20 % of MTG was inactivated when low pressures (< 300 MPa) were used at pH 4 and 5, whereas a higher pressure (above 400 MPa) was needed to obtain a similar inactivation at pH 6 or 7. MTG pressure-inactivation followed first-order kinetics under all tested conditions. Inactivation rate constants decreased with increasing pressure at constant temperature and pH 4, with a positive activation volume, while the opposite was verified for the other pH values. Both activation energy and volume were dependent on pH. Overall, MTG can be considered relatively resistant to pressure, particularly near its optimal pH.

The influence of HPP (200 – 600 MPa; 5 – 15 min) was also evaluated, applied individually or in combination with MTG (up to 30 $U \cdot g^{-1}$), on selected properties of pea (PPI) and soy (SPI) protein isolates with concentrations between 1 and 9 % (w/v). For a protein concentration of 1 % (w/v), HPP increased the protein solubility of both isolates when applied individually. This effect was more pronounced for SPI, particularly at pH 7 and 8. Similarly, the protein surface hydrophobicity also increased with HPP for proteins from both sources, increasing, in general, with increasing pressure and holding time. On the contrary, the content of free sulfhydryl groups decreased with HPP for proteins from both sources. The effects of HPP on the emulsifying properties of the protein isolates, considering both the whole and soluble protein fractions, were dependent on pH and HPP conditions (pressure, holding time). HPP appeared to have minimal effects on the surface tension of both proteins and the general absence of negative effects on emulsifying activity results from HPP-induced protein aggregation effects. On the other hand, MTG individual treatments had no significant effects on the studied properties. For the other protein concentrations studied, HPP increased the solubility of proteins when there were at low initial concentrations, decreasing it when they were in the higher concentration range analysed. Regardless of the concentration, HPP decreased the content of free sulfhydryl groups for pea proteins, however, had the contrary effect on soy proteins. Comparably to the solubility, the surface hydrophobicity increased in low protein concentrations and the contrary was verified in high protein concentrations. MTG decreased solubility and increased the content of free sulfhydryl groups of both proteins. The enzyme decreased the surface hydrophobicity of soy proteins and of the pea proteins, but only when these were within the higher concentration range analysed.

When combined, HPP and MTG appear to have antagonistic effects on the solubility and content of free sulfhydryl groups and synergistic effects on viscosity. The obtained results indicate that simultaneous HPP and MTG treatments can be used to modify the proteins' structure and consequently tailor their techno-functional properties.

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## LIST OF PUBLICATIONS

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### POSTER COMMUNICATIONS IN SCIENTIFIC MEETINGS:

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 1-anilino-8-naphthalene-sulfonate | ANS |
| Activation energy | $E_a$ |
| Activation volume | $V_a$ |
| Analysis of variance | ANOVA |
| Apparent viscosity | $\eta_a$ |
| Complex shear modulus | G* |
| Complex viscosity | $\eta*$ |
| Consistency coefficient | $K$ |
| Dilution factor | $DF$ |
| Dimensionless coded forms of variables | $x_1$, $x_2$, $x_3$ |
| Disulfide bonds | S-S |
| Emulsifying activity index | EAI |
| Emulsifying stability index | ESI |
| Enzyme’s activity at time 0 | $A_0$ |
| Enzyme’s activity at time t | $A_t$ |
| Enzyme’s residual activity | $A_r$ |
| First-order inactivation rate constant | $k$ |
| Flow behaviour index | $n$ |
| Frequency | $\omega$ |
| Frequency factor | $A$ |
| High-density polyethylene | HDPE |
| High pressure processing | HPP |
| Initial protein concentration | $c$ |
| Loss modulus | G” |
| Microbial transglutaminase | MTG |
| Model constructed for pea protein isolate free sulfhydryl groups | $PPI_{SH}$ |
| Model constructed for pea protein isolate solubility | $PPI_{SOL}$ |
| Model constructed for pea protein isolate surface hydrophobicity | $PPI_{H0}$ |
| Model constructed for soy protein isolate free sulfhydryl groups | $SPI_{SH}$ |

| | |
|---|---|
| Model constructed for soy protein isolate solubility | $SPI_{SOL}$ |
| Model constructed for soy protein isolate surface hydrophobicity | $SPI_{H0}$ |
| Models' independent variables | $\beta_0$; $\beta_i$; $\beta_{ii}$; $\beta_{ij}$ |
| Models' number of independent variables | *n* |
| Models' response | *Y* |
| Molecular weight | MW |
| Oil volumetric fraction | $\Theta$ |
| Optical path | $\varphi$ |
| Pea protein isolate | PPI |
| Phase difference angle | $\delta$ |
| Pressure | P |
| Reference pressure | $P_{ref}$ |
| Regression coefficient | $R^2$ |
| Relative thixotropic structural breakdown | *RTB* |
| Rotations per minute | rpm |
| Shear rate | $\dot{\gamma}$ |
| Sodium dodecyl sulfate | SDS |
| Soy protein isolate | SPI |
| Standard error | SE |
| Storage modulus | G' |
| Sulfhydryl groups | SH |
| Surface hydrophobicity | $H_0$ |
| Surface tension | γ |
| Temperature | T |
| Texture profile analysis | TPA |
| Time | t |
| Time interval | $\Delta t$ |
| Universal gas constant | *R* |
| Water holding capacity | WHC |

# CHAPTER 1

## General Introduction, Objectives and Thesis Structure

## 1.1. GENERAL INTRODUCTION AND OBJECTIVES

Food ingredients, and their functional and technological properties, are important attributes affecting product design and consumer acceptability. Although foods are complex multicomponent dispersed systems, their structure depends mainly on the interactions that take place between macromolecular components. Food ingredients' technological properties can be tentatively tailored in many ways, including different chemical/physical methods.

In this work, a ‘clean’ and ‘green’ approach was used to manipulate protein-protein interactions, intending to obtain additional knowledge that allows to tailor desirable functional properties for the treated vegetable protein systems. Two main methods were explored aiming for complementary and synergic effects to be obtained: high pressure processing (HPP) and enzymatic cross-linking using a microbial transglutaminase (MTG).

The main rationale of this project was to develop novel and tailored crosslinking and aggregation mechanisms between vegetable proteins, from soybean and pea isolates, developing structures with desirable properties and stability. Therefore, the main objective of this work was the optimization of combined treatments by HPP and crosslinking with MTG for tailoring food structure based on protein matrices. Synergistic mechanisms involving the effect of HPP on protein conformation and accessibility of target amino acids and the formation of covalent bonds between the protein chains, catalysed by MTG under pressure, were evaluated by analysing a set of key parameters indicative of protein structure and functionality.

Different conditions were tested regarding the proteins (protein type, concentration, and solvent pH), the HPP conditions (pressure level and holding time), catalytic conditions with the MTG (substrate/enzyme ratio and time) and how both treatments can be combined to achieve the desired results.

In this work, several specific objectives were pursuit:

- To assess the resistance of MTG to HPP inactivation;
- To evaluate how HPP conditions influence the structure, aggregation and technological properties of soybean and pea proteins;
- To evaluate how the treatments with MTG influence the crosslinking and technological properties of the proteins under study;

- To determine how the proteins' susceptibility to the enzymatic crosslinking is affected by the native state of the protein and conformational changes introduced by the HPP treatments;
- Establish synergistic or antagonistic relationships between combined treatment conditions (HPP+MTG), structural and conformational changes and aggregation of the protein systems.

## 1.2. THESIS STRUCTURE

To pursue the established specific objectives, this thesis was structured as follows (Figure 1.1).

First, it is presented a literature review regarding the topics approached on this work, namely on HPP and its effects on plant-based proteins, MTG, and the combination of HPP and MTG (**Chapter 2**).

Combining MTG treatments and HPP may be an interesting tool to modify proteins' properties, still, it was necessary to gather knowledge regarding the behaviour not only of the target proteins under pressure but also of the MTG. To provide insight on the pressure resistance of MTG and inactivation kinetics involved, an integrated study using response surface methodology to determine the effect of several combined factors (pressure, holding time, temperature) at different pH values (4 – 7) was performed (**Chapter 3**). The results obtained in this study were considered to further studies.

Afterward, there was a need to assess the individual effects of HPP on selected properties of pea and soy proteins. To that end, the effects of different processing parameters, *i.e.* pressure and holding time on some technological characteristics of pea and soy protein isolates at three different pH values were evaluated (**Chapter 4)**. A similar study was performed regarding the individual effects of MTG on the same characteristics of the proteins, which revealed the difficulty of MTG to crosslink commercial proteins isolates (**Annex C**).

Subsequently, it was evaluated the effects of simultaneously applied HPP and MTG crosslink on some key technological properties of the protein isolates. A response surface methodology was used to determine the effect of several combined factors, like pressure, holding time, and MTG concentration. The influence of the concentration of protein was also

brought into consideration when studying the effects of HPP and MTG applied simultaneously (**Chapter 5**).

Lastly, in **Chapter 6**, some general conclusions are presented, highlighting the most important results obtained in this work. Some future work is also suggested that could complement this work.

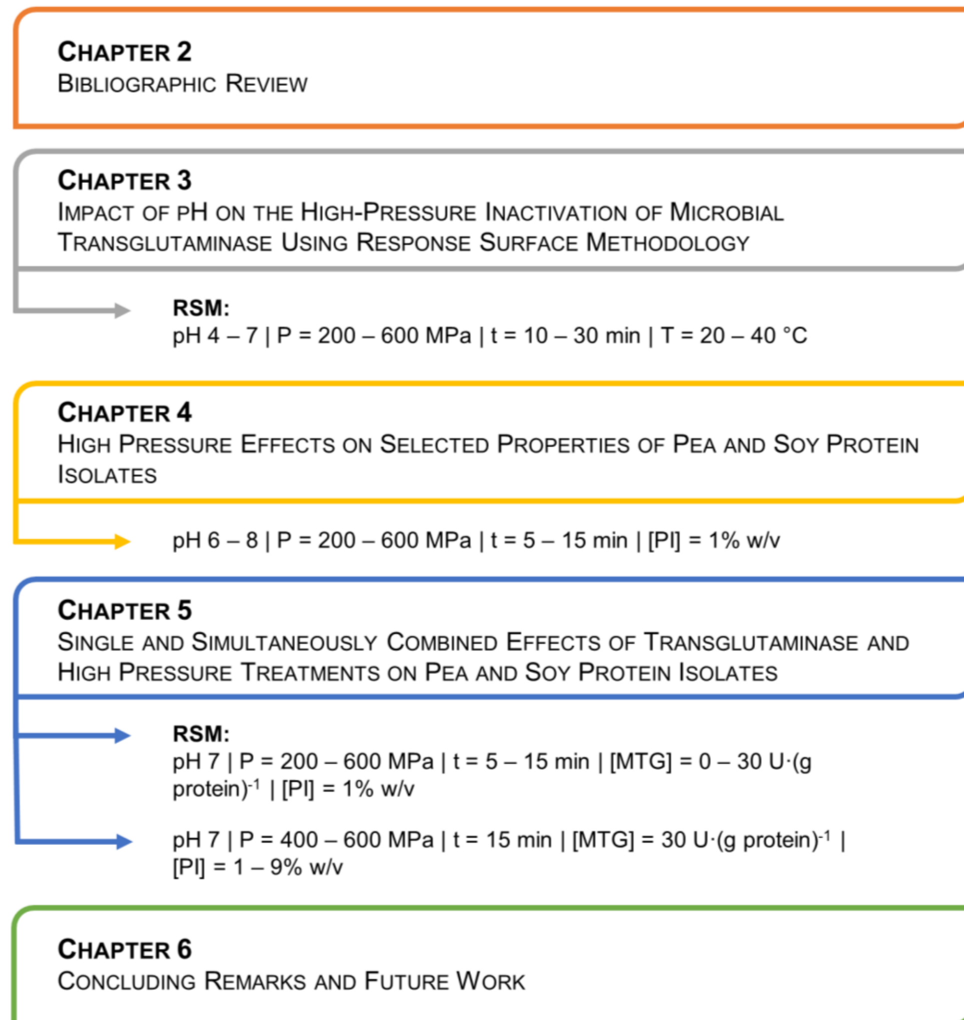



**Figure 1.1.** Thesis structure overview

# CHAPTER 2

## Bibliographic Review

# INTRODUCTION

Proteins are critical macronutrients in human nutrition that play important roles in the body. However, replacement of animal proteins as the main source of protein-rich foods for those of plant origin is becoming essential, considering that animal husbandry typically relies on extensive and intensive systems that have high costs and an extreme impact on the environment. In fact, plant proteins are a more sustainable protein source since their cultivation is less intensive to the environment than animal agriculture (Wu et al., 2014).

Additionally to the proteins' nutritional role as a source of amino acids, they provide several technical functions in the quality and stability of food products and are key components in many food processes. They can stabilise foams and emulsions or interact with each other to aggregate or create networks that may result in gels. There are different mechanisms affecting their main techno-functional properties: i) hydration can influence solubility or wettability, ii) hydrophobicity, hydrophilicity and charge distribution can impact gelling, emulsifying and foaming capabilities, iii) protein structure such as conformation, size and amino acid composition can modify rheological characteristics, *i.e.* adhesiveness, viscosity or gelation (Lam and Nickerson, 2013; Mirmoghtadaie et al., 2016). Furthermore, food contains several proteins that are known to cause allergic reactions when ingested. Altering the structure and/or conformation of allergenic proteins may reduce these unwanted effects (Somkuti and Smeller, 2013).

## 2.1. HIGH PRESSURE PROCESSING

Techno-functional properties of proteins can be modified by several methods (chemical, physical or enzymatic). Since the structure, conformation, and physicochemical properties of proteins can be affected by pressure, high-pressure processing (HPP) can be a useful tool to tailor these properties of food proteins (Mirmoghtadaie et al., 2016). Protein functionality is also strongly dependent on the interactions with other food components. It is worth to mention the importance of protein-polysaccharide interactions to understand structure and functionality of complex multicomponent food systems and to design products with desired structure and

consumer acceptance (Monteiro et al., 2013; Turgeon et al., 2007), which are also affected by HPP treatments (Galazka et al., 2000a; Knorr et al., 2006).

HPP is a non-thermal technology mainly used in the food industry as a cold pasteurisation process. A pressure level ranging from 400 to 600 MPa is generally applied for a few minutes to foods regardless their shape and size. HPP does not considerably affect physicochemical properties like colour or flavour and has a small impact on bioactive compounds while inactivating pathogens and vegetative spoilage microorganisms (Balasubramaniam et al., 2015). Moreover, other food applications have been developed in the last years, such as food preservation under pressure (Fernandes et al., 2015), improvement of biotechnological processes (Mota et al., 2013) and modification of biopolymers structural and functional properties (Knorr et al., 2006).

As stated, an important HPP application is the modification of macromolecules, namely proteins. Protein denaturation induced by high pressure was discovered a long time ago, in 1914, by Bridgman, who observed the coagulation of albumen when subjected to pressure, that presented a similar, although not identical, appearance to a hard-boiled egg (Bridgman, 1914). Since then, several studies regarding the effects of HPP on proteins have been made, but still a better knowledge on the subject is necessary, especially regarding the effects on the structure and techno-functional properties of proteins from less studied sources (*e.g.* plants, insects, algae), the interaction between proteins and other molecules, and how HPP impacts proteins when in complex food matrixes.

Proteins under high pressure are governed by the Le Châtelier's principle, which states that pressure shifts equilibrium towards the state that occupies less volume, and accelerates processes where the transition state has a smaller volume than the ground state (Winter et al., 2007). The partial specific volume of a protein is the sum of three contributions: (i) the volume of the constitutive atoms, (ii) the volume of the cavities formed due to imperfect atomic packing, and (iii) the change in volume of the water due to hydration on the protein surface (Kauzmann, 1959). Pressure acts on the proteins' partial molar volume thus, when pressure is applied the conformational equilibrium is shifted in favour of a lower volume conformer (Akasaka, 2003a), where the resulting effects may be reversible or non-reversible (Balny and Masson, 1993).

Proteins' structure may have two responses to pressure, namely general compression (*i.e.* elastic effects, usually reversible) and conformation changes (*i.e.* plastic effects – mostly irreversible). The first is a linear response to pressure that usually occurs at lower pressures, typically < 200 MPa, and consists in a general compression within the sub-ensemble of

conformer (*i.e.* reduction of volume without changing the conformation). Pressure has a small effect on the primary structure of proteins since the primary chemical bonds are very little compressible, therefore their contribution to the reduction of volume is very small. However, there is an increase in the side chain packing, the hydrogen bonds' distances are shortened and slight changes occur in the torsion angles of the polypeptide chain (Akasaka, 2003a, 2014).

The second response involves changes in the conformation of the proteins, which occur predominantly at higher pressures and consists in the change from a high-volume conformation to a low-volume conformation following a non-linear response to pressure. The protein's most compressible parts are the cavities followed by the hydrogen bonds. Consequently, the collapse of cavities due to water penetration, accompanied by their hydration and the shortening of the hydrogen bonds are the most relevant consequences of pressure (Akasaka, 2006). These effects promote intermolecular interactions that destabilise the tertiary structure and lead to the eventual unfolding of the protein (Somkuti and Smeller, 2013; Winter et al., 2007). Thus, proteins may change their native conformation into locally unfolded intermediates and/or complete unfolded conformations (Akasaka, 2003a, 2014). Unfolded intermediates may have larger cavities collapsed although keeping the smaller cavities intact, resulting in an intermediate volume, whereas a complete unfolded protein with all the cavities collapsed will have the lowest volume (Akasaka, 2003b; Boonyaratanakornkit et al., 2002). After the release of pressure, the proteins' structures will frequently be different from the native structures, leading to altered properties such as foaming, emulsification or gelation (Ledward, 2000).

An alternative approach to modify/enhance techno-functional properties of proteins is through enzymatic hydrolysis, which can be improved and/or accelerated by pressure (Akasaka et al., 2008). The breakage of the peptide links produces smaller peptides units that are more soluble, which can modify emulsification, foaming and gel formation. Additionally, small peptides resultant from hydrolysis may present bioactivity (Day, 2013).

As mentioned above, HPP can induce permanent changes in the structure of proteins, thus changing their allergenicity. This subject has been recently reviewed (Somkuti and Smeller, 2013) and therefore will not be approached in this review. Similarly, the effects of HPP on enzymatic activity are out of the scope of this review and therefore will not be discussed in detail. Detailed information on this topic can be found elsewhere (Chakraborty et al., 2014; Eisenmenger and Reyes-De-Corcuera, 2009; Terefe et al., 2014).

## 2.2. EFFECTS OF HPP ON PROTEINS' STRUCTURE

The structure of proteins is affected by pressure at different levels of their macromolecular organisation depending on several factors, such as temperature, pH or protein source. In general, the primary structure of proteins is not affected by pressures below 2 GPa (Winter et al., 2007), since the compressibility of covalent bonds is often negligible (Boonyaratanakornkit et al., 2002). On the contrary, the quaternary structure is primarily sustained by hydrophobic interactions that are very sensitive to pressure (Balny and Masson, 1993; Boonyaratanakornkit et al., 2002), and therefore lower pressure values (100-200 MPa) are ordinarily sufficient to dissociate multi-subunit complexes into the oligomeric protein constituents, a phenomenon accompanied by a negative volume change.

### 2.2.1. GENERAL MACROMOLECULAR STRUCTURE

Usually, dissociation of protein subunits is the first step in protein denaturation induced by HPP, an effect that is strongly dependent on protein native structure, ionic conditions (pH, ionic strength) and protein concentration. Typically, the unfolding degree of a protein chain gradually increases with rising pressure, accompanied by an increase in disordered structure, as reported for example for the unfolding of walnut (Qin et al., 2013) and sweet potato proteins (Khan et al., 2013). In a general way, pressures above 350 MPa may result in protein rearrangements and/or aggregation (Liu et al., 2013), with important consequences for their techno-functional properties.

Different types of proteins are known to have different susceptibilities to pressure. For instance, chaperones of conarrachin from peanut denature at 150 MPa, whereas arachin maintains its conformation above 200 MPa (He et al., 2014). Also, cowpea proteins start denaturing at 200 MPa, with a denaturing degree of 41%, reaching 66% at 400-600 MPa, showing a higher resistance to pressure-induced denaturation than most proteins (Peyrano et al., 2016). Most sensitive fractions of amaranth proteins (albumins and a minor globulin-7S) were unfolded at 200 MPa, while the other fractions (globulin-11S, globulin-P and glutelins) unfolded above 400 MPa (Condés et al., 2012). Vicilin (7S) fraction of kidney bean protein is very compact and thus not very susceptible to HPP, although some unfolding may occur under pressure but only at relatively high pressures (Yin et al., 2008). Pressure dissociation of pea's

vicilin quaternary structure was shown to increase with increasing pressure, being the protein completely dissociated at 240 MPa, a process that exhibited a low dependence on protein concentration but was markedly pH-dependent and significantly inhibited by addition of salts (Pedrosa & Ferreira, 1994).

Other vegetable proteins showed different pressure critical values to achieve complete dissociation, *e.g.* the dissociation of the subunits of 11S globulin of *Vicia faba* occurred at 200 MPa (Galazka et al., 2000b), the required pressure for the dissociation of soy's glycinin was 350 MPa (Ahmed et al., 2007) whereas lentil proteins were not dissociated until pressure up to 300 MPa (Garcia-Mora et al., 2015).

Changes in the tertiary structure of proteins can be observed above 200 MPa, although there are some cases were higher pressures (*i.e.* 400-800 MPa) are necessary. This generally happens when the hydrophobic effects do not dominate volume and compressibility changes during the denaturation. It is important to notice that pressure-induced denaturation of proteins is a complex process that involves many intermediate forms (Balny and Masson, 1993).

Among vegetable proteins, soy proteins are probably those that have been more extensively studied when subjected to HPP. As mentioned above, soy proteins typically lose their native structure and unfold for pressures around 350 MPa. As expected, changes in the tertiary structure are accompanied by exposure of hydrophobic sites within the protein structure, an effect that is enhanced with increasing pressure (Alvarez et al., 2008).

Regarding the main soy protein fractions, it is generally accepted that HPP treatments promote the unfolding of the β-conglycinin and glycinin fractions, and the dissociation of the glycinin. However, there are some conflicting results that reflect the complex effects of HPP and their dependence on a variety of other factors, including the particular HPP conditions and mainly, protein concentration, solvent and ionic effects related to the protein environment. At first, since β-conglycinin does not have S-S bonds, it would be expected to be more affected by the pressure than glycinin in which the S-S bonds play an important role in the native conformation. In fact, some studies have demonstrated this general trend showing that glycinin denatures gradually with increasing pressure, whereas the unfolding of β-conglycinin was almost complete at 400 MPa (Molina et al., 2001; Puppo et al., 2004). Contrarily, other studies (Añón et al., 2011; Speroni et al., 2010) reported a complete denaturation of glycinin at 400 MPa but an incomplete denaturation of β-conglycinin, even at 600 MPa. The different protein concentrations used in different works may explain the apparent discordant results. Studies performed with lower protein concentrations (Añón et al., 2011; Wang et al., 2008) have

suggested that glycinin was easier denatured by pressure than β-conglycinin, whereas the contrary was observed while using higher protein concentrations (Molina et al., 2002). In fact, denaturation is known to be influenced by protein concentration, being generally accepted that higher protein concentrations minimise proteins' denaturation (Condés et al., 2012, 2015). Nevertheless, other factors including structural differences between proteins and even small variations of the ionic conditions of the protein environment (pH, ionic strength) may originate different molecular flexibility and compressibility what necessarily result in different pressure sensitivities.

### 2.2.2. SECONDARY STRUCTURE

Changes at the level of the secondary structure, which usually result in non-reversible denaturation, generally take place at higher pressures (*e.g.* > 400 MPa), although some exceptions have been observed. It is noteworthy that the changes in the secondary structure are also dependent on the compression rate and on the extent of the secondary structure rearrangements (Balny and Masson, 1993). Usually, β-sheet regions are less prone to deformation, and thus less sensitive to pressure, than are the α-helices (Kundrot and Richards, 1988). Table 2.1. provides a general overview of the effects of HPP on the secondary structure of plant proteins. The effect of pressure on the secondary structures of proteins clearly depends on the type of protein, concentration and environment conditions. Often, when there is a decrease in a certain secondary structure, this reduction is accompanied by the increase of another, or vice versa, suggesting that frequently there is a conversion from one secondary conformer to another.

Some aspects are worth to mention from the studies performed so far. For instance, in the particular case of soy proteins, where there is more information available, some apparent contrasting results were reported. For example, the rupture of α-helix structures have been reported even at relatively low pressures (*i.e.* 200 MPa) (Puppo et al., 2004; Tang and Ma, 2009; Yang et al., 2014). This is in agreement with the higher susceptibility of the α-helix structures to pressure, as mentioned above, although Li et al. (2012) observed an increase of α-helix after treatment at 300 and 500 MPa. For β-sheet structures, the available results either suggest a decrease of the β-sheet structures (Alvarez et al., 2008; Li et al., 2012; Yang et al., 2014) or their increase (Puppo et al., 2004; Tang and Ma, 2009) when the proteins were subjected to HPP.

**Table 2.1.** Effects of pressure on the secondary structure of plant proteins.

| Protein or source | Pressure (MPa) | α-helix | β-sheet | β-turn | random coil | Reference |
|---|---|---|---|---|---|---|
| ginko seeds protein 5% (pH 8) | 100 - 700 | ↓ 100 and 200 MPa<br>↓↓ 300 - 700 MPa | = 100 and 200 MPa<br>↑ 300 and 400 MPa<br>↓↓ 500 - 700 MPa | NA | ↑ 100 - 400 MPa<br>↑↑ 500 - 700 MPa | Zhou et al., 2016a |
| rapeseed 1% (pH 7.5) | 200 - 600 | ↑↑ 200 and 600 MPa<br>↑ 400 MPa | ↑↑ 200 and 600 MPa<br>↓ 400 MPa | ↑ 200 - 600 MPa | ↓ 200 and 600 MPa<br>↓↓ 400 MPa | He et al., 2013 |
| phytohemagglutinin 10% (pH 7.4) | 150 - 450 | ↓ 150, 350 and 450 MPa<br>↓↓ 250 MPa | ↑ 150, 350 and 450 MPa<br>↑↑ 250 MPa | NA | NA | Lu et al., 2015 |
| phytohemagglutinin 10% (pH 7.4) | 50 – 450 | ↓ 50 – 450 MPa | ↑ 50 – 450 MPa | NA | ↑ 50 – 450 MPa | Liu et al., 2013 |
| sesame 10% (pH 2) | 100 – 500 | ↓ 100 - 300 MPa<br>↓↓ 400 - 500 MPa | ↑↑ 100 - 500 MPa | ↓↓ 100 - 500 MPa | = 100 MPa<br>↓ 200 – 500 MPa | Achouri & Boye, 2013 |
| sesame 10% (pH 7) | 100 - 500 | ↓ 100 and 200 MPa<br>↑ 300-500 MPa | ↑ 100-500 MPa | = | ↓ 100 and 200 MPa<br>↑ 300-500 MPa | Achouri & Boye, 2013 |
| soy protein 15% (pH 3-7) | 250 | = | ↓ | ↓ | ↑ | Alvarez et al., 2008 |
| soy protein 1% (pH 6.8) | 300; 500 | ↑ 300 and 500 MPa | ↓↓ 300 MPa<br>↓ 500 MPa | ↑ 300 MPa<br>↑↑ 500 MPa | ↓ 300 and 500 MPa | Li et al., 2012 |
| soy protein 1% (pH 3; 8) | 200 - 600 | ↓ 200 - 600 MPa | ↑ 200 - 600 MPa;<br>(pH 3) = 400 and 600 MPa | NA | ↑ 200 - 600 MPa | Puppo et al., 2004 |
| soy protein 1, 3 and 5% | 200 – 600 | ↓ 200 – 600 MPa | ↑ 200 – 600 MPa | ↑ 200 – 600 MPa | ↑ 200 – 600 MPa | Tang & Ma, 2009 |
| soy β-conglycinin 5% (pH 6.4 – 8.4) | 100 - 500 | ↑ 100 - 500 MPa | = 200 and 300 MPa<br>↓ 400 and 500 MPa | = 200 MPa<br>↓ 300 - 500 MPa | = 200 MPa<br>↓ 300 - 500 MPa | Wang et al., 2011 |
| soy β-conglycinin 0.1% (pH 7.4) | 300; 500 | ↓ 500 MPa | = | NA | ↑ 500 MPa | Zhang et al., 2010 |
| soy glycinin 5% (pH 6.4 – 8.4) | 100 - 500 | ↑ 100 - 500 MPa | ↑ 100 - 500 MPa | ↑ 100 - 500 MPa | ↓ 100 - 400 MPa<br>= 500 MPa | Wang et al., 2011 |

NA = Not available; ↓ - decrease; ↓↓ - accentuated decrease; = - no relevant changes; ↑ - increase; ↑↑ - accentuated increase

**Table 2.1**. Effects of pressure on the secondary structure of plant proteins (*continued*).

| Protein or source | Pressure (MPa) | α-helix | β-sheet | β-turn | random coil | Reference |
|---|---|---|---|---|---|---|
| soy glycinin<br>0.1% (pH 7.4) | 300; 500 | ↓ 500 MPa | ↓ 500 MPa | NA | ↑ 500 MPa | Zhang et al., 2003 |
| soy glycinin<br>10 and 30% (pH 7) | 600 | ↓ | ↓ | ↓ | ↑↑ | Savadkoohi et al., 2014 |
| soy glycinin<br>60% (pH 7) | 600 | ↓ | ↓ | ↑ | ↑ | Savadkoohi et al., 2014 |
| soy glycinin<br>80% (pH 7) | 600 | = | = | = | = | Savadkoohi et al., 2014 |
| soybean seeds | 100 - 500 | ↓ 100 – 500 MPa | ↓↓ 100 – 500 MPa | ↑ 100 – 500 MPa | ↑↑ 100 – 500 MPa | Yang et al., 2014 |
| sweet potato<br>1% (pH 3) | 200 - 600 | ↓ 200 MPa<br>↓↓ 400 and 600 MPa | ↑↑ 200 and 600 MPa<br>↑ 400 MPa | ↓↓ 200 MPa<br>= 400 MPa<br>↓ 600 MPa | = 200 - 600 MPa | Khan et al., 2015b |
| sweet potato<br>1% (pH 6) | 200 - 600 | ↓↓ 200 and 400 MPa<br>↑↑ 600 MPa | ↑ 200 MPa<br>↑↑ 400 MPa<br>↓↓ 600 MPa | ↓ 200 MPa<br>↓↓ 400 MPa<br>↑↑ 600 MPa | ↑↑ 200 MPa<br>↑ 400 MPa<br>↓ 600 MPa | Khan et al., 2015b |
| sweet potato<br>1% (pH 9) | 200 - 600 | ↓ 200 and 600 MPa<br>↑↑ 400 MPa | ↑↑ 200 MPa<br>↓ 400 and 600 MPa | ↓↓ 200 MPa<br>↑ 400 MPa<br>= 600 MPa | ↓ 200 MPa<br>= 400 MPa<br>↑ 600 MPa | Khan et al., 2015b |
| sweet potato<br>3% (pH 7) | 200 - 600 | = | = 200 MPa<br>↑ 400 and 600 MPa | ↑ 200 MPa<br>= 400 and 600 MPa | ↓ 200 - 600 MPa | Sun et al., 2014 |

NA = Not available; ↓ - decrease; ↓↓ - accentuated decrease; = - no relevant changes; ↑ - increase; ↑↑ - accentuated increase

The reasons for these discrepancies are not yet fully understood, since the processing conditions (protein concentration, pH, temperature, holding pressure and time) were comparable in most studies. Some explanations may be related to the soy cultivar, the method used for the extraction/purification of the proteins and/or the compression rates employed. Nonetheless, there appears to be a consensus regarding random coils, since most studies described a concomitant increase of these unordered structures with the decrease of ordered structures (Alvarez et al., 2008; Puppo et al., 2004; Tang and Ma, 2009; Yang et al., 2014). Overall, for soy proteins, pressure increases the content of random coils, whereas some ordered structures may be destroyed and others restored depending on processing conditions.

It is also relevant to mention that the effects of HPP on the protein's secondary structure are as well dependent on the pH. In general, more pronounced changes in the protein's structure occur at alkaline and neutral pH than at acidic pH (Achouri and Boye, 2013; Puppo et al., 2004). Interesting studies on this subject were carried out by Khan et al. (2015a) who investigated the HPP effects (200 to 600 MPa for 15 minutes at 20 °C) on the secondary structure of sweet potato protein at pH 3, 6 and 9. Generally, at pH 3, the content of α-helix and β-turn decreased while the content of β-sheet increased with rising pressure. A similar pattern was observed at pH 6 below 600 MPa, except for the increase of random coil. At 600 MPa both α-helix and β-turn structures increased, contrarily to what was verified at pH 3. More pronounced differences were observed at pH 9, with a significant increase of α-helix and β-turn and a decrease of β-sheet already observed at lower pressures (at 400 MPa and above) (Khan et al., 2015a).

Unfortunately, none of these studies established a well-founded interpretation to explain the influence of pH, and therefore of the number and distribution of charges on the proteins upon the observed effect of HPP on the protein structure. Further studies in this area could make an important contribution to the monitoring of environmental conditions, ionisation of protein and manipulation of the structural changes resulting from HPP.

### 2.2.3. SURFACE HYDROPHOBICITY

Another important factor that affects the technological functions of proteins is their surface hydrophobicity ($H_0$), which is strongly related to the structural particularities of each protein (Zhang et al., 2003). Increases in the $H_0$ are related to the exposition of the side chain of

aromatic amino acids, *i.e.* the higher the $H_0$ the higher the amount of hydrophobic groups exposed to the outside of the protein (He et al., 2014).

As expected different effects of HPP have been reported, depending on treatment conditions (pressure, time, and temperature), macromolecular characteristics of the protein, ionic conditions and composition of the medium, but in general HPP lead to an increase of protein surface hydrophobicity due to the resulting conformational changes.

Worth to mention the initial studies regarding the effect of pressure carried out by Ledward and co-workers (Galazka et al., 1999b, 2000b). Relatively low pressure, between 150 and 200 MPa, led to an increase of the $H_0$ of the 11S globulin of *Vicia faba* and no major changes were reported at lower or higher pressures. The $H_0$ of both glycinin and β-conglycinin also increased for relatively low pressures (> 200/300 MPa) (Wang et al., 2011; Zhang et al., 2003), apparently with β-conglycinin being slightly more sensitive to pressure regarding the changes in surface hydrophobicity (Zhang et al., 2010). However, in many cases, the increase in $H_0$ can be observed only at higher pressure levels, as illustrated by the studies on soymilk proteins (Kajiyama et al., 1995), peanut's arachin (Zhao et al., 2015), ginkgo seeds protein's isolates (Zhou et al. 2016a), kidney bean proteins (Yin et al., 2008) and walnut isolates (Qin et al., 2013), though in the later the $H_0$ decreased at 600 MPa (being still higher than control samples).

Based on available studies, we can assume that in most cases there will be a certain pressure value above which treatment by HPP will have no significant effects or may even lead to reduced hydrophobicity, an effect that is most likely due to increasing aggregation associated to conformational changes (dissociation of protein subunits, changes in the tertiary and secondary structures, increasing exposure of hydrophobic groups, etc.) promoted by higher pressures. To document this important feature regarding the existence of an intermediate pressure for which the effect on $H_0$ is maximum one can refer, among many others, the works of Li et al. (2012) with soy proteins, who reported an increase of the $H_0$ particularly at 300 MPa, and later that of Yang et al. (2014) who described an increase of $H_0$ up to 200 MPa, but a decrease when higher pressure values were used. Also, pressure up to 400 MPa increased $H_0$ of rapeseed proteins, not being further increased with higher pressures (He et al., 2013), whereas for peanut proteins a pressure up to 100 MPa increased $H_0$ and higher pressure values led to lower $H_0$ (though even so higher than untreated samples) (He et al., 2014).

HPP holding time also impacts on $H_0$. An increase in the holding time up to 15 min led to an increase of $H_0$ for soy (Li et al., 2012) and rapeseed proteins (Wang et al., 2015), although higher times may lead to a further decrease of this parameter.

Considering the combined effect of pH and pressure, Puppo et al. (2004) reported an increase of $H_0$ of soy proteins at pH 3, particularly at high pressures. However, at pH 8 there was a slight decrease at 200 MPa followed by an increase at 400 MPa. The $H_0$ of soluble proteins of soymilk increased with pressure, both at pH 6 and 7 (Lakshmanan et al., 2006). In the particular case of the $H_0$ of glycinin and β-conglycinin, it increased with pressure both at pH 6.5 and 7.5 (more markedly at pH 6.5) (Molina et al., 2001). Khan et al. (2015a and b) verified that sweet potato proteins treated at pH 3 presented an increase of the $H_0$, particularly at 400 MPa. However, the contrary was observed at higher pH, where a decrease of $H_0$ (regardless the applied pressure) at pH 6 and a decrease with increasing pressure at pH 9 was verified, what was not in agreement with the previous studies of Puppo et al. (2004). This may indicate that at higher pressures and pH values, the interactions between proteins, or proteins and solvent, are promoted thus reducing the $H_0$ (Khan et al., 2015a).

The effect of HPP on $H_0$ is also affected by the protein concentration. At 2% protein, the $H_0$ of sweet potato proteins increased with pressure, particularly at 400 MPa, whereas at higher concentrations (*i.e.* 4 and 6%) $H_0$ increased only for 400 MPa, not being significantly affected at 600 MPa and even showing lower values at 200 MPa (Khan et al., 2013). For soy proteins, low concentrations (*i.e.* 1 and 3%) led to an increase of $H_0$ with increasing pressure up to 600 MPa, whereas at 5% protein the increase was verified only for 400 MPa (Wang et al., 2008). Once again protein concentration is a determining factor in the elucidation of the pressure effects on $H_0$, due to its influence on the aggregation of proteins. At a lower concentration (*e.g.* <3%) there are fewer protein-protein interactions and the amount of exposed hydrophobic groups depends on the pressure. However, at higher concentrations (*e.g.* >5%) the proteins form aggregates more easily reducing the exposure of hydrophobic groups (Wang et al., 2008).

#### 2.2.4. Sulfhydryl groups

Sulfhydryl (SH) groups and disulphide bonds (S-S) play important roles in the conformation, stability and technological properties of proteins. They are weak secondary covalent bonds and help maintain proteins' tertiary structure, therefore their changes are essential to manipulate the said properties (Bulaj, 2005). Thiol groups in proteins occur as free

sulfhydryl groups (SH) in the cysteine residues or as disulphide bonds (S-S) in oxidised cysteines. As discussed above HPP can cause important changes at different levels of proteins' structural organisation, and therefore it is not surprising that HPP can lead to changes in the amount of free and/or available (reactive) SH groups (Table 2.2.).

The expected effects are again related to conformational changes that occur in the protein as a result of the increasing pressure. Thus, up to a pressure value which favours the unfolding of the protein, there is often an increase in the amount of available SH groups, whereas for higher values of pressure one can expect an absence of effect or even a decline in the free sulfhydryl groups due to the increased propensity for protein aggregation. As discussed before, pressure up to 300 MPa may lead to the dissociation/unfolding of the proteins, and the rupture of S-S bonding may be implicated in this mechanism, therefore exposing SH residues. Still, higher pressure values or longer holding times may promote hydrophobic interactions that can lead to S-S exchange, or the formation of new S-S bonds by oxygen-catalysed oxidation of the free SH groups and subsequent aggregation/re-association of unfolded proteins (Kajiyama et al., 1995; Yin et al., 2008; Li et al., 2012).

It is worth emphasize that the influence of pressure on the content of SH is strongly dependent on the type and concentration of the protein, source cultivars, pH, and HPP conditions, which may affect differently either the unfolding or the protein aggregation processes, which in turn influence the content of SH groups (Li et al., 2012). In what concerns the effect of protein concentration, one may conclude that it does not have a great impact on the SH content, at least for concentrations below 10%. Still, as already mentioned, higher concentrations of protein may have a protective effect against denaturation, consequently resulting in fewer effects in what regards the SH content.

Concerning the influence of pH, and taking soy proteins as an example, while at pH 8 HPP led to a decrease in the SH content with increasing pressure, at pH 3 the content of free SH increased at 200 MPa but decreased at higher pressures (*i.e.* 400 and 600 MPa) (Puppo et al., 2004).

**Table 2.2.** Effects of HPP on the content of available sulfhydryl groups for plant proteins

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Sulfhydryl group content | Reference |
|---|---|---|---|---|---|
| amaranth | 5 | NA[a] | 200; 400; 600 MPa<br>5 min / NA | increased with pressure; however, decreased with increasing pressure | Condés et al., 2015 |
| amaranth | 1; 5; 10 | NA | 200; 400; 600 MPa<br>5 min / RT[b] | at 1% protein increased with increasing pressure; at 5% increased, particularly at 200 MPa; at 10% had no effects | Condés et al., 2012 |
| ginko seeds | 5 | 8 | 100 to 700 MPa<br>20 min / 20 ºC | increased with increasing pressure | Zhou et al., 2016a |
| kidney bean | 3 | 7 | 200; 400; 600 MPa<br>20 min / 25 ºC | increased with increasing pressure (S-S increased with increasing pressure) | Yin et al., 2008 |
| peanut | 5 | NA | 50; 80; 100; 150; 200 MPa<br>5 min / 25 ºC | increased with increasing pressure (S-S increased with increasing pressure) | He et al., 2014 |
| peanut arachin | 1 | 7.5 | 200; 300; 400; 500; 600 MPa<br>20 min / 25 ºC | decreased with increasing pressure up to 500 MPa | Zhao et al., 2015 |
| rapeseed | 1 | 7 | 200; 400; 600 MPa<br>15 min / 25 ºC | increased at 200 MPa; decreased at 400 and 600 MPa | He et al., 2013 |
| soy | 1 | 6.8 | 200; 300; 400; 500 MPa<br>5; 10; 15; 20 min / 15 ºC | increased with increasing pressure up to 300 and decreased afterwards; increased with increasing time up to 15 min and decreased afterwards | Li et al., 2012 |
| soy | 1 | 6.8 | 350 MPa / 16 min / 20 ºC | increased | Li et al., 2016 |
| soy | 1 | 3; 8 | 200; 400; 600 MPa<br>10 min / 20 ºC | at pH 3 increased up to 200 MPa and decreased afterwards; at pH 8 decreased with increasing pressure | Puppo et al., 2004 |
| soy | 0.32-3.68 | 2.66-6.84 | 198; 300; 450; 600; 702 MPa<br>20 min / RT | decreased | Torrezan et al., 2007 |
| soy | 1 | 7 | 200; 400; 600 MPa<br>15 min / 25 ºC | at pH 1 and 3 increased with pressure, but decreased with increasing pressure; at pH 5 decreased with increasing pressure above 400 MPa | Wang et al., 2008 |

[a]NA = Not available; [b]RT = Room temperature

**Table 2.2.** Effects of HPP on the content of available sulfhydryl groups for plant proteins (*continued*)

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Sulfhydryl group content | Reference |
|---|---|---|---|---|---|
| soy milk | 5 | 6.54 | 300; 500 MPa<br>10; 30 min / 23 ºC | decreased | Kajiyama et al., 1995 |
| soy glycinin | NA[a] | NA | 100; 200; 300; 400; 500 MPa<br>10; 20; 30 min / RT [b] | increased with increasing pressure (up to 400 MPa) and time | Zhang et al., 2003 |
| soy β-conglycinin | NA | NA | 100; 200; 300; 400; 500 MPa<br>10; 20; 30 min / RT | decreased with 100 MPa; increased with increasing pressure and time | Zhang et al., 2010 |
| walnut | 1 | 7 | 300; 400; 500; 600 MPa<br>20 min / RT | increased with pressure above 400 MPa (particularly at 400 MPa) | Qin et al., 2013 |

[a]NA = Not available; [b]RT = Room temperature

## 2.3. EFFECTS OF HPP ON PROTEINS' TECHNO-FUNCTIONAL PROPERTIES

### 2.3.1. SOLUBILITY

The solubility of proteins is a very important parameter that conditions most of the proteins' techno-functional properties. In fact, individualization of protein molecules and their interaction with the solvent, usually aqueous, are decisive steps for their stabilising, thickening and gelling capabilities. This parameter may be influenced by several external factors such as pH, ionic strength, and additives (Baier and Knorr, 2015).

From the studies performed so far, one may infer that HPP usually leads to a loss of protein solubility, particularly at higher pressures. This will be disadvantageous for most intended applications, which may bring additional problems of loss of functionality for these proteins, often already compromised during the isolation and drying procedures. For instance, pressure up to 200-300 MPa does not usually affect the solubility of most proteins, as demonstrated for pinto bean (Garcia-Mora et al., 2016) or lentil proteins (Garcia-Mora et al., 2015). However, higher pressures (*i.e.* > 400 MPa) have been reported to decrease the solubility of proteins from several sources (Chapleau and de Lamballerie-Anton, 2003; Condés et al., 2015; Garcia-Mora et al., 2015; Qin et al., 2013; Zhao et al., 2015). The reduction of solubility is mainly associated with the formation of insoluble macro-aggregates (Condés et al., 2015).

Table 2.3. presents an overview of how the pressure affects the solubility of plant proteins, illustrating the general expected trend but also some relevant exceptions. Nonetheless, there are particular cases in which pressure increased the protein's solubility. For instance, Achouri & Boye (2013) reported that pressure between 100 and 500 MPa, mainly at lower pH, increased the solubility of sesame proteins, as well as Yin et al. (2008) who reported an increase in solubility of kidney bean proteins for higher pressure values (400-600 MPa). In both cases the observed trend was attributed to an increase in protein-solvent interactions promoted by the dissociation of aggregates and proteins' unfolding.

In the case of soy proteins, pressure seems to have small effects on the protein's solubility (Añón et al., 2012), or in some cases even increasing it, contrarily to what happens to most proteins. Pressure up to 400 MPa seems to increase the solubility of soy proteins, particularly at 200-300 MPa, possibly due to the partial unfold of the proteins that led to changes

of the proteins' surface charge, enhancing the protein-water interactions (Yang et al., 2014). Li et al. (2011) also found a greater solubility of soy proteins treated at 200-300 MPa and increasing time, not being affected by further increasing pressure, except at pH 6.8 where the solubility dropped to values close to the untreated samples. Once again, the observed effect at lower pressure was attributed to enhanced interactions between the protein and solvent, thus increasing solubility, whereas higher pressures may expose hydrophobic residues increasing intermolecular interactions and the formation of aggregates.

Contradictory results are found regarding the pressure effect on β-conglycinin solubility. Some studies suggested that pressures up to 600 MPa do not significantly affect (in some cases even decrease) the solubility (Añón et al., 2012; Molina et al., 2001), whereas Speroni et al. (2009) stated that the protein solubility increased with increasing pressure up to 600 MPa. The apparent discrepancy can be attributed to the differences in protein concentration, pH and processing conditions.

Regarding glycinin at pH 6.5 (Molina et al., 2001) or 8 (Añón et al., 2012) no effects were noticeable, however at pH 7.5 a complex behaviour was described. At a lower pressure (*i.e.* 200 MPa) protein partial unfolding led to an increase in the interactions between the protein and the solvent increasing the solubility, whereas at 400 MPa a more pronounced unfolding occurred, exposing more hydrophobic residues, thus promoting aggregation and subsequent loss in solubility. Higher pressures (*i.e.* 600 MPa) may have caused further dissociation of subunits promoting more interactions between proteins and solvent, therefore increasing the protein's solubility (Molina et al., 2001).

The differences in the effect of HPP on different proteins are most likely related to differences in the type and nature of the proteins, as well as their conformational stability (Garcia-Mora et al., 2015).

**Table 2.3.** Effects of HPP on the solubility of plant proteins

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Solubility | Reference |
|---|---|---|---|---|---|
| almond milk | NA | NA | 150; 300; 450; 600 MPa / 5 min / 30 ºC | 150 MPa had no effects; decreased with increasing pressure stabilised ≥400 MPa) | Dhakal et al. 2014 |
| amaranth | 5 | 5 | 200; 400; 600 MPa / 5 min / NA [a] | 200 MPa had no effects; 400 and 600 MPa decreased | Condés et al. 2015 |
| cowpea | 10 | 8; 10 | 200; 400; 600 MPa / 5 min / 20 ºC | 200 and 400 MPa decreased; 600 MPa had no effects | Peyrano et al. 2016 |
| kidney bean | 3 | 7 | 200; 400; 600 MPa / 20 min /25 ºC | 200 MPa had no effects; 400 and 600 MPa increased | Yin et al. 2008 |
| lentil | 2 | 8 | 100; 200; 300; 400; 500 MPa / 15 min / 40 ºC | 100-300 MPa had no effects; 400 and 500 MPa decreased | Garcia-Mora et al. 2015 |
| lupin | 1.5 | 7 | 200; 400; 600 MPa / 10 min / 10 ºC | 400 and 600 MPa decreased | Chapleau et al. 2003 |
| peanut arachin | 1 | 7.5 | 200; 300; 400; 500; 600 MPa / 20 min / 25 ºC | decreased with increasing pressure | Zhao et al. 2015 |
| pinto bean | 3.2 | 8 | 100; 200; 300 MPa/ 15 min / 50 ºC | had no effects | Garcia-Mora et al. 2016 |
| potato | 1 | 7 | 200; 400; 600 MPa / 10 min / 20; 40 ºC | at 20 ºC had no effects; at 40 ºC 600 MPa decreased | Baier & Knorr 2015 |
| potato patatin | 1 | 7 | 200; 400; 600 MPa / 10 min / 20; 40 ºC | at pH 7 had no effects; at pH 6 and 20 ºC 400 MPa decreased; at pH 6 and 40 ºC decreased with increasing pressure | Baier & Knorr 2015 |
| sesame | 1 | 2; 5 | 100; 200; 300; 400; 500 MPa / 10 min / RT [b] | at pH 2 and 5, decreased for 100 MPa but increased for 200-500 MPa; at pH 7, increased for 100-500 MPa; at pH 10, decreased for 100 MPa but was not affected for 200-500 MPa | Achouri & Boye 2013 |

[a] NA = Not available; [b] RT = Room temperature;

**Table 2.3.** Effects of HPP on the solubility of plant proteins (*continued*)

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Solubility | Reference |
|---|---|---|---|---|---|
| soy | 0.32 - 3.68 | 2.66 - 6.84 | 198; 300; 450; 600; 702 MPa<br>20 min / RT [b] | had no effects | Torrezan et al., 2007 |
| soy | 1 | 8 | 200; 400; 600 MPa<br>10 min / NA [a] | had no effects | Añón et al., 2012 |
| soy | 1 | 3; 6.8 | 200; 300; 400; 500 MPa<br>5; 10; 15; and 20 min / 15 ºC | at pH 3 increased with pressure up to 300 MPa and decreased at higher pressures; increased with increasing holding time until 15min; at pH 6.8 increased up to 300 MPa, stabilised at higher pressures, and increased with increasing holding time | Li et al., 2011 |
| soy | 1 | 5.9 - 8 | 600 MPa<br>5 min / 20 ºC | at pH 5.9 had no effects; at pH ≥6.4 increased | Manassero et al., 2015 |
| soy | 1 | 3 | 200; 400; 600 MPa<br>10 min / 20 ºC | at pH 3, increased for all pressures; at pH 8, increased slightly for 200 and 400 MPa; 600 MPa had no effects | Puppo et al., 2004 |
| soy | 1; 3; 5 | 7 | 200; 400; 600 MPa<br>15 min / 25 ºC | at 1 and 3% had no effects; at 5% decreased for 400 MPa | Wang et al., 2008 |
| soy | 10 | 8 | 300; 600 MPa<br>10 min / 20 ºC | increased with increasing pressure | Speroni et al., 2009 |
| soy | 10 | 6.5; 7.5 | 200; 400; 600 MPa<br>15 min / 20 ºC | at pH 6.5 decreased above 400 MPa; at pH 7.5 had no effects | Molina et al., 2001 |
| soy | NA | NA | 100; 200; 300; 400; 500 MPa<br>20 min / 25 ºC | From 100 to 400 MPa increased, particularly at 200 and 300 MPa; 500 MPa had no effects | Yang et al., 2014 |

[a] NA = Not available; [b] RT = Room temperature;

**Table 2.3.** Effects of HPP on the solubility of plant proteins (*continued*)

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Solubility | Reference |
|---|---|---|---|---|---|
| soy β-conglycinin | 10 | 6.5; 7.5 | 200; 400; 600 MPa 15 min / 20 ºC | had no effects | Molina et al., 2001 |
| soy β-conglycinin[c] | 1 | 8 | 200; 400; 600 MPa 10 min / NA[a] | had no effects | Añón et al., 2012 |
| soy β-conglycinin[c] | 10 | 8 | 300; 600 MPa 10 min / 20 ºC | increased with increasing pressure | Speroni et al., 2009 |
| soy glycinin | 10 | 6.5; 7.5 | 200; 400; 600 MPa 15 min / 20 ºC | at pH 6.5 had no effects but at pH 7.5 increased at 200 and 600 MPa | Molina et al., 2001 |
| soy glycinin[c] | 1 | 8 | 200; 400; 600 MPa 10 min / NA | had no effects | Añón et al., 2012 |
| soy glycinin[c] | 10 | 8 | 300; 600 MPa 10 min / 20 ºC | increased with increasing pressure | Speroni et al., 2009 |
| sweet potato | 2; 4; 6 | NA | 200; 400; 600 MPa 15 min / 25 ºC | decreased more markedly at 400 MPa | Khan et al., 2013 |
| walnut | 1 | 7 | 300; 400; 500; 600 MPa 20 min / RT[b] | decreased with increasing pressure up to 500 MPa (500 MPa = 600 MPa | Qin et al., 2013 |

[a] NA = Not available; [b] RT = Room temperature; [c] Enriched fraction.

### 2.3.2. Water holding capacity

Another important parameter in proteins' functionality is the water holding capacity (WHC) that together with solubility belong to the protein technological properties related to hydration (Li et al., 2011), and plays an important role in the texture, including tenderness and juiciness, of many food products.

Most studies performed so far on this topic have shown that HPP usually leads to an increase in WHC, what may positively impact the textural attributes of many protein-rich food commodities. For example, HPP in the range 300-700 MPa originated soy protein gels with improved WHC (especially for β–conglycinin) (Molina et al., 2002), and HPP from 50 to 200 MPa (5 min) gradually increased the WHC of peanut isolate proteins (He et al., 2014).

WHC is directly related to the protein conformation and its capacity to interact with the solvent (water). Therefore, as observed for other protein properties, HPP conditions need to be carefully controlled and selected in order to achieve the desirable effects. As expected, WHC depends on HPP levels (Speroni and Añón, 2013). In fact, WHC can be improved for lower pressures and time levels, but this property often declines at higher levels, as demonstrated by Li et al. (2011) for soy proteins.

The pH value at which the proteins are extracted also influenced the WHC of cowpea protein isolates. For samples extracted at pH 10, increasing pressure increased the WHC suggesting that the changes induced in the proteins originated a more polar environment. For those protein samples extracted at pH 8, HPP at low pressure (*i.e.* 200 MPa) improved WHC, 400 MPa did not have a significant impact on WHC and for higher pressure (*i.e.* 600 MPa) this parameter decreased (Peyrano et al., 2016). The authors suggested that the increase of WHC with pressure was due to unfolding-induced exposure of polar amino acids, and thus to a more polar environment for the protein facilitating the interactions with the solvent, whereas the decline observed for 600 MPa was related to a more extensive denaturation and higher surface hydrophobicity.

### 2.3.3. Aggregation

Protein aggregation is a complex issue of technological relevance. Some important functional properties of proteins are strongly dependent on "controlled" aggregation processes that occur, for example, at interfaces or in the bulk during protein gelation. Considering the

structural and conformational changes that HPP causes on proteins, as discussed above, it is not surprising that these changes result in the disruption of certain intermolecular interactions and/or willingness of protein molecules for the establishment of new intermolecular interactions, thus influencing protein aggregation. Naturally, how HPP influences protein aggregation will depend on protein structure, besides depending, of course, on the actual HPP conditions.

Several studies, mainly using size exclusion chromatography or light-scattering methods, have provided information regarding the complex influence of HPP on protein aggregation. For soy proteins, probably the most studied proteins in this area, pressures up to 200 MPa seem to have little effect on protein aggregation. On the contrary, pressures above 400 MPa have shown to promote the dissociation of existing aggregates, though inducing the formation of new ones. In fractions constituted mainly of glycinin, a lower pressure (*i.e.* 200 MPa) promoted the dissociation of aggregates, while 400 and 600 MPa promoted both dissociation and aggregation, more evidently at 600 MPa. On the other hand, for fractions with a high content of β-conglycinin, the rise of pressure only induced aggregation. Generally, the higher the amount of glycinin the higher the propensity to the formation of high molecular weight aggregates induced by HPP, maybe due to the moieties of β-conglycinin that possibly restrain the formation of aggregates (Añón et al., 2011, 2012; Puppo et al., 2004; Tang and Ma, 2009). Results obtained by Kajiyama et al. (1995) indicated that some soy proteins dissociated into their subunits when subjected HPP, however, some of them re-associated and formed larger aggregates. Aggregates formed at 300 MPa via S-S bonds were also observed by Li et al. (2011). Yang et al. (2014) suggested that lower pressure treatments, up to 200 MPa, dissociate soy proteins into smaller unities, whereas higher pressures (*i.e.* 300-500 MPa) induce proteins' aggregation. Increasing pressures led to glycinin aggregation, whereas β-conglycinin aggregation occurred only above 600 MPa, with the possible formation of S-S bonds (Molina et al., 2002).

A similar behaviour was reported for lupin proteins where aggregation was also verified above 400 MPa, mainly for the α-conglutin (11S) fraction. The effect was accentuated with the increase of pressure and also impacted on the β-conglutin (7S) fraction at 600 MPa (Chapleau and de Lamballerie-Anton, 2003).

Also, although pressures up to 200 MPa did not induce the aggregation of pinto bean proteins, 300 MPa promoted the aggregation of the higher molecular weight proteins (*i.e.* linoleate 9S lipoxygenase 1, legumin and the subunits of phaseolin) (Garcia-Mora et al., 2016). Likewise, proteins from almond milk (Dhakal et al., 2014), amaranth (Condés et al., 2012) and

cowpea (Peyrano et al., 2016) aggregated with increasing pressure, particularly above 400 MPa. Moreover, HPP promoted the aggregation of 11S globulin of *Vicia faba* (Galazka et al., 2000b), kidney bean proteins (Liu et al., 2013; Yin et al., 2008), rapeseed protein isolates (He et al., 2013) and sweet potato protein (Khan et al., 2014).

Overall, HPP may induce changes in non-covalent interactions that could change protein's secondary, tertiary and quaternary structures, and be involved in the formation of new bonds leading to protein aggregation. Possibly some dissociation also occurs but generally, it is largely exceeded by new aggregation processes (Chapleau and de Lamballerie-Anton, 2003; Galazka et al., 2000b).

### 2.3.4. EMULSIFYING AND FOAMING PROPERTIES

Proteins are generally used as stabilisers for food emulsions and foams due to their amphiphilic nature, surfactant activity and capability to adsorb to interfaces. Different proteins are more or less efficient at forming and stabilising emulsions and foams, and even the same protein may exhibit a different interfacial behaviour depending on the properties and composition of the medium. In general, however, one may expect that proteins will help both the formation and stabilisation of disperse systems, in the first case mainly by decreasing the interfacial tension between the two phases, in the second case mainly by forming a viscoelastic film at the interface that will act as a physical protective barrier.

Some of the structural and functional properties of proteins discussed above are directly related to the emulsifying (or foaming) capacity and stability of emulsions (or foams) where proteins are present. As mentioned before HPP can change the structure of proteins at different levels, their hydrodynamic volume and surface hydrophobicity/hydrophilicity, and consequently their solubility, propensity to adsorb on interfaces and availability to interact with themselves or with other constituents of the medium. Surface hydrophobicity, solubility and the resulting capability to decrease the interfacial tension will be crucial for the formation of the emulsion, namely to the amount of a certain phase that may be dispersed within another and for the average size of the droplets obtained for a particular dispersion process and composition. Molecular flexibility, capacity to partial denature, adsorb and interact on the interface are decisive for emulsion and foam stability.

Therefore, HPP appears as a good strategy to also manipulate the surfactant properties of the proteins and their ability to facilitate the formation of dispersed food systems such as

emulsions and foams, and to stabilise them. This approach is in fact well documented in the literature, especially for model oil-in-water emulsions (Table 2.4.).

It is advantageous that a particular emulsifying agent originates dispersed droplets with the smallest possible diameter to thereby decrease the emulsion destabilization processes such as coalescence and creaming. Different results have been reported for the HPP effects on emulsion droplet diameters depending on the protein, solution conditions, and applied pressure (magnitude and duration). The application of HPP (up to 250 MPa for 20 min) on 11S globulin of *Vicia faba* (Galazka et al., 1999b, 2000b) resulted in larger average droplet diameters as the pressure increased, which was prejudicial to the emulsion stability despite the lower surface tension induced by the treated proteins. This may have been the result of extensive protein aggregation induced by S-S bonds. However, a beneficial decrease in average droplet diameter was reported for other proteins and processing conditions, for example for soy protein (pH 8) treated by HPP (200-600 MPa, 10 min) (Puppo et al., 2005), thus improving the emulsifying properties of the treated proteins. Others have reported no significant changes in the droplet diameter as a result of HPP, for example, for both glycinin and β-conglycinin (Puppo et al., 2011). Reduction of the droplet size by increasing pressures has been associated to a decrease of flocculation and creaming processes and hence to an increase in emulsion stability (Chapleau and de Lamballerie-Anton, 2003). Increased emulsifying capacity of the proteins by HPP has been related to the resultant partial unfolding and exposure of hydrophobic groups, consequently increasing the interfacial activity of the proteins (Lakshmanan et al., 2006; Li et al., 2011). It is pertinent to recall that the interfacial activity of proteins is strongly related with their capability to change conformation and to adsorb to the interface. Therefore, it is perfectly acceptable that the alterations in protein conformation, namely at the level of its tertiary and quaternary structures, caused by HPP, will facilitate the adsorption process and the expected 'surface denaturation'.

**Table 2.4.** Effects of HPP on the emulsifying activity and stability index of plant proteins

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Emulsifying activity index | Emulsifying stability index | Reference |
|---|---|---|---|---|---|---|
| ginko seeds | 0.25; 1.25 | 8 | 100 – 700 MPa 20 min / 20 ºC | increased with increasing pressure and decreased with increasing protein concentration | NA [a] | Zhou et al., 2016a |
| kidney bean | 3 | 7 | 200; 400; 600 MPa 20 min / 25 ºC | increased with 200 and 400 MPa, decreased with 600 MPa | increased with increasing pressure up to 400 MPa, 600 MPa had no significant effects | Yin et al., 2008 |
| peanut arachin | 1 | 7.5 | 200; 300; 400; 500; 600 MPa 20 min / 25 ºC | increased with pressure above 400 MPa, particularly at 400 MPa | increased slightly with 200 and 300 MPa and decreased with increasing pressure above 400 MPa | Zhao et al., 2015 |
| soy protein | 1 | 3; 6.8 | 200; 300; 400; 500 MPa 5; 10; 15; and 20 min / 15 ºC | increased up to 300 MPa, decreasing afterwards; increased with increasing holding time up to 15 min | decreased with increasing pressure and holding time | Li et al., 2011 |
| soy protein | 0.50; 0.75 | 6.5; 7.5 | 200; 400; 600 MPa 15 min / 20 ºC | increased slightly with increasing pressure up to 400 MPa, decreasing afterwards | decreased with increasing pressure | Molina et al., 2001 |
| soy protein | 0.3 - 3.7 | 2.7 - 6.8 | 198; 300; 450; 600; 702 MPa 20 min / RT [b] | higher values between pH 4.3 and 5.2, for lower pressures near 4.3 and higher pressures near 5.2 | NA | Torrezan et al., 2007 |
| soy protein | 1; 3; 5 | 7 | 200; 400; 600 MPa 15 min / 25 ºC | Increased, but no significant effects of the pressure value | at 1% decreased with 200 and 600 MPa; at 3 and 5% decreased with increasing pressure | Wang et al., 2008 |
| soy β-conglycinin | 0.50; 0.75 | 6.5; 7.5 | 200; 400; 600 MPa 15 min / 20 ºC | increased at 200 and 600 MPa and decreased at 400 MPa | decreased with increasing pressure | Molina et al., 2001 |
| soy glycinin | 0.50; 0.75 | 6.5; 7.5 | 200; 400; 600 MPa 15 min / 20 ºC | increased slightly with increasing pressure up to 400 MPa, decreasing afterwards | decreased with increasing pressure at pH 6.5 and at 0.50%; increased with 200 and 400 MPa at pH 7.5 and 0.75% | Molina et al., 2001 |

[a] NA = Not available; [b] RT = Room temperature;

**Table 2.4.** Effects of HPP on the emulsifying activity and stability index of plant proteins (*continued*)

| Protein or source | Conc. (%) | pH | HPP Conditions (Pressure /time /temperature) | Emulsifying activity index | Emulsifying stability index | Reference |
|---|---|---|---|---|---|---|
| sweet potato | 1 | 7 | 200; 400; 600 MPa 20 min / 25 ºC | increased with increasing pressure | had no significant effects | Khan et al., 2015 |
| sweet potato | 1 | 3; 7; 8 | 200; 400; 600 MPa 15 min / 25 ºC | Increased at pH 3, butdecreased with increasing pressure; at pH 7 increased with increasing pressure; at pH 8 increased, particularly at 200 MPa | at pH 3 increased with increasing pressure; at pH 7 increased, particularly at 200 MPa; at pH 8 had no significant effects | Khan et al., 2014 |
| sweet potato | 1 | 3; 6; 9 | 200; 400; 600 MPa 15 min / 25 ºC | at pH 3 had no significant effects; at pH 6 increased with increasing pressure; at pH 9 decreased with increasing pressure | at pH 3 decreased with increasing pressure; at pH 6 increased with increasing pressure; at pH 9 increased at 400 and 600 MPa | Khan et al., 2015b |
| sweet potato | 2; 4; 6 | NA [a] | 200; 400; 600 MPa 15 min / 25 ºC | at 2% and 6% had no significant effects; at 4% decreased at 600 MPa | at 2% and 6% decreased with increasing pressure; at 4% increased with increasing pressure above 400 MPa | Khan et al., 2013 |

[a] NA = Not available; [b] RT = Room temperature;

Type and extent of the structural changes caused by HPP on proteins and how these changes are reflected in the protein's ability to adsorb on the interface are important aspects to be considered. Kinetic aspects are also relevant; if HPP promotes interactions between proteins and aggregation that occur extensively and quickly prior to occur interfacial adsorption, the result will be negative for the emulsifying or foaming capacity of the protein; if on the contrary, HPP promotes increased interactions between the adsorbed proteins, at the interface level, for example by increasing hydrophobic interactions, it can lead to an adsorbed protein film with improved viscoelastic properties, and thus to an increase of the emulsion/foam stability.

Table 2.4. compiles the effects of HPP on two of the most used parameters to evaluate the ability of a protein to facilitate the formation and stabilise an emulsion: the emulsifying activity index (EAI) and the emulsion stability index (ESI). Typically, treatments at lower to intermediate pressures (*e.g.* 200 – 400 MPa) seem to increase EAI values, as demonstrated by several studies (Li et al., 2011; Yin et al., 2008; Zhou et al., 2016a). However, higher pressures or holding times do not appear to have additional effects (Mirmoghtadaie et al., 2016) and in some situations may even decrease the EAI and/or ESI (Wang et al., 2008).

Overall HPP can improve the emulsifying activity of proteins but, in most cases, not the emulsion stability (Molina et al., 2001), most likely due to the exposition of hydrophobic groups and/or changes in the molecular flexibility that may lead to increased interactions between the adsorbed proteins, as mentioned previously, thus resulting in altered emulsifying properties (Li et al., 2011; Zhou et al., 2016a).

As expected, changes in the net charge and degree of ionisation of the proteins will influence the structural changes they undergo under HPP and consequently, also how the HPP will affect their interfacial behaviour. In fact, results obtained from several studies support this expectation. Puppo et al. (2005) have shown that soy proteins treated by HPP at pH 8 originated emulsions with lower droplet sizes than the untreated proteins, but were also characterised by an increased depletion flocculation, contrarily to what was observed for the proteins treated at pH 3. In contrast, studies conducted with sweet potato protein revealed a reduction of the droplet diameter at high pressures combined with acidic conditions, whereas no considerable impact was verified under alkaline conditions (Khan et al., 2015a).

In the low pH range (*c.a.* 4) the EAI values for emulsions prepared with HPP-treated soy proteins were reported to be higher at low pressure while in the near-neutral pH range, the maximum EAI was at the middle range of the pressure treatment (Torrezan et al., 2007). For

sweet potato proteins, Khan et al. (2015b) reported significant increases in EAI at pH 3 (200 and 600 MPa) and pH 6 (400 and 600 MPa), but a significant decrease at pH 9 and 600 MPa and no noteworthy effects for lower pressures. The authors suggested that protein's aggregation could have impaired the EAI at pH 9 due to the loss of protein at the oil–water interface, whereas at lower pH the unfolding of the protein and the exposure of hydrophobic groups increased their emulsifying capacity. The combination of the pH value and the applied pressure also presented an impact on the emulsion stability, since, at pH 6 and 9, pressures of 400 and 600 MPa were beneficial to the ESI whereas at pH 3 and 600 MPa was disadvantageous. As discussed above and also supported by the authors' conclusions, denaturation of the proteins may have exposed hydrophobic groups that decreased the surface tension thus improving the ESI, however, if aggregation occurs it may decrease molecular flexibility leading to a lower ESI (Khan et al., 2015a).

Partial unfolding of the proteins and a controlled increase of their surface hydrophobicity can also improve their adsorption at gas-liquid interfaces, intermolecular interactions and their capacity to decrease interfacial tensions. Therefore, provided that treatment conditions are properly selected, HPP can also increase the foaming properties of proteins. The application of 200 MPa on 11S globulins of *Vicia faba* reduced the surface tension of their solutions, resulting in improved foaming capacity (Galazka et al., 2000b). The foaming capacity of HPP-treated soy proteins increased with pressure and holding time, particularly at 300 MPa and 15 min, however, the foaming stability decreased with increasing pressure or holding time (Li et al., 2011). The authors suggested that these results may be related to an improved solubility in the aqueous phase at the specified conditions that led to a higher adsorption of the proteins to the air-water interface. Conversely, higher pressure/holding times led to a higher unfolding degree and subsequent aggregation or gelation that reduced the proteins flexibility thus reducing the foaming capacity. Baier & Knorr (2015) studied the impact of HPP on the foaming properties of potato protein isolate and verified that the foaming stability increased up to 117% with HPP, although it was not long term stable, probably due to enhanced unfolded and protein-protein interactions promoted by HPP. The loss of stability may be related to a change in the hydrogen bonds between proteins and proteins and solvent, decreasing the intermolecular interactions between the adsorbed proteins over time. Moreover, increasing pressure led to an increased foaming capacity of walnut protein isolate that peaked at 500 MPa, slightly decreasing at 600 MPa, whereas the foaming stability increased with the pressure increase up to 600 MPa (Qin et al., 2013).

Reported results reinforce the importance of proper control of the HPP conditions for each type of protein, knowledge of the relationship between structural changes and consequent changes in the functional properties of proteins, in this particular case the changes to their surfactant properties and those related to the interfacial adsorption processes, in order to achieve the desired results.

### 2.3.5. Gelation

Gelation of globular proteins, naturally present in certain food products or intentionally added as additives, plays a key role for the texture of many food products. The gelation process is now well-understood, especially that induced by heating (Clark, 1998; Foegeding, 2006). The physical gel results from the establishment of a protein three-dimensional network after partial unfolding of the native protein structures, which expose reactive groups on the protein chains and allows for “new” intermolecular interactions. The structural changes needed for gelation to occur and the structure of the resulting gels depend on a complex set of factors, related to the protein itself (native structure, concentration), the ionic characteristics and composition of the medium and on the physical or chemical stimulus that led to gelation. At the molecular level, although there are changes among the secondary structures, significant amounts of ordered structures (α-helix and β-sheets) typically still remain within the gel.

Not surprisingly, the structural changes that might be caused by HPP, under certain conditions, may also lead to protein gelation. As discussed above, hydrophobic interactions, hydrogen bonds and electrostatic effects among the native proteins are altered by high pressure treatments, leading to unfolding of the proteins and dissociation of polymeric structures and to their partial denaturation, thus enabling the occurrence of aggregation, formation of a three-dimensional protein network (gel) or of a precipitate.

There are already numerous studies that demonstrate this possibility, making the HPP also an effective strategy to manipulate this important protein functional property (Galazka et al., 2000c). Here we discuss some important contributions to understand the effect of HPP on the gelation behaviour of vegetable proteins. One well-accepted general trend is that the structural changes induced by heat are different from those induced by pressure, therefore the pressure or heat induced denaturation mechanisms are different, and so there will be the establishment of different intermolecular interactions that are responsible for the aggregation and gelation processes.

Although different physical methods can be used to characterize the gelation process at different length and time scales, oscillatory shear rheology has been extensively used to characterize gel formation and the final viscoelastic properties of the gels (Lopes da Silva and Rao, 2007), including, as testified below, many of the studies on the protein gelation induced by HPP. Therefore, it worth a brief reference to this method and the rheological parameters of interest thus obtained, for the discussion that follows.

The principle is based on the application of a sinusoidal strain (or alternatively an applied sinusoidal stress) and on the measurement of the material's response (a sinusoidal stress, or strain, respectively). The tests are typically performed at low strain amplitudes within the linear viscoelastic regime, thus assuming that the measurements will not alter the internal network structure of the tested material. In general, for a viscoelastic material, the applied strain (or stress) wave will have a phase difference, the angle $\delta$ ($0 < \delta < 90°$) relatively to the resulting stress (or strain) wave. A common parameter of interest corresponds to tan $\delta$, the loss tangent, which is a measure of the viscous/elastic character of the material (the higher tan $\delta$, the higher the viscous character of the sample) at frequency $\omega$. From dynamic rheological tests performed in the linear viscoelastic range, the storage modulus (the elastic in-phase component), $G'$, and the loss modulus (the viscous out-of-phase component), $G''$, can be obtained and are defined as:

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad (2.1)$$

Where $G^*$ is the complex shear modulus. It can be demonstrated that tan $\delta = G''/G'$ and $\eta^* = G^*/\omega$, where $\eta^*$ is the complex viscosity, another useful parameter. The storage modulus is a measure of the elastic response of the material, thus related to the energy stored during the sample solicitation, whereas the loss modulus is a measure of the viscous response of the material, and is related to the energy dissipated during the oscillatory solicitation.

It is worth mentioning that the studies already performed involved essentially two strategies: (1) the formation of a protein gel subjecting a protein solution/dispersion to HPP, and (2) performing a pre-treatment by HPP of a protein solution below gelling concentration, recovering the treated protein, and studying its gelation behaviour by heating.

As expected, concentration has a significant effect in producing pressure-induced gels. Regardless of the gelation mechanism, the minimum protein concentration ($C_g$) necessary to obtain a gel is an important fundamental and technological parameter. This critical

concentration has been assessed for pressure-induced protein gels and also for pre-treated proteins by HPP subjected to heat-induced gelation. In general, HPP decreases the minimum protein concentration necessary for heat-induced gelation to occur, as it was observed, for example, for rapeseed proteins (He et al., 2013).

One of the first reports on gelation of a plant protein by high pressure dates back to more than two decades, showing that soy proteins (17%) originate self-supporting gels when treated by HPP above 300 MPa at 'room temperature' (Okamoto et al., 1990). This gelling behaviour was confirmed later by other authors (Dumoulin et al., 1998; Molina et al., 2002), as well as the fact that the gels obtained by high-pressure treatment are generally softer, but more deformable without breaking than those obtained under similar conditions but by heating the soy protein solutions.

Dynamic rheology was used to evaluate the effects of HPP (pressures up to 800 MPa, 20 or 60 ºC, and treatment times of 20 or 50 min) on soy proteins dispersions prepared at 20 % (w/w) (Apichartsrangkoon, 2003). The non-treated samples of the so-called soy dough originated already a viscoelastic behaviour typically of a solid-like system. The author observed an increase in both viscoelastic moduli (*G'*, *G"*) with increasing pressure and/or temperature, but the effects were relatively small and qualitatively the general viscoelastic behaviour did not change. The results obtained are probably influenced by sample composition (*e.g.*, presence of starch and minerals in the commercial soy protein concentrates or isolates) and/or possible partial denaturation of the proteins during obtaining of samples, since many of them are derived from wastes after processing the vegetable raw materials, thus allowing for protein aggregation even for the unpressurized samples at room temperature. Results should be regarded with caution if one intends to achieve a deeper explanation at the molecular level for the observed effects.

Similar viscoelastic behaviour was reported by Ahmed et al. (2007) also for soy protein dispersions. Mechanical spectra obtained for non-pressurized samples (protein concentration from 10 to 20%) showed already a solid-like behaviour, with *G'* higher than *G"* for about one decade and showing a small dependency on oscillatory frequency. Once again the effects of HPP (350-650 MPa, 15 min, 22-25 ºC) were small, no significant differences were observed in the phase angle ($\delta$ as a function of frequency), and then in the relative viscous (or solid) character of the samples, neither on the magnitude of the $\eta^*$ or of its dependence on oscillatory frequency, comparing pressurised and non-pressurised samples. However, the general trend was contrary to that obtained by Apichartsrangkoon (2003), as the pattern observed by

increasing the pressure was dependent on protein concentration, but for most samples *G′* decreased as the pressure increased. Pressure-induced gels were less rigid (lower *G′*) and with a more pronounced viscous character (higher tan $\delta$) than those obtained by heating, for the same soy protein sample and concentration.

A more complex study was carried out by Alvarez et al. (2008) involving additional variables: pH (3–7), sugar (5% w/v), $CaCl_2$ (5% w/v), besides soy protein concentration, pressure values and holding times, and processing temperature. As expected and in accordance with the previous studies discussed before, protein concentration had a strong influence on both storage (*G′*) and viscous (*G″*) moduli, increasing with concentration. Changes on the viscoelastic behaviour of the soy protein dispersions caused by HPP were attributed to the protein structural changes already discussed (§ 2.2.2), namely the increase in hydrophobicity, the relative increase in the proportion of random coil, and the decrease in β-sheet content. Evaluation of the changes caused by the HPP on the viscoelastic behaviour of the protein dispersions is however restricted since the authors limited the dynamic rheological tests to one constant frequency. Nevertheless, for 5 and 10% protein, the viscoelastic moduli were very low and *G″* was higher than *G′* irrespective of the applied pressure (250-650 MPa). For higher protein concentrations, the moduli increased significantly as the pressure increased; at 15% soy protein, a relatively low-pressure treatment of 250 MPa achieved the cross-over of *G′* over *G″*, likely meaning that a gel was obtained. The pH had an interesting influence on the observed HPP effect on the viscoelastic behaviour of the soy protein dispersions, attributed to changes in the protein solubility: for pH in the range 3-4, the 15% protein control samples (before pressure treatment) showed a solid-like behaviour ($\delta < 45^o$), contrarily to the samples treated at 250 MPa (liquid-like behaviour, $\delta \sim 50^o$); for pH in the range 5-7, there was a tendency to change from liquid-like ($\delta \sim 60^o$) to solid-like behaviour ($\delta \sim 40^o$ for pH 7 and 5, and $\delta \sim 53^o$ for pH 6) after pressure treatment. The effects of the HPP holding time was dependent on pH; increasing time can either influence viscoelastic properties positively (pH 3) or negatively (pH 7).

Others have shedding light on the role of the main soy protein fractions in the pressure-induced gelation. Speroni et al. (2009) observed that dispersions of β-conglycinin (10% (w/v), pH 8) when pressurised at 600 MPa presented a weak gel-like behaviour (relatively low moduli and small difference between them, with *G′* still showing a small frequency dependency at high frequencies, meaning that some molecular rearrangements are still occurring), contrary to

when submitted to 300 MPa (semi-dilute macromolecular solution behaviour), whereas the glycinin fraction maintained the behaviour of a semi-diluted macromolecular system (*G″* higher than *G′* at low frequencies, and a cross-over point at an intermediate frequency) even at 600 MPa. One can then expect that the β-conglycinin fraction will play the most important role in the pressure-induced gelation of soy protein mixtures (*e.g.*, isolates).

Influence of HPP on the viscoelastic properties of hydrated gluten protein complex was also studied. Hydrated gluten is a high viscoelastic material with peculiar rheological behaviour and an enormous importance in the bread making quality of wheat, dough performance and texture of baked products.

Apichartsrangkoon et al. (1999) analysed the effect of HPP (changing pressures, temperatures and holding times) on the viscoelastic behaviour of hydrated wheat gluten. Summing up the more important results, particularly for the different pressures and holding times, compared to the behaviour of the hydrated gluten sample not subjected to HPP: (1) At the lower pressure (200 MPa) the gluten had viscoelastic properties similar to those of the non-pressurised sample, corresponding essentially to a viscous liquid-like behaviour (low *G′* and *G″* moduli and increased tan $\delta$ values); (2) At 400 MPa, both *G′* and *G″* intercepted for higher values, and their slopes upon frequency were slightly lower, meaning that the interactions between the proteins were somewhat enhanced; (3) At 600 MPa, the general rheological profile was qualitatively similar to that observed at lower pressures, although the *G′* and *G″* intercepts were higher and the system showed lower tan $\delta$ values; (4) At 800 MPa, the previously mentioned changes were increased, especially for the higher holding times, suggesting a less liquid-like behaviour and the formation of more entanglements between the protein chains, probably induced by increasing hydrogen bonding. Increasing the temperature to 60 ºC during the HPP led, in general, to the increase of the *G′* and *G″*, with *G′* being substantially higher than *G″* within the analysed frequency range. The slopes were also markedly decreased, particularly for *G′*, suggesting a clear increasing in crosslinking and structuring of the protein network with increasing temperature and time.

In general, similar results were achieved when wheat gluten, gliadin, and glutenin were submitted to HPP. At 500 MPa and the same temperature HPP increased *G'* and *G''* of gluten and glutenin, with *G'* being higher than *G''*. No major effects were verified after HPP treatment of gliadin. Increased pressure and temperature induced a substantial strengthening of gluten,

although, under more severe conditions (*i.e.* 800 MPa and 60 ºC), gluten cohesiveness was lost (Kieffer et al., 2007), contrary to that reported by Apichartsrangkoon et al. (1999).

The study of the effect of HPP on the rheological behaviour of a mixture of vegetable proteins is important in order to understand the behaviour of more complex food systems. One of the few examples in this area was the study of the viscoelastic behaviour of gluten-soy proteins, for different protein proportions, when subjected to HPP (700 MPa, 50 min, 20 and 60 ºC) (Apichartsrangkoon and Ledward, 2002). Interesting, no specific interactions were detected between the proteins in the mixtures and the influence of pressure and temperature was much more pronounced on the gluten than on the soy protein component. All the systems exhibited a solid-like character ($G'>G''$) after pressure treatment. For the gels with high concentrations of gluten, both $G'$ and $G''$ tended to increase, whereas in the gels having high concentrations of soy protein they increased only slightly, with increasing pressure and temperature.

Studies regarding the effect of HPP on the viscoelastic behaviour and gelation of other vegetable proteins in aqueous solution are very scarce. Peyrano et al. (2016) showed that the minimum protein concentration necessary to obtain a thermal-induced gel of cowpea proteins was around 10% for a pre-pressurised protein sample at 200 MPa, although to obtain a firm auto-supported gel it was needed at least a protein concentration of 12 %, whereas this critical concentration was higher for untreated samples. Firmness of the cowpea protein gels increased with increasing pressure and concentration.

### 2.3.6. TEXTURE

Compared to the dynamic rheological tests discussed before (§ 2.3.5) used to characterise gel formation and the viscoelastic properties of the gels, instrumental evaluation of texture usually involves the study of the mechanical properties of the material using higher strains, what can be advantageous to evaluate consumer perception and acceptance. Therefore, the texture characteristics of the protein gels and how they can be changed by HPP are of relevant importance, despite that only a few studies have addressed this subject, especially for vegetable proteins.

Naturally, the instrumental texture characteristics and the sensory perception of texture are related to the structure and rheological properties of the protein gel (Rao and Lopes da Silva, 2007), and the changes brought in by HPP are thus related to the changes in protein structure and gelling properties discussed in the previous sections. For a better understanding

of the meaning of texture parameters discussed below, and on the methods used to obtain them, namely texture profile analysis (TPA), the reader may find helpful to consult one of the many literature sources available on the subject (*e.g.*, Pons and Fiszman, 1996).

As discussed above, self-supporting gels of soy proteins can be formed by HPP (above 200-300 MPa). Dumoulin et al. (1998) and Molina et al. (2002) have studied the textural properties of these kinds of gels and have reached, essentially, concordant results. Gel hardness significantly increased as pressure increased at a given temperature and treatment time, but the pressure-induced gels were in generally softer than heat-induced gels used as controls. Cohesiveness and springiness were only marginally affected by pressure and of a similar magnitude to those observed for heat-induced gels. Gel gumminess increased only slightly with pressure and was also lower than that of the heat-induced gels (Dumoulin et al, 1998).

Another approach was followed by Li et al. (2011), who studied textural characteristics of heat-induced gels (16% soy protein), obtained from soy protein solutions (1%, pH 6.8) treated by HPP (200-500 MPa, 5-20 min), thus allowing to evaluate the effect of prior treatment by HPP on the behaviour of the protein subjected to the same gelation mechanism. Hardness, springiness and adhesiveness of the gels obtained from HPP-treated proteins were significantly lower than those obtained for the non-pressurized samples, but no significant differences were observed for cohesiveness. Hardness, adhesiveness, and springiness also increased with increasing of pressure or time, although the effects were small.

Different results were obtained for rapeseed proteins also pre-treated by HPP or heating (He et al., 2013). Heat-induced gels obtained from the HP-treated samples showed significantly higher values for hardness, adhesiveness, springiness, and cohesiveness than those of native or pre-heat-treated rapeseed proteins. Also, increasing the pressure of the samples' pre-treatment from 200 to 600 MPa increased these textural parameters.

Influence of HPP on the textural properties of hydrated gluten protein complex was also studied. Apichartsrangkoon et al. (1998) analysed the effect of HPP on hardness and modulus of elasticity of hydrated wheat gluten (37.5% w/w) samples. The magnitude of applied pressure, time, and temperature were evaluated. Hardness increased sharply at high pressures and temperatures, and a high positive correlation was observed between the hardness of the samples and their moduli of elasticity. Important findings were related to the correlation of increasing hardness and some structural changes, namely the increase in the degree of disulphide bonding.

## 2.4. EFFECT OF OTHER FOOD COMPONENTS ON TECHNOLOGICAL PROPERTIES OF HPP-TREATED PLANT PROTEINS

Foods are complex systems where proteins interact with other components present in the matrix. It is important to understand how the presence of other food constituents and additives may influence the effects of HPP on structure and technological properties of proteins, although there are only a few studies regarding this subject, and most of them addressing soy proteins. This is clearly an area where further research is still needed.

The presence of minerals (type of ion, concentration) and how they interact with proteins was one of the most studied cases, as could be expected considering their important effects on proteins' conformation and techno-functional properties. The influence of minerals depends on their concentration, pH, type of protein and pressure level.

One of the first studies about this topic was from Pedrosa and Ferreira (1994), who showed that the presence of salts (namely LiCl, KCl, or NaCl) and glycerol inhibited the dissociation of pea's vicilin induced by HPP. The authors suggested that this effect was due to the exclusion of the solvent from the protein solvation layer and the increase in the surface tension, and/or because the ions can influence the water's organisation around the protein's polar groups and change the chemical potential of the dissociated subunits in order to favour the oligomer.

The decrease of electrostatic repulsions between protein chains and stabilisation of hydrophobic interactions by certain salts, *e.g.* NaCl, was also suggested as the reason for the thermal stabilisation of HPP-treated soybean proteins, namely by favouring the formation of hydrogen bonds (Añón et al., 2011). Furthermore, the addition of NaCl also protects soy proteins against pressure-induced denaturation, especially in the case of the glycinin fraction (Añón et al., 2011). Similar effects were observed in the presence of calcium (Speroni et al., 2010).

The influence of salts on protein solubility is complex, depending on several factors including type and concentration of the salt, and pH. Without going into details out of the scope of the presence discussion, it is enough to consider that there is a strong correlation between protein solubility and the intermolecular interactions between the proteins in solution, and that the protein solubility is expected to decrease when the protein-protein interactions become less repulsive. Typically, the effects of anions are more drastic than those of cations, but depending

on the anion/cation we may find a decrease in protein solubility (salting-out effects by those named “kosmotropes”) or an increase in protein solubility (salting-in effect, caused by the “chaotropes”).

Under conditions that led to the decrease of protein’s solubility due to the presence of salts (*e.g.* certain concentrations of $CaCl_2$, $MgCl_2$, and FeSO4), the effect can be partially reversed by HPP, as verified for soy protein isolate, glycinin, and β-conglycinin (Añón et al., 2012; Manassero et al., 2015), particularly at higher pressures and lower salt concentrations. HPP treatments increase the solubility of cation-added soybean proteins and/or modify the structure of insoluble aggregates induced by high-pressure, namely decreasing their size, thus improving the stability of cation-added soy protein dispersions.

Complex effects of calcium and sucrose, including some competition effects between both, have been described by Alvarez et al. (2008) regarding the influence of these additives upon the HPP effects on the viscoelastic behaviour of pressure-induced soy protein gels. At pH 7, a protection effect of the additives was observed against the increase of the viscoelastic parameters ($G'$ and $G''$ modulus) induced by pressure at short holding times, whereas at pH 3 they have observed a negative effect of the additives for short holding times, but positive for longer pressurising times.

The importance of protein-polysaccharide interactions to understand structure and functionality of complex multicomponent food systems and to design products with desired structure and consumer acceptance, or novel textures is well recognised (Dickinson, 2006; Turgeon et al., 2007). For the present discussion is important to highlight that polysaccharides influence the effects that HPP may have on proteins.

Studies of HPP effects on protein-polysaccharide mixtures are also scarce, especially considering vegetable proteins, but in general polysaccharides seems to play a protective role upon the protein regarding the changes caused by the high pressure, with two important consequences already reported: i) a decrease in protein aggregation during or after HPP and ii) improvement of the emulsifying efficiency and stability of the HP-treated proteins (Galazka et al., 1999a, 2000a, 2000b).

Taking into account the studies performed with plant proteins it is noteworthy that the presence of ι-carrageenan decreases the unfold and aggregation of 11S globulin *Vicia faba* brought about by HPP, due to the formation of electrostatic polysaccharide-protein complexes at low ionic strength and high pH that protected the protein against loss of functionality during or after high-pressure treatment (Galazka et al., 2000b). The addition of carrageenan to 11S

also reduced the denaturation temperature, as well as the protein surface hydrophobicity. Further studies showed that carrageenan's addition to the native protein led to emulsions with smaller droplets whose size decreased with the increase in polysaccharide concentration and extent of HPP. Furthermore, the presence carrageenan led to a significant improvement in creaming stability (Galazka et al., 1999b). The application of pressure to the 11S and carrageenan mixture led to higher values of surface tension than 11S alone, which is another indication of the complexation between the protein and polysaccharide in bulk solution. This perturbs the dynamic equilibrium of protein between bulk and interface, favouring the bulk. Thus, complexation reduces the number and availability of hydrophobic groups on the protein for lowering free energy at the air-water interface (Galazka et al., 2001).

The effects of HPP on emulsifying properties of sweet potato protein in the presence of guar gum were also investigated. Emulsions prepared with this protein-polysaccharide mixture pre-treated by HPP revealed an increase in ESI in addition to a considerable reduction in the creaming rate, when compared with the behaviour of the pressurised protein alone (Khan et al., 2015b). Additionally, the protein-polysaccharide mixture originated emulsions with higher viscosity and non-Newtonian behaviour, as could be anticipated due to the presence of the thickening polysaccharide.

Overall, it appears that the addition of polysaccharides to plant proteins is a reliable method to minimise loss of functionality caused by HPP, namely to decrease protein aggregation and to improve emulsifying efficiency.

## 2.5. EFFECTS OF HPP ON PROTEINS' IN-VITRO DIGESTIBILITY

Proteins and peptides may strongly influence functional and biological properties of food products. In fact, several peptides are known to present bioactivity, such as antihypertensive, antioxidant, immunomodulatory, antimicrobial, and antithrombotic properties, among others. These peptides are usually inactive within the protein sequence, however, they can be produced through several methods, including *in-vitro* hydrolysis by proteolytic enzymes (Carrasco-Castilla et al., 2012). As previously discussed, HPP can modify the structure of proteins, which can lead to an enhancement in the efficiency of protein hydrolysis, mainly by exposing inaccessible sites. Consequently, the application of pressure can improve the digestibility of proteins and/or enhance the production of peptides with higher bioactivity and

lower allergenicity. For instance, Chao et al. (2013) showed that HPP pre-treatment of pea proteins at 200 MPa enhanced hydrolysis by alcalase, possibly as a result of an increase of exposed hydrophobic groups, enabling the production of peptides with enhanced ACE- and renin-inhibitory activity comparatively to untreated samples. Also, Girgih et al. (2015) have recently shown that the HPP pretreatment of pea protein dispersions was superior to thermal pretreatment in facilitating enzymatic release of antioxidant peptides by action of alcalase and Zhou et al. (2016a,b) have demonstrated that HPP can enhance the hydrolysis of ginkgo seed proteins and reduce the antigenicity of the resultant hydrolysates.

Generally, there are two main approaches using HPP to enhance enzymatic proteolysis: i) pre-treatment of the proteins with HPP prior to the enzymatic hydrolysis or ii) HPP assisted enzymatic hydrolysis.

### 2.5.1. Enzymatic hydrolysis of HPP pre-treated plant proteins

Regarding soy proteins, a broad range of pressure values seemed to improve proteolysis. Peñas et al. (2004) performed one of the first studies in this area by evaluating the effect of HPP on soybean whey proteins when they are hydrolysed under or after the pressure treatment (100–200 MPa). They verified an enhancement of hydrolysis of the pressure pre-treated proteins using trypsin, pepsin and chymotrypsin, particularly when the pre-treatment occurred at 100 MPa.

Higher pressures (400-600 MPa, 20 min, 20 ºC) also yielded an improved *in-vitro* digestibility of soy protein isolate, after digestion with pepsin and pancreatin (Dan et al., 2010). The previous HPP treatments resulted in higher amounts of peptides with molecular weight lower than 3 kDa, although the peptide profile was similar to the one obtained with non-treated proteins. Li et al. (2011) also studied the effect of HPP (300 MPa) on *in-vitro* digestibility of soy proteins using pepsin and trypsin, and described an improved digestibility of the treated samples comparatively to the untreated ones.

There are several studies that described the HPP pre-treatments impact in the *in-vitro* digestibility of other plant proteins. De Lamballerie-Anton et al. (2002) showed that an increase in pressure during the HPP pre-treatment can lead to an increased hydrolysis of lupin proteins by pepsin. In this case, the hydrolysis was enhanced with a pre-treatment at 500 MPa, whereas the 200 MPa treatment only marginally increased the *in-vitro* digestion. Correia et al. (2011) also observed that mild pressure (300 MPa for 5 or 15 min) enhance the *in-vitro* digestibility of

sorghum proteins by pepsin, with the pressure treatment to be performed either before or after cooking. On the other hand, Qin et al. (2013) verified that different pressures (300-600 MPa) during the HPP pre-treatment enhanced the *in-vitro* digestibility of walnut protein isolate using trypsin, but no significant differences were verified among the different pressures used. Similarly, HPP pre-treatment of ginkgo seed proteins improved the extent of the hydrolysis by papain, alcalase or pepsin, mostly at 300 and 400 MPa (Zhou et al., 2016b); the effect of HPP was dependent on type of protease and both the degree of hydrolysis and the peptide profile of the hydrolysates were dependent on the applied pressure.

Summarising, HPP may have positive effects on protein digestibility, as demonstrated above, by causing protein unfolding and exposure of more cleavage sites, which are quantitatively dependent on the type of protein and degree of structural and conformational changes induced by the pressure treatment. However, some negative effects have been also reported, especially for higher pressure values, associated with increased protein-protein interactions and the incidence of aggregation, which cause lower enzyme accessibility to the hydrolysis sites, thus resulting in a decrease in digestibility. Several studies also illustrate this behaviour, which emphasises once again the importance of adequate monitoring the HPP conditions to achieve the desired effects on protein functionality.

For example, *in vitro* trypsin digestibility of kidney bean proteins decreased when previously treated by HPP at pressures above 200 MPa and for long incubation times (*e.g.*, 120 min) (Yin et al., 2008). Also, Zhang et al. (2012) showed that previous treatments (before hydrolysis) at low pressures (100-200 MPa) did not significantly influence the digestibility of chickpea proteins by alcalase, whereas mild pressure (300 and 400 MPa) increased the hydrolysis rate, but higher values (500 and 600 MPa) decreased the degree of hydrolysis. Similar pre-treatment conditions (400 and 600 MPa) also appear to decrease the hydrolysis of pea proteins by alcalase (Chao et al., 2013).

The inability of HPP pre-treatments to yield higher digestibility was also verified for sweet potato proteins digested with pepsin and pancreatin, contrarily to what was observed by thermal pre-treatments (Sun et al., 2014). The differences between both pre-treatments were explained by the different structural changes caused by heat or high pressure and protein aggregation mediated by covalent disulphide bonds, promoted by HPP, which caused the cleavage sites to be inaccessible to the digestive enzymes, thus explaining the little or no effect of HPP on protein digestibility.

Overall, HPP pre-treatments can be advantageous (by exposing inaccessible cleavage sites) or disadvantageous (by preventing enzymatic access to cleavage sites due to protein-protein interactions) for the hydrolysis of plant proteins, to increase the degree of hydrolysis and to produce peptides with improved functional and/or bioactive properties, depending on the protein source, treatment conditions and protease used. However, due to the variability of these results available in the literature, more studies are paramount in order to better understand the mechanisms involved.

### 2.5.2. HPP ASSISTED ENZYMATIC HYDROLYSIS

Proteolysis can also be assisted by HPP (*i.e.* by applying pressure in the presence of the enzyme, during the hydrolysis). In this case, one of the main expected differences comparing with the previous discussed studies is that now the effects of HPP upon the enzyme itself will play an important role.

HPP (100-300 MPa for 15 min) assisted enzymatic hydrolysis (using alcalase, neutrase, corolase 7089 or corolase PNL) of soybean whey was performed by Peñas et al. (2006). The authors described an overall increase in the proteolytic activity of all the enzymes under pressure, particularly at 200 and 300 MPa. There was also an enhancement of HPP assisted hydrolysis soybean whey proteins using trypsin, pepsin or chymotrypsin at 100 MPa (Peñas et al., 2004).

Zhang et al. (2012) assessed the HPP assisted digestibility of chickpea protein isolates using alcase (100-300 MPa for 10-30 min). The activity of alcase increased with lower pressure values (*i.e.* 100 to 200 MPa) but decreased at 300 MPa. Additionally, the degree of hydrolysis also increased with pressurising time. This was likely related to the increased activity of alcase promoted by the pressure up to 200 MPa or increased exposure of cleavage sites by HPP (or a combined effect). Worth to mention that hydrolysis at 200 MPa for 20 min also yielded peptides with higher antioxidant activity.

Garcia-Mora et al. (2015) studied the enzymatic proteolysis (alcalase, savinase, protamex and corolase 7089) under pressure (100-500 MPa for 15 min) of lentil proteins to assess the release of ACE’s inhibitory and antioxidant peptides. Pressure up to 300 MPa enhanced the hydrolytic efficiency of protamex, savinase and corolase 7089 and hydrolysis at 300 MPa resulted in a complete proteolysis by all enzymes yielding peptides with molecular

weights lower than 3 kDa. The peptides with higher ACE inhibitory effects and antioxidant activity resulted from proteolysis with savinase at 300 MPa.

In opposition to HPP pre-treatments, HPP assisted hydrolysis results are less variable and it appears that low/mild pressures (200–300 MPa) enhance the hydrolysis, likely resulting from the combined partial unfold of the substrate (protein) and higher accessibility of the target bonds, and an enhanced proteolytic activity of the enzyme, whereas higher pressures could inhibit the proteases and simultaneously contribute for substrate aggregation and lower accessibility to the cleavage sites (Garcia-Mora et al., 2015; Peñas et al., 2006; Zhang et al., 2012).

## 2.6. MICROBIAL TRANSGLUTAMINASE

The enzymatic crosslinking of food proteins is an attractive methodology to manipulate food structure. To this purpose, microbial transglutaminase (MTG) (EC 2.3.2.13) is being widely used in the food industry. The first sources of this enzyme were plant and animal tissues, however, due to the lower amounts produced and expensive separation and purification operations, MTG is now typically produced for industrial applications through fermentation processes using *Streptomyces mobaraensis bacterium*. It was first produced at an industrial scale by the Japanese company Ajinomoto Co. in collaboration with Amano Enzyme Co. (Duarte, Matte, Bizarro, & Ayub, 2020). MTG has been accepted as a safe substance for human ingestion, having a GRAS (Generally Recognized as Safe) status by the FDA (Food and Drugs Administration) since 1998 (Gaspar & de Góes-Favoni, 2015). MTG is an extracellular enzyme, with a molecular mass of approximately 38 kDa. It is active in a wide pH interval, from 5 to 8, and over an extensive temperature range, up to 70 ºC. Furthermore, MTG does not require $Ca^{2+}$ for its action, contrary to the transglutaminases from plant/animal origins (Ando et al., 1989; Yokoyama, Nio, & Kikuchi, 2004).

Overall, MTG can change proteins' functional properties by incorporating amines, promoting intra- and intermolecular crosslinks or by deamination (Jong & Koppelman, 2002). It catalyzes the acyl transfer reaction between the γ-carboxyamide group of protein-bound glutamine residues and primary amines, preferentially the ε–amino group of lysine residues. This reaction may lead to the formation of intra- and/or intermolecular crosslinks between proteins (Partschefeld, Richter, Schwarzenbolz, & Henle, 2007). The formation of these

crosslinks between proteins may cause changes in their techno-functional properties. These polymerizations may result in changes in the molecule's hydrophobicity and solubility, therefore affecting gelation, emulsification, foaming, viscosity and water-holding capacity (Gaspar & de Góes-Favoni, 2015). When lysine residues, free lysine or primary amines are absent in system, water may become the acyl acceptor and promotes hydrolytic deamidation of the glutamine and asparagine residues, converting them into glutamic acid and aspartic acid, respectively (Ando et al., 1989). This results in a change in the overall charge of the protein, leading to changes in its surface hydrophobicity and solubility, thus altering its techno-functional properties, particularly emulsification, foaming and gelation (Jong & Koppelman, 2002). The resulting proteins have lower isoelectric points, which increases their solubility in more acidic environments (Babiker, 2000; Renzetti, Dal Bello, & Arendt, 2008). Additionally, this increase in negative charge also increases the amphiphilic nature of the proteins. These changes, alongside the formation and maintenance of smaller droplets at the water/oil interface also promoted by MTG, reduces surface tension and facilitates the binding ability to water, increasing the protein emulsifying capacity (Agyare, Addo, & Xiong, 2009; Babiker, 2000).

Along with changing the techno-functional of proteins, MTG can also change the appearance and texture of high protein-based foods. The crosslinking promoted by MTG results in the formation of high molecular weight polymers. These can reduce water mobility in the protein network, leading to a larger flow resistance, thus increasing viscosity and giving the products an improved consistency (Gaspar & de Góes-Favoni, 2015). Many foods with certain physical characteristics are produced by protein gelation, *e.g.* yogurt, cheese, surimi, *etc.* The crosslink promoted by MTG enables the formation of highly elastic and irreversible gels, using different substrates, even at relatively low protein concentrations (de Góes-Favoni & Bueno, 2014). Consequently, the action of MTG promotes changes in the appearance and texture of food systems such as fish, meat, and dairy (Motoki & Seguro, 1998). Although most studies have been directed to these food systems, the influence of MTG crosslinking on technical and physiological functionality of vegetable proteins was already reported (Babiker, 2000; Dube, Schäfer, Neidhart, & Carle, 2007; Schäfer, Zacherl, Engel, Neidhart, & Carle, 2007; Tang, Li, & Yang, 2006). The extent of said crosslinking and consequent polymerization depends on environmental factors, such temperature, pH and presence and/or absence of enzyme inhibitors (*e.g.* $Cu^{2+}$, $Zn^{2+}$, $Pb^{2+}$, $Li^{2+}$, and $Mg^{2+}$), and the ability of the MTG's active site to bind to glutamine residues, which depends on the structure and conformation of the target protein(s). Therefore, despite the ease of MTG to crosslink various proteins, non-globular proteins are

more easily accessible to MTG crosslinking activity than globular proteins (Lorenzen, Schlimme, & Roos, 1998; Sharma, Lorenzen, & Qvist, 2001).

To overcome this limitation some non-conventional methods of food processing, such as HPP, ultrasonication, microwaves or ultraviolet light irradiation, have been considered to modify the quality and functionality of protein-based food products (Gharibzahedi et al., 2018).

### 2.6.1. MICROBIAL TRANSGLUTAMINASE COMBINED WITH HPP

Of particular interest to the current study, is the combination of HPP and MTG catalytic activity, using HPP as pre-treatment to change protein conformation, or by the concomitant use with the enzyme during processing, similarly to what have been done for other enzymes (§2.5). As previous discussed, high-pressure may change the structural organization of proteins (Ledward, 2000) and thus can expose MTG's target residues, otherwise inaccessible within the protein tertiary structure. Consequently, there is an interest in combining HPP and MTG to tailor protein-based food characteristics, mainly textural properties.

MTG is relatively resistant to pressure, retaining a high percentage of its activity after HPP, particularly at pH 6 and 7. For instance, being submitted to 600 MPa for 30 min, MTG's retained more than 40 % of its activity (Queirós, Gouveia, Saraiva, & Lopes-da-Silva, 2019). Nonetheless, different degrees of inactivation of MTG were described for pressures of 500 MPa and above (Lauber, Krause, Klostermeyer, & Henle, 2003; Lauber, Noack, Klostermeyer, & Henle, 2001; Lee & Park, 2002; Nonaka et al., 1997), depending on the medium used, HPP and environmental conditions. It was also reported that the MTG high pressure inactivation followed first-order kinetics (Queirós et al., 2019). MTG's stability under pressure is due to its structure, which is constituted by eleven α-helix and eight β-strands, seven of which form a β-sheet in the center of the enzyme that is surrounded by the α-helices (Kashiwagi et al., 2002). Being less compressible, β-sheet regions are less susceptible to deformation, and thus less sensitive to pressure (Kundrot & Richards, 1988), explaining MTG's stability under pressure. Still, at pressure levels above 600 MPa, the tertiary and secondary structures may be affected, as these pressure levels lead to the unfold of the enzyme and convert α-helix to unordered structures, where β-sheet structures seem to be more pressure stable (Menéndez et al., 2006). Therefore, higher pressure levels can cause conformational changes on the MTG's surface, *i.e.* in the α-helical areas, leading to conformational deviations and consequent inactivation (Menéndez, Rawel, Schwarzenbolz, & Henle, 2006).

MTG is not only resistant to pressure, it can also maintain its activity under pressure, as exemplified by the crosslinking of casein monomers, β-lactoglobulin and β-casein (Lauber, Krause, Klostermeyer, & Henle, 2003; Lauber, Noack, Klostermeyer, & Henle, 2001), especially at pressures around or lower than 400 MPa. Furthermore, HPP may increase the polymerization activity of MTG on different proteins, *e.g.* bovine serum albumin, ovalbumin, γ-globulin and lysozyme (Nonaka, Ito, Sawa, Motoki, & Nio, 1997). It is notable that MTG was not able to polymerize β-lactoglobulin at atmospheric pressure, whereas polymerization occurred with the simultaneous application of HPP and MTG (Lauber et al., 2001). As previously mentioned, the protein unfolding caused by pressure may expose some hydrophobic regions. The MTG presence in the vicinity of these regions may significantly develop crosslinking reactions through inter and intramolecular bonds resulting in higher molecular weight peptide chains with different functional properties (Gharibzahedi et al., 2018). Therefore, MTG and HPP simultaneous treatments may be an appropriate tool to improve the techno-functional properties of proteins with no pre-treatment or the use of reducing agents (Partschefeld et al., 2007). For instance, simultaneous application of HPP and MTG has already been reported to increase water holding capacity in gels from chicken meat and egg (Trespalacios & Pla, 2007a, 2007b), fish (Cando, Borderías, & Moreno, 2016) and yogurt (M. S. Tsevdou, Eleftheriou, & Taoukis, 2013). Still, most studies regarding simultaneous application of HPP and MTG were performed on animal protein-based foods, such as fish (Cardoso, Mendes, Saraiva, Vaz-Pires, & Nunes, 2010), meat (Trespalacios & Pla, 2007b) and dairy (Tsevdou, Eleftheriou, & Taoukis, 2013; M. Tsevdou et al., 2013), with very little information being available on its effects on proteins of plant origin.

## 2.7. CONCLUSIONS

The impact of HPP on the structure and conformation, techno-functional properties and digestibility of plant proteins, and on the catalytic activity of enzymes with interest to also modify protein functionality, was revised in this chapter. HPP can tailor plant proteins' properties since different processing conditions may induce different unfolding paths with a different exposition of buried sites. The level of exposure of these sites dictates the behaviour of the proteins, inducing their aggregation by promoting protein-protein interactions, or their solubility through the promotion of protein-solvent interactions. Moreover, the exposure of hydrophobic sites may

be also helpful to improve interfacial properties. Therefore, HPP may allow altering the solubility and surface hydrophobicity of proteins by modifying the balance between the partial unfolding and aggregation. Plus, the combination of HPP and other parameters such as pH or temperature, can be useful to create novel protein ingredients that may have improved properties, form new aggregates or gels, or present different thermal stabilities. It is also promising to alter food textural properties by addition of HPP-treated proteins and these properties may be further improved by mixing the proteins with additives that may further improve their functionality. Furthermore, interesting effects are found when HPP is applied to improve enzymatic hydrolysis, allowing to obtain new peptides with improved bioactive functionality.

These features may provide alternative solutions and applications for more sustainable ingredients. However, how the changes in protein structure promoted by HPP will affect the techno-functional properties of the protein is a complex subject and strongly dependent on several factors not easily controlled, mainly the type of protein and its native characteristics, the composition of the medium, and HPP conditions.

The current challenges are mainly related to the correlation among this complex set of factors: how changes in protein functionality correlate with structural and conformational changes, and in turn how these changes correlate with the HPP conditions (pressure, time and temperature) and medium composition (*e.g.* pH, ionic strength and co-solutes) to achieve the desired objectives. In fact, further research is necessary to better understand the complex processes proteins undergo when subjected to high pressure, in order to overcome the challenge to efficiently exploit this technology to improve food quality and consumer acceptance. There is much that is not yet fully understood in this field.

The enzymatic modification and polymerization of proteins has been increasingly used in the food industry to change their techno-functional properties. The use of MTG permits these modifications to be efficient, safe, and highly controlled. HPP induced protein denaturation combined with subsequent enzymatic crosslinking may allow to tailor-make desirable structural and mechanical changes in the protein matrix. However, despite what is already known regarding HPP effects on protein functionality and the MTG effects on protein crosslinking and structure development, extensive and fundamental knowledge concerning physical and enzymatic treatments combined effects on vegetable proteins' complex structure and functionality are still needed.

## 2.8. REFERENCES

Achouri, A., & Boye, J. I. (2013). Thermal processing, salt and high pressure treatment effects on molecular structure and antigenicity of sesame protein isolate. *Food Research International.* 53: 240–251.

Agyare, K. K., Addo, K., & Xiong, Y. L. (2009). Emulsifying and foaming properties of transglutaminase-treated wheat gluten hydrolysate as influenced by pH, temperature and salt. *Food Hydrocolloids*, *23*(1), 72–81.

Ahmed, J., Ayad, A., Ramaswamy, H. S., Alli, I., & Shao, Y. (2007). Dynamic viscoelastic behavior of high pressure treated soybean protein isolate dispersions. *International Journal of Food Properties.* 10: 397–411.

Akasaka, K. (2014). Pressure and protein dynamism. *High Pressure Research.* 34: 222–235.

Akasaka, K. (2006). Probing conformational fluctuation of proteins by pressure perturbation. *Chemical Reviews*. 106: 1814–1835.

Akasaka, K. (2003a). Exploring the entire conformational space of proteins by high-pressure NMR. *Pure and Applied Chemistry.* 75: 927–936.

Akasaka, K. (2003b). Highly fluctuating protein structures revealed by variable-pressure nuclear magnetic resonance. *Biochemistry* 42: 10875–10885.

Akasaka, K., Nagahata, H., Maeno, A., & Sasaki, K. (2008). Pressure acceleration of proteolysis: A general mechanism. *Biophysics (Oxf).* 4: 29–32.

Alvarez, P. A., Ramaswamy, H. S., & Ismail, A. A. (2008). High pressure gelation of soy proteins: Effect of concentration, pH and additives. *Journal of Food Engineering.* 88: 331–340.

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., & Motoki, M. (1989). Purification and characteristics of a novel transglutaminase derived from microorganisms. *Agricultural and Biological Chemistry*, *53*(10), 2613–2617.

Añón, M. C., De Lamballerie, M., & Speroni, F. (2012). Effect of high pressure on solubility and aggregability of calcium-added soybean proteins. *Innovative Food Science and Emerging Technologies.* 16: 155–162.

Añón, M. C., De Lamballerie, M., & Speroni, F. (2011). Influence of NaCl concentration and high pressure treatment on thermal denaturation of soybean proteins. *Innovative Food Science and Emerging Technologies.* 12: 443–450.

Apichartsrangkoon, A. (2003). Effects of high pressure on rheological properties of soy protein gels. *Food Chemistry.* 80: 55–60.

Apichartsrangkoon, A., & Ledward, D. A. (2002). Dynamic viscoelastic behaviour of high pressure treated gluten–soy mixtures. *Food Chemistry.* 77: 317–23.

Apichartsrangkoon, A., Ledward, D. A., Bell, A. E., & Brennan, J. G. (1998). Physicochemical properties of high pressure treated wheat gluten. *Food Chemistry.* 63: 215–220.

Apichartsrangkoon, A., Bell, A. E., Ledward, D. A., & Schofield, J. D. (1999). Dynamic viscoelastic behavior of high-pressure-treated wheat gluten. *Cereal Chemistry.* 76: 777–782.

Babiker, E. E. (2000). Effect of transglutaminase treatment on the functional properties of native and chymotrypsin-digested soy protein. *Food Chemistry*, *70*(2), 139–145.

Baier, A. K., & Knorr, D. (2015). Influence of high isostatic pressure on structural and functional characteristics of potato protein. *Food Research International.* 77: 753–761.

Balasubramaniam, V. M., Martínez-Monteagudo, S. I., & Gupta, R. (2015). Principles and application of high pressure–based technologies in the food industry. *Annual Review of Food Science and Technology.* 6: 435–462.

Balny, C., & Masson, P. (1993). Effects of high pressure on proteins. *Food Reviews International.* 9: 611–628.

Boonyaratanakornkit, B. B., Park, C. B., & Clark, D. S. (2002). Pressure effects on intra- and intermolecular interactions within proteins. *Biochimica et Biophysica Acta* 1595: 235–249.

Bridgman, P. W. (1914). The coagulation of albumen by pressure. *Journal of Biological Chemistry.* 18: 511–512.

Bulaj, G. (2005). Formation of disulfide bonds in proteins and peptides. *Biotechnology Advances.* 23: 87–92.

Cando, D., Borderías, A. J., & Moreno, H. M. (2016). Combined effect of aminoacids and

microbial transglutaminase on gelation of low salt surimi content under high pressure processing. *Innovative Food Science and Emerging Technologies*, *36*, 10–17.

Cardoso, C. L., Mendes, R. O., Saraiva, J. a., Vaz-Pires, P. R., & Nunes, M. L. (2010). Quality Characteristics of High Pressure-Induced Hake ( Merluccius capensis ) Protein Gels with and without MTGase. *Journal of Aquatic Food Product Technology*, *19*(3–4), 193–213.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Gutiérrez-López, G. F., and Dávila-Ortiz, G. (2012). Use of proteomics and peptidomics methods in food bioactive peptide science and engineering. *Food Engineering Reviews.* 4: 224–243.

Chakraborty, S., Kaushik, N., Rao, P. S., & Mishra, H. N. (2014). High-pressure inactivation of enzymes: a review on its recent applications on fruit purees and juices. *Comprehensive Reviews in Food Science and Food Safety.* 13: 578–596.

Chao, D., He, R., Jung, S., & Aluko, R. E. (2013). Effect of pressure or temperature pretreatment of isolated pea protein on properties of the enzymatic hydrolysates. *Food Research International.* 54: 1528–1534.

Chapleau, N., & de Lamballerie-Anton, M. (2003). Improvement of emulsifying properties of lupin proteins by high pressure induced aggregation. *Food Hydrocolloids.* 17: 273–280.

Clark, A. H. (1998). Gelation of globular proteins. In: Functional Properties of Food Macromolecules, pp. 77-142. Hill, S. E., Ledward, D. A., and Mitchell, J. R., Eds., 2nd ed. Aspen Publishers, Gaithersburg, USA.

Condés, M. C., Añón, M. C., & Mauri, A. N. (2015). Amaranth protein films prepared with high-pressure treated proteins. *Journal of Food Engineering.* 166: 38–44.

Condés, M. C., Speroni, F., Mauri, A., & Añón, M. C. (2012). Physicochemical and structural properties of amaranth protein isolates treated with high pressure. *Innovative Food Science and Emerging Technologies.* 14: 11–17.

Correia, I., Nunes, A., Saraiva, J. A., Barros, A. S., & Delgadillo, I. (2011). High pressure treatments largely avoid/revert decrease of cooked sorghum protein digestibility when applied before/after cooking. *LWT - Food Science and Technology.* 44: 1245–1249.

Dan, S., Shujun, L., Laurie, M. H., Fengmin, Z., Lanfang, Z., Xiaopeng, Z., Wei, L., & Youfu, C. (2010). Effects of high hydrostatic pressure on digestibility of soy protein. *International*

*Agrcultural Engeneering Journal.* 19: 49–58.

Day, L. (2013). Proteins from land plants – Potential resources for human nutrition and food security. *Trends in Food Science & Technology.* 32: 25–42.

de Góes-Favoni, S. P., & Bueno, F. R. (2014). Microbial Transglutaminase: General Characteristics and Performance in Food Processing Technology. *Food Biotechnology*, *28*(1), 1–24.

De Lamballerie-Anton, M., Delépine, S., & Chapleau, N. (2002). High pressure effect on meat and lupin protein digestibility. *High Pressure Research.* 22: 649–652.

Dickinson, E. (2006). Colloid science of mixed ingredients. *Soft Matter*, 2: 642-652.

Dhakal, S., Liu, C., Zhang, Y., Roux, K. H., Sathe, S. K., & Balasubramaniam, V. M. (2014). Effect of high pressure processing on the immunoreactivity of almond milk. *Food Research International.* 62: 215–222.

Duarte, L., Matte, C. R., Bizarro, C. V., & Ayub, M. A. Z. (2020). Review transglutaminases: part II—industrial applications in food, biotechnology, textiles and leather products. *World Journal of Microbiology and Biotechnology*, *36*(1), 1–20.

Dube, M., Schäfer, C., Neidhart, S., & Carle, R. (2007). Texturisation and modification of vegetable proteins for food applications using microbial transglutaminase. *European Food Research and Technology*, *225*(2), 287–299.

Dumoulin, M., Ozawa, S., & Hayashi, R. (1998). Textural properties of pressure-induced gels of food proteins obtained under different temperatures including subzero. *Journal of Food Science.* 63: 92–95.

Eisenmenger, M. J., & Reyes-De-Corcuera, J. I. (2009). High pressure enhancement of enzymes: A review. *Enzyme and Microbial Technology.* 45: 331–347.

Fernandes, P. A. R., Moreira, S. A., Fidalgo, L. G., Santos, M. D., Queirós, R. P., Delgadillo, I., & Saraiva, J. A. (2015). Food preservation under pressure (hyperbaric storage) as a possible improvement/alternative to refrigeration. *Food Engineering Reviews.* 7: 1–10.

Foegeding, E. A. (2006). Food biophysics of protein gels: a challenge of nano and macroscopic proportions. *Food Biophysics.* 1: 41–50.

Galazka, V., Dickinson, E., & Ledward, D. (1999b). Emulsifying behaviour of 11S globulin *Vicia faba* in mixtures with sulphated polysaccharides: comparison of thermal and high-pressure treatments. *Food Hydrocolloids.* 13: 425–435.

Galazka, V. B., Dickinson, E., & Ledward, D. (2001). Effect of high-pressure on surface behaviour of adsorbed films formed from mixtures of sulfated polysaccharides with various proteins. *Innovative Food Science and Emerging Technologies.* 1: 177–185.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000a). Effect of high pressure on ovalbumin-polysaccharide interactions. *High Pressure Research.* 19: 125–130.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000b). Influence of high pressure on interactions of 11S globulin *Vicia faba* with ι-carrageenan in bulk solution and at interfaces. *Food Hydrocolloids.* 14: 551–560.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000c). Influence of high pressure processing on protein solutions and emulsions. *Current Opinion in Colloid & Interface Science.* 5: 182-187.

Galazka, V. B., Smith, D., Ledward, D. A., & Dickinson, E. (1999a). Interactions of ovalbumin with sulphated polysaccharides: effects of pH, ionic strength, heat and high pressure treatment. *Food Hydrocolloids.* 13: 81–88.

Garcia-Mora, P., Peñas, E., Frias, J., Gomez, R., & Martinez-Villaluenga, C. (2015). High-pressure improves enzymatic proteolysis and the release of peptides with angiotensin I converting enzyme inhibitory and antioxidant activities from lentil proteins. *Food Chemistry.* 171: 224–232.

Garcia-Mora, P., Peñas, E., Frias, J., Zieliński, H., Wiczkowski, W., Zielińska, D., & Martínez-Villaluenga, C. (2016). High-pressure-assisted enzymatic release of peptides and phenolics increases angiotensin converting enzyme I inhibitory and antioxidant activities of pinto bean hydrolysates. *J. Agric. Food Chemistry.* 64: 1730–1740.

Gaspar, A. L. C., & de Góes-Favoni, S. P. (2015). Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review. *Food Chemistry*, *171C*, 315–322.

Gharibzahedi, S. M. T., Roohinejad, S., George, S., Barba, F. J., Greiner, R., Barbosa-Cánovas, G. V., & Mallikarjunan, K. (2018). Innovative food processing technologies on

the transglutaminase functionality in protein-based food products: Trends, opportunities and drawbacks. *Trends in Food Science and Technology*, *75*(December 2017), 194–205.

Girgih, A. T., Chao, D., Lin, L., He, R., Jung, S., & Aluko, R. E. (2015). Enzymatic protein hydrolysates from high pressure-pretreated isolated pea proteins have better antioxidant properties than similar hydrolysates produced from heat pretreatment. *Food Chemistry.* 188: 510–6.

He, R., He, H.-Y., Chao, D., Ju, X., & Aluko, R. (2013). Effects of high pressure and heat treatments on physicochemical and gelation properties of rapeseed protein Isolate. *Food Bioprocess Technology.* 7: 1344–1353.

He, X.-H., Liu, H.-Z., Liu, L., Zhao, G.-L., Wang, Q., & Chen, Q.-L. (2014). Effects of high pressure on the physicochemical and functional properties of peanut protein isolates. *Food Hydrocolloids.* 36; 123–129.

Jong, G. H. A. de, & Koppelman, S. J. (2002). Transglutaminase Catalyzed Reactions: Impact on Food Applications. *Journal of Food Science*, *67*(8), 2798–2806.

Kajiyama, N., Isobe, S., Uemura, K., & Noguchi, A. (1995). Changes of soy protein under ultra-high hydraulic pressure. *International Journal of Food Science & Technology.* 30: 147–158.

Kashiwagi, T., Yokoyama, K., Ishikawa, K., Ono, K., Ejima, D., Matsui, H., & Suzuki, E. (2002). Crystal Structure of Microbial Transglutaminase from Streptoverticillium mobaraense. *Journal of Biological Chemistry*, *277*(46), 44252–44260.

Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. *Advances in Protein Chemistry*. 14: 1-63.

Khan, N. M., Mu, T. H., Sun, H. N., Zhang, M., & Chen, J. W. ( 2015a). Effects of high hydrostatic pressure on secondary structure and emulsifying behavior of sweet potato protein. *High Pressure Research.* 35: 189–202.

Khan, N. M., Mu, T.-H., Sun, H.-N., Zhang, M., & Chen, J.-W. (2015b). Effects of high hydrostatic pressure on emulsifying properties of sweet potato protein in model protein – hydrocolloids system. *Food Chemistry*. 169: 448–454.

Khan, N. M., Mu, T. H., Zhang, M., & Arogundade, L. A. (2014). The effects of pH and high

hydrostatic pressure on the physicochemical properties of a sweet potato protein emulsion. *Food Hydrocolloids.* 35: 209–216.

Khan, N. M., Mu, T. H., Zhang, M., & Chen, J. W. (2013). Effects of high hydrostatic pressure on the physicochemical and emulsifying properties of sweet potato protein. *International Journal of Food Science & Technology.* 48: 1260–1268.

Kieffer, R., Schurer, F., Kohler, P., & Wieser, H. (2007). Effect of hydrostatic pressure and temperature on the chemical and functional properties of wheat gluten: Studies on gluten, gliadin and glutenin. *Journal of Cereal Science.* 45: 285–292.

Knorr, D., Heinz, V., & Buckow, R. (2006). High pressure application for food biopolymers. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics.* 1764: 619–631.

Kundrot, C. E., & Richards, F. M. (1988). Effect of hydrostatic pressure on the solvent in crystals of hen egg-white lysozyme. *Journal of Molecular Biology.* 200: 401–410.

Lakshmanan, R., de Lamballerie, M., & Jung, S. (2006). Effect of soybean-to-water ratio and pH on pressurized soymilk properties. *Journal of Food Science.* 71, 384–391.

Lam, R. S. H., & Nickerson, M. T. (2013). Food proteins: A review on their emulsifying properties using a structure–function approach. *Food Chemistry.* 141: 975–984.

Lauber, S., Krause, I., Klostermeyer, H., & Henle, T. (2003). Microbial transglutaminase crosslinks β-casein and β-lactoglobulin to heterologous oligomers under high pressure. *European Food Research and Technology*, *216*, 15–17.

Lauber, S., Noack, I., Klostermeyer, H., & Henle, T. (2001). Oligomerization of β-lactoglobulin by microbial transglutaminase during high pressure treatment. *European Food Research and Technology*, *213*(3), 246–247.

Ledward, D. A. (2000). Effects of pressure on protein structure. *High Pressure Research.* 19: 1–10.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2012). Effects of high hydrostatic pressure treatment on allergenicity and structural properties of soybean protein isolate for infant formula. *Food Chemistry.* 132: 808–814.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2011). Effects of high hydrostatic pressure on some

functional and nutritional properties of soy protein isolate for infant formula. *Journal of Agricultural and Food Chemistry.* 59: 12028–12036.

Li, H., Zhu, K., Zhou, H., Peng, W., & Guo, X. (2016). Comparative study of four physical approaches about allergenicity of soybean protein isolate for infant formula. *Food and Agricultural Immunology.* 68: 1–20.

Liu, C., Zhao, M., Sun, W., & Ren, J. (2013). Effects of high hydrostatic pressure treatments on haemagglutination activity and structural conformations of phytohemagglutinin from red kidney bean (*Phaseolus vulgaris*). *Food Chemistry.* 136: 1358–63.

Lopes da Silva, J. A., & Rao, M. A. (2007). Rheological behavior of food gels. In: Rheology of Fluid and Semisolid Foods: Principles and Applications, pp. 339-401. Rao, M. A., Ed., 2nd ed. Springer Science+Business Media LLC, NY, USA.

Lorenzen, P. C., Schlimme, E., & Roos, N. (1998). Crosslinking of sodium caseinate by a microbial transglutaminase. *Nahrung*, *42*(3–4), 151–154.

Lu, Y., Liu, C., Zhao, M., Cui, C., & Ren, J. (2015). Structure and activity changes of phytohemagglutinin from red kidney bean ( *Phaseolus vulgaris* ) affected by ultrahigh-pressure treatments. *Journal of Agricultural and Food Chemistry.* 63: 9513–9519.

Manassero, C. A., Vaudagna, S. R., Añón, M. C., & Speroni, F. (2015). High hydrostatic pressure improves protein solubility and dispersion stability of mineral-added soybean protein isolate. *Food Hydrocolloids.* 43, 629–635.

Menéndez, O., Rawel, H., Schwarzenbolz, U., & Henle, T. (2006). Structural changes of microbial transglutaminase during thermal and high-pressure treatment. *Journal of Agricultural and Food Chemistry*, *54*, 1716–1721.

Mirmoghtadaie, L., Aliabadi, S. S., & Hosseini, S. M. (2016). Recent approaches in physical modification of protein functionality. *Food Chemistry.* 199: 619–627.

Molina, E., Defaye, A. B., & Ledward, D. A. (2002). Soy protein pressure-induced gels. *Food Hydrocolloids.* 16: 625–632.

Molina, E., Papadopoulou, A., & Ledward, D. A. (2001). Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. *Food Hydrocolloids.* 15: 263–269.

Monteiro, S. R., Rebelo, S., da Cruz e Silva, O. B., & Lopes da Silva, J. A. (2013). The influence of galactomannans with different amount of galactose side chains on the gelation of soy proteins at neutral pH. *Food Hydrocolloids.* 33: 349–360.

Mota, M. J., Lopes, R. P., Delgadillo, I., & Saraiva, J. A. (2013). Microorganisms under high pressure — Adaptation, growth and biotechnological potential. *Biotechnology Advances.* 31: 1426–1434.

Okamoto, M., Kawamura, Y., & Hayashi, R. (1990). Application of high pressure to food processing: textural comparison of pressure and heat-induced gels of food proteins. *Agricultural and Biological Chemistry.* 54: 183-189.

Partschefeld, C., Richter, S., Schwarzenbolz, U., & Henle, T. (2007). Modification of β-lactoglobulin by microbial transglutaminase under high hydrostatic pressure: Localization of reactive glutamine residues. *Biotechnology Journal*, *2*(4), 462–468.

Pedrosa, C., & Ferreira, S.T. (1994). Deterministic pressure-induced dissociation of vicilin, the 7S storage globulin from pea seeds: effects of pH and cosolvents on oligomer stability. *Biochemistry* 33: 4046–4055.

Peñas, E., Préstamo, G., & Gomez, R. (2004). High pressure and the enzymatic hydrolysis of soybean whey proteins. *Food Chemistry.* 85: 641–648.

Peñas, E., Préstamo, G., Polo, F., & Gomez, R. (2006). Enzymatic proteolysis, under high pressure of soybean whey: Analysis of peptides and the allergen Gly m 1 in the hydrolysates. *Food Chemistry.* 99: 569–573.

Peyrano, F., Speroni, F., & Avanza, M. V. (2016). Physicochemical and functional properties of cowpea protein isolates treated with temperature or high hydrostatic pressure. *Innovative Food Science and Emerging Technologies.* 33: 38–46.

Pons, M., & Fiszman, S. M. (1996). Instrumental texture profile analysis with particular reference to gelled systems. *Journal of Texture Studies.* 27: 597–624.

Puppo, C., Chapleau, N., Speroni, F., De Lamballerie-Anton, M., Michel, F., Añón, C., & Anton, M. (2004). Physicochemical modifications of high-pressure-treated soybean protein isolates. *Journal of Agricultural and Food Chemistry.* 52: 1564–71.

Puppo, M. C., Beaumal, V., Speroni, F., de Lamballerie, M., Añón, M. C., & Anton, M. (2011).

β-Conglycinin and glycinin soybean protein emulsions treated by combined temperature–high-pressure treatment. *Food Hydrocolloids.* 25: 389–397.

Puppo, M. C., Speroni, F., Chapleau, N., De Lamballerie, M., Añón, M. C., & Anton, M. (2005). Effect of high-pressure treatment on emulsifying properties of soybean proteins. *Food Hydrocoloidsl.* 19: 289–296.

Qin, Z., Guo, X., Lin, Y., Chen, J., Liao, X., Hu, X., & Hu, J. (2013). Effects of high hydrostatic pressure on physicochemical and functional properties of walnut (*Juglans regia* L.) protein isolate. *Journal of the Science of Food and Agriculture.* 93: 1105–1111.

Queirós, R. P., Gouveia, S., Saraiva, J. A., & Lopes-da-Silva, J. A. (2019). Impact of pH on the high-pressure inactivation of microbial transglutaminase. *Food Research International*, *115*(August 2018), 73–82.

Rao, M. A., & Lopes da Silva, J. A. (2007). Role of rheological behavior in sensory assessment of foods and swallowing. In: Rheology of Fluid and Semisolid Foods: Principles and Applications, pp. 403-426. Rao, M. A., Ed., 2nd ed. Springer Science+Business Media LLC, NY, USA.

Renzetti, S., Dal Bello, F., & Arendt, E. K. (2008). Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free flours treated with a microbial transglutaminase. *Journal of Cereal Science*, *48*(1), 33–45.

Savadkoohi, S., Bannikova, A., Mantri, N., & Kasapis, S. (2014). Structural modification in condensed soy glycinin systems following application of high pressure. *Food Hydrocolloids.* 53: 115–124.

Schäfer, C., Zacherl, C., Engel, K. H., Neidhart, S., & Carle, R. (2007). Comparative study of gelation and cross-link formation during enzymatic texturisation of leguminous proteins. *Innovative Food Science and Emerging Technologies*, *8*(2), 269–278.

Sharma, R., Lorenzen, P. C., & Qvist, K. B. (2001). Influence of transglutaminase treatment of skim milk on the formation of ε-(γ-glutamyl)lysine and the susceptibility of individual proteins towards crosslinking. *International Dairy Journal*, *11*(10), 785–793.

Somkuti, J., & Smeller, L. (2013). High pressure effects on allergen food proteins. *Biophysical Chemistry.* 183: 19–29.

Speroni, F., & Añón, M. C. (2013). Cold-set gelation of high pressure-treated soybean proteins. *Food Hydrocoloidsl.* 33: 85–91.

Speroni, F., Añón, M. C., & de Lamballerie, M. (2010). Effects of calcium and high pressure on soybean proteins: A calorimetric study. *Food Research International.* 43: 1347–1355.

Speroni, F., Beaumal, V., de Lamballerie, M., Anton, M., Añón, M. C., & Puppo, M. C. (2009). Gelation of soybean proteins induced by sequential high-pressure and thermal treatments. *Food Hydrocolloids.* 23: 1433–1442.

Speroni, F., Jung, S., & de Lamballerie, M. (2010). Effects of calcium and pressure treatment on thermal gelation of soybean protein*. Journal of Food Science.* 75: 30-38.

Sun, M., Mu, T., Sun, H., & Zhang, M. (2014). Digestibility and structural properties of thermal and high hydrostatic pressure treated sweet potato (*Ipomoea batatas* L.) protein. *Plant Foods for Human Nutrition.* 69: 270–275.

Tang, C. H., Li, L., & Yang, X. Q. (2006). Influence of transglutaminase-induced cross-linking on in vitro digestibility of soy protein isolate. *Journal of Food Biochemistry*, *30*, 718–731.

Tang, C.H., & Ma, C. Y. (2009). Effect of high pressure treatment on aggregation and structural properties of soy protein isolate. *LWT - Food Science and Technology.* 42: 606–611.

Terefe, N. S., Buckow, R., & Versteeg, C. (2014). Quality-related enzymes in fruit and vegetable products: effects of novel food processing technologies, part 1: High-pressure processing. *Critical Reviews in Food Science and Nutrition.* 54: 24–63.

Torrezan, R., Thamb, W., Bell, A. E., Frazier, R. A., & Cristianini, M. (2007). Effects of high pressure on functional properties of soy protein. *Food Chemistry.* 104: 140–147.

Trespalacios, P., & Pla, R. (2007a). Simultaneous application of transglutaminase and high pressure to improve functional properties of chicken meat gels. *Food Chemistry*, *100*(1), 264–272.

Trespalacios, P., & Pla, R. (2007b). Synergistic action of transglutaminase and high pressure on chicken meat and egg gels in absence of phosphates. *Food Chemistry*, *104*(4), 1718–1727.

Tsevdou, M. S., Eleftheriou, E. G., & Taoukis, P. S. (2013). Transglutaminase treatment of

thermally and high pressure processed milk: Effects on the properties and storage stability of set yoghurt. *Innovative Food Science & Emerging Technologies*, *17*, 144–152.

Tsevdou, M., Soukoulis, C., Cappellin, L., Gasperi, F., Taoukis, P. S., & Biasioli, F. (2013). Monitoring the effect of high pressure and transglutaminase treatment of milk on the evolution of flavour compounds during lactic acid fermentation using PTR-ToF-MS. *Food Chemestry*, *138*(4), 2159–2167.

Turgeon, S. L., Schmitt, C., & Sanchez, C. (2007). Protein-polysaccharide complexes and coacervates. *Current Opinion in Colloid and Interface Science.* 12: 166–178.

Wang, J. M., Yang, X. Q., Yin, S. W., Zhang, Y., Tang, C. H., Li, B. S., Yuan, D. B., & Guo, J. (2011). Structural rearrangement of ethanol-denatured soy proteins by high hydrostatic pressure treatment. *Journal of Agricultural and Food Chemistry.* 59: 7324–7332.

Wang, X. S., Tang, C. H., Li, B. S., Yang, X. Q., Li, L., & Ma, C. Y. (2008). Effects of high-pressure treatment on some physicochemical and functional properties of soy protein isolates. *Food Hydrocolloids.* 22: 560–567.

Wang, Z., Ju, X., He, R., Yuan, J., & Aluko, R. E. (2015). Effect of high pressure treatment on rapeseed protein microparticle properties and gastrointestinal release behavior of the encapsulated peptides. *Food Research International.* 77: 549–555.

Winter, R., Lopes, D., Grudzielanek, S., & Vogtt, K. (2007). Towards an understanding of the temperature/pressure configurational and free-energy landscape of biomolecules. *Journal of Non-Equilibrium Thermodynamics.* 32: 41–97.

Wu, G., Fanzo, J., Miller, D. D., Pingali, P., Post, M., Steiner, J. L., & Thalacker-Mercer, A. E. (2014). Production and supply of high-quality food protein for human consumption: Sustainability, challenges, and innovations. *Annals of the New York Academy of Sciences.* 1321: 1–19.

Yang, H., Yang, A., Gao, J., & Chen, H. (2014). characterization of physicochemical properties and IgE-binding of soybean proteins derived from the HHP-treated seeds. *Journal of Food Science* 79: C2157–C2163.

Yin, S. W., Tang, C. H., Wen, Q. B., Yang, X. Q., & Li, L. (2008). Functional properties and in vitro trypsin digestibility of red kidney bean (*Phaseolus vulgaris* L.) protein isolate: Effect

of high-pressure treatment. *Food Chemistry.* 110: 938–945.

Yokoyama, K., Nio, N., & Kikuchi, Y. (2004). Properties and applications of microbial transglutaminase. *Applied Microbiology and Biotechnology*, *64*(4), 447–454.

Zhang, H., Li, L., & Mittal, G. S. (2010). Effects of high pressure processing on soybean beta-conglycinin. *Journal of Food Process Engineering.* 33: 568–583.

Zhang, H., Li, L., Tatsumi, E., & Kotwal, S. (2003). Influence of high pressure on conformational changes of soybean glycinin. *Innovative Food Science and Emerging Technologies.* 4: 269–275.

Zhang, T., Jiang, B., Miao, M., Mu, W., & Li, Y. (2012). Combined effects of high-pressure and enzymatic treatments on the hydrolysis of chickpea protein isolates and antioxidant activity of the hydrolysates. *Food Chemistry.* 135: 904–912.

Zhao, J., Zhou, T., Zhang, Y., Ni, Y., & Li, Q. (2015). Optimization of arachin extraction from defatted peanut (*Arachis hypogaea*) cakes and effects of ultra-high pressure (UHP) treatment on physiochemical properties of arachin. *Food and Bioproducts Processing.* 95: 38–46.

Zhou, H., Wang, C., Ye, J., Chen, H., Tao, R., & Cao, F. (2016a). Effects of high hydrostatic pressure treatment on structural, allergenicity, and functional properties of proteins from ginkgo seeds. *Innovative Food Science and Emerging Technologies.* 34: 187–195.

Zhou, H., Wang, C., Ye, J., Tao, R., Chen, H., & Cao, F. (2016b). effects of enzymatic hydrolysis assisted by high hydrostatic pressure processing on the hydrolysis and allergenicity of proteins from ginkgo seeds. *Food and Bioprocess Technology.* 9: 839–848.

# CHAPTER 3

## Impact of pH on the High-Pressure Inactivation of Microbial Transglutaminase Using Response Surface Methodology

## 3.1. INTRODUCTION

Microbial transglutaminase (MTG) (EC 2.3.2.13) is an enzyme that catalyzes the acyl transfer reaction between the γ-carboxyamide group of protein-bound glutamine residues and primary amines, preferentially the ε–amino group of lysine residues. As previously discussed (§2.6), the formation of additional intra- and/or intermolecular crosslinks between proteins, promoted by MTG during food processing, may cause changes in the techno-functional properties of proteins, and consequently in the appearance and texture of food systems (Motoki & Seguro, 1998). Several reviews have been written over the past few years discussing the characteristics and applications of MTG on food products (de Góes-Favoni & Bueno, 2014; Gaspar & de Góes-Favoni, 2015; Kieliszek & Misiewicz, 2014).

The compact structure of certain proteins (*e.g.*, β-lactoglobulin or lysozyme) often leads to a reduced accessibility of MTG to their reactive residues (glutamines and lysines). High-pressure changes the structural organization of proteins (Ledward, 2000) and thus can expose otherwise inaccessible MTG's target residues, which are buried within the protein tertiary structure. Hence, there has been a growing interest in combining high-pressure processing (HPP) with MTG to optimize the enzyme action and to achieve the desired improvement of food characteristics, mainly textural properties. HPP treatments typically increase the polymerization activity of MTG towards different proteins (Nonaka, Ito, Sawa, Motoki, & Nio, 1997). This strategy was already successfully tested in egg (Ma, Lozano-Ojalvo, Chen, Lopez-Fandiño, & Molina, 2015) and dairy proteins (Menéndez, Schwarzenbolz, Partschefeld, & Henle, 2009), and also in fish (Herranz, Tovar, Borderias, & Moreno, 2013) and meat products (Trespalacios & Pla, 2007).

The successful application of MTG-HPP combined strategies requires in-depth knowledge of the effect of HPP on MTG activity. The part of the work described in this chapter aimed to contribute to the deepening of knowledge in this area. HPP effects on the catalytic activity of enzymes are diverse and dependent on a set of factors, which include the origin of the enzyme, nature of substrates, medium composition (pH, salts, food matrix) and processing conditions (pressure, temperature, time, single- or multi-cycle processes). In general, due to the structural changes caused by high pressures, there is a change in enzyme functionality (catalytic activity and substrate specificity) that in most cases results in decreased activity, especially for pressures above 400 MPa. Nevertheless, enzyme activation has been described,

especially for relatively low pressures (up to 300 MPa) (Chakraborty, Kaushik, Rao, & Mishra, 2014; Eisenmenger & Reyes-De-Corcuera, 2009; Mozhaev, Lange, Kudryashova, & Balny, 1996).

Regarding MTG stability under high pressure, the enzyme typically showed a moderate resistance to pressure if compared to other enzymes (*e.g.*, polygalacturonase from tomato which can be almost fully inactivated at around 400 MPa (Rodrigo et al., 2006) whereas peroxidase from horseradish requires 1 GPa for a ~40% inactivation (Prestamo, Arabas, Fonberg-Broczek & Arroyo, 2001). Different degrees of inactivation of MTG were reported for pressures of 500 MPa and above (Lauber, Krause, Klostermeyer, & Henle, 2003; Lauber, Noack, Klostermeyer, & Henle, 2001; Lee & Park, 2002; Nonaka et al., 1997), depending on the HPP time and environmental conditions.

Pressure inactivation of enzymes occurs accordingly to a complex mechanism where the major driven force is the volume reduction. The increase of pressure leads to the loss of empty spaces between protein chains (cavities), exposing otherwise unexposed side chains to the solvent that are consequently hydrated, causing proteins to unfold (Meersman, Smeller, & Heremans, 2006), and in the case of enzymes, rendering them inactive. The inactivation process associated to protein unfolding is accompanied by conformational changes at different levels and of variable extent, depending on the enzyme, the environment and the HPP conditions (Boonyaratanakornkit, Park, & Clark, 2002). Different mechanisms for pressure-induced stabilization and activation of enzymes have been described as well, related to changes in intermolecular interactions, mainly stabilization of hydrogen bonds, disruption of bound water and hydration of charged groups (Eisenmenger & Reyes-De-Corcuera, 2009).

The purpose of this part of the work was to provide insights on the effect of several combined factors (pressure, holding time, and temperature) on the inactivation of MTG at different pH values, in order to further optimize the application of the enzyme to modify protein functionality, in combination with HPP, as it will be described in chapters 5 and 6.

## 3.2. MATERIALS AND METHODS

### 3.2.1. MATERIALS

Activa® Transglutaminase (100 U·g$^{-1}$; 1 U is defined as the amount of enzyme that catalyzes the formation of 1 μmol hydroxamate from hydroxylamine and carbobenzoxy-L-glutaminylglycine per min at pH 6.0, 37°C) was a kind gift from Ajinomoto Foods Europe SAS (Hamburg, Germany). γ-Glutamyl donor substrate (Z-glutaminylglycin (Z-Gln-Gly)) was obtained from Sigma-Aldrich (Steinheim, Germany). Hydroxylamine was obtained from Merck Millipore (Darmstadt, Germany). Glutathione was purchased from Chemopharma (Vienna, Austria). All other reagents were of analytical grade.

### 3.2.2. Experimental Design and modelling

The experimental design adopted to analyze the effect of high-pressure processing conditions (pressure, temperature, holding time) on the inactivation of MTG at different pH (4, 5, 6 and 7) was a face-centred composite design. Previous studies on MTG inactivation served as the basis for the screening of the factors and corresponding levels. The chosen lower limit for pressure was 200 MPa, considering that MTG is resistant to pressure and very little inactivation is observable below this value (Lauber et al., 2001; Menéndez et al., 2006), whereas 600 MPa was chosen as the upper limit, since it is the maximum pressure for industrial HPP equipment (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015). Regarding the temperature, the lower level was defined as 20 ºC, as one of the main advantages of HPP is being a non-thermal technology, and the upper level was set at 40 ºC since it is near MTG's optimal temperature at atmospheric pressure (Ando et al. 1989). Concerning the holding time, the lower level was defined as 10 min since the extent of the MTG inactivation below this time is small (Lauber et al. 2001; Menéndez et al. 2006), and the upper level was defined as 30 min since it is generally accepted that HPP treatments during longer times are commercially impracticable.

A set of 20 experiments, including 6 replicates at the central point, were performed in randomized order. The samples were treated in triplicates and analyzed in duplicates. The response, *i.e.* the residual activity ($A_r$), was defined as (Eq. 3.1):

$$A_r = \frac{A_t}{A_0} \quad (3.1)$$

Where $A_t$ is the activity at time $t$ and $A_0$ the activity of the untreated enzyme. At the end of the experiment, 60 observations of the residual enzymatic activity $A_r$ were obtained for each

pH level, by setting different experimental values of pressure (P), temperature (T) and treatment time (t).

For the description of the response, $A_r$, a quadratic polynomial equation (Eq. 3.2) and its subsets were evaluated. The general formulation of the model was as follows:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i<j} \sum \beta_{ij} x_i x_j \quad (3.2)$$

Where $Y$ is the response (dependent variable), $n$ is the number of independent variables, $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are model coefficients, and $x_i$ and $x_j$ are the independent variables (Baş & Boyacı, 2007). In this work, one regression model was constructed for each pH level, by setting $Y = A_r$ at the corresponding pH, considering $n$=3 with $x_1$, $x_2$, and $x_3$ representing dimensionless coded forms of pressure, temperature, and holding time, respectively.

### 3.2.3. Sample Preparation

Solutions of 0.04 g·mL-1 of Activa® transglutaminase were prepared in McIlvaine buffer at acidic (4, 5 and 6) and neutral pH (7), placed in polyethylene tubes and kept at 4 °C until HPP (up to 24 h).

### 3.2.4. Pressure Treatments

The MTG samples were treated at the conditions defined in Table 3.1., using a hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United Kingdom). This equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature. The pressurization fluid was a mixture (60:40) of water and propylene glycol. The vessel and samples were previously conditioned for 15 min to the temperature of the treatment. The increase of temperature due to adiabatic heating was approximately 4 °C for every 100 MPa, decreasing in about 5 min to the set temperature. The compression and decompression rates were *c.a.* 250 $MPa \cdot min^{-1}$ and 450 $MPa \cdot min^{-1}$, respectively.

Table 3.1. Levels of independent variables per the experimental design and resultant $A_r$ at different pH values

| Real | | | $A_r$ | | | |
|---|---|---|---|---|---|---|
| **P (MPa)** | **T (ºC)** | **t (min)** | **pH 4** | **pH 5** | **pH 6** | **pH 7** |
| 200 | 20 | 10 | 0.843 ± 0.009 | 0.995 ± 0.006 | 0.931 ± 0.022 | 0.963 ± 0.003 |
| 200 | 20 | 30 | 0.604 ± 0.004 | 0.916 ± 0.010 | 1.016 ± 0.024 | 1.069 ± 0.004 |
| 200 | 30 | 20 | 0.496 ± 0.013 | 0.908 ± 0.035 | 0.946 ± 0.018 | 0.964 ± 0.035 |
| 200 | 40 | 10 | 0.500 ± 0.034 | 0.901 ± 0.027 | 0.945 ± 0.018 | 0.993 ± 0.015 |
| 200 | 40 | 30 | 0.362 ± 0.018 | 0.868 ± 0.024 | 0.914 ± 0.005 | 0.998 ± 0.020 |
| 400 | 20 | 20 | 0.381 ± 0.009 | 0.698 ± 0.009 | 0.861 ± 0.018 | 0.916 ± 0.016 |
| 400 | 30 | 10 | 0.338 ± 0.009 | 0.656 ± 0.007 | 0.845 ± 0.017 | 0.982 ± 0.001 |
| 400 | 30 | 20 | 0.308 ± 0.034 | 0.638 ± 0.020 | 0.880 ± 0.008 | 0.933 ± 0.019 |
| 400 | 30 | 20 | 0.277 ± 0.016 | 0.657 ± 0.011 | 0.881 ± 0.029 | 0.953 ± 0.014 |
| 400 | 30 | 20 | 0.277 ± 0.013 | 0.658 ± 0.002 | 0.899 ± 0.006 | 0.906 ± 0.016 |
| 400 | 30 | 20 | 0.278 ± 0.007 | 0.639 ± 0.014 | 0.887 ± 0.005 | 0.961 ± 0.026 |
| 400 | 30 | 20 | 0.288 ± 0.019 | 0.642 ± 0.022 | 0.903 ± 0.015 | 0.958 ± 0.025 |
| 400 | 30 | 20 | 0.282 ± 0.017 | 0.625 ± 0.014 | 0.903 ± 0.023 | 0.941 ± 0.025 |
| 400 | 30 | 30 | 0.249 ± 0.011 | 0.398 ± 0.012 | 0.861 ± 0.004 | 0.943 ± 0.009 |
| 400 | 40 | 20 | 0.248 ± 0.032 | 0.426 ± 0.011 | 0.853 ± 0.018 | 0.965 ± 0.042 |
| 600 | 20 | 10 | 0.242 ± 0.010 | 0.146 ± 0.002 | 0.281 ± 0.024 | 0.805 ± 0.015 |
| 600 | 20 | 30 | 0.333 ± 0.003 | 0.119 ± 0.002 | 0.198 ± 0.010 | 0.645 ± 0.011 |
| 600 | 30 | 20 | 0.239 ± 0.029 | 0.121 ± 0.007 | 0.207 ± 0.003 | 0.729 ± 0.039 |
| 600 | 40 | 10 | 0.215 ± 0.027 | 0.107 ± 0.007 | 0.213 ± 0.004 | 0.759 ± 0.007 |
| 600 | 40 | 30 | 0.214 ± 0.012 | 0.089 ± 0.003 | 0.111 ± 0.004 | 0.439 ± 0.013 |

$x_1$, $x_2$, and $x_3$ represent dimensionless coded forms of pressure, temperature, and holding time, respectively. $A_r$ values are presented as a mean ± standard deviation (n=3).

### 3.2.5. Enzyme Activity Measurement

After the HPP treatments, the samples were kept at 4 ºC, and the MTG activity was measured using the method described by Folk and Cole (1966) with a few modifications. MTG solution (50 µL) in McIlvaine buffer was added to 100 µL of substrate solution (containing 0.1 M hydroxylamine, 10 mM glutathione, 30 mM CBZ-L-glutaminylglycine, in McIlvaine buffer at pH 6.0) and incubated 10 min at 37 ºC. Then the reaction was stopped using 100 µL of ferric chloride-trichloracetic acid reagent (1 vol 12% HCl, 1 vol 12% trichloroacetic, and 1 vol 5% ferric trichloride solution in 0.1M HCl) and the resultant color was measured at 525 nm using a spectrophotometer (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA). The enzyme activity was expressed as the change in absorbance per unit of time (min) and per unit volume (mL) of the enzyme solution (4 U MTG/mL).

### 3.2.6. Kinetic Data Calculation

Inactivation of MTG followed a first-order kinetics, with the decrease of enzyme activity with time being described by,

$$A_t = A_0 e^{-tk} \tag{3.3}$$

and thus

$$lnA_t = ln\, A_0 - kt \tag{3.4}$$

$$\ln A_r = -k \times t \tag{3.5}$$

where $A_t$ is the enzymatic activity at time $t$; $A_0$ is initial activity, $A_r$ is the residual enzymatic activity ($A_t/A_0$), $t$ is the treatment time and $k$ is the first-order inactivation rate constant.

The inactivation first-order rate constants ($k$, $min^{-1}$) were computed from $A_r$ generated by the respective model at different pressure/temperature combinations.

The rate constant was used to describe the temperature and pressure dependence of the MTG inactivation kinetics. The temperature dependence was characterized using the linear form of the Arrhenius equation (Eq. 3.6):

$$\ln k = \ln A - \frac{E_a}{R}\left(\frac{1}{T}\right) \tag{3.6}$$

where $A$ is the frequency factor, $E_a$ is the activation energy ($J \cdot mol^{-1}$), $T$ is the experimental temperature (K), and $R$ is the universal gas constant (8.314 $J \cdot mol^{-1} \cdot K^{-1}$).

The pressure dependence was characterized using the linear form of the Eyring equation (Eq. 3.7):

$$\ln k = \ln k_{ref} - \frac{V_a}{R \times T}\left(P - P_{ref}\right) \quad (3.7)$$

where $P$ and $P_{ref}$ are experimental and reference pressures (MPa), respectively, $V_a$ is the activation volume ($cm^3 \cdot mol^{-1}$), $k$ and $k_{ref}$ are the inactivation rates ($min^{-1}$) at $P$ and $P_{ref}$ (0.1 MPa), respectively, $T$ is the experimental temperature (K) and $R$ is the universal gas constant (8.314 $J \cdot mol^{-1} \cdot K^{-1}$).

### 3.2.7. Statistical Analysis

The regression models were constructed using a stepwise procedure where the variables were included or removed from the model based on its *p*-value (and a significance of $\alpha$=0.05). The iterative method ended when no more variables were eligible for inclusion or removal. The coefficients of the model were estimated by maximum likelihood. Their standard errors and *p*-values were used to inspect the statistical significance of the coefficients.

The model summary statistics included model significance, model goodness of fit and model predictive ability. The statistical significance of the regression models was assessed through an analysis of variance (ANOVA) and the evaluation of the F statistics and its significance. The goodness of fit was evaluated by the coefficient of determination ($0\% \leq R^2 \leq 100\%$) and the $R^2$ adjusted for the number of terms in the model (adjusted $R^2$). Furthermore, the model predictive ability was quantified from predicted $R^2$ obtained as the $R^2$ evaluated from residuals of observations not considered in the construction of the model (*i.e.*, each observation was removed from the dataset, the regression model was estimated and the corresponding residual was evaluated). Note that predicted $R^2$ is expected to be always lower than $R^2$, and that a value markedly lower than $R^2$ suggests model overfitting to the data.

Finally, the variance of the data was decomposed into the contribution of the model terms (discriminating between the contribution of linear, quadratic and 2-way interaction terms, which sums up to $R^2$) and error terms (distinguishing lack of fit and pure error). The lack of fit of the

models was also investigated as experimental data contained replicate measures, where differences between replicate measures are assumed to represent pure error in the analysis.

All statistical analyses were performed with Minitab v17 (PA, USA) and Microsoft Excel 2010 (Microsoft Office System, USA), considering a statistical significance of $\alpha = 0.05$.

## 3.3. RESULTS AND DISCUSSION

### 3.3.1. REGRESSION MODELLING

The $A_0$ (mean ± standard deviation) of MTG at the different pH levels (4, 5, 6, and 7) was $2.07 \times 10^{-3} \pm 4.68 \times 10^{-5}$, $5.16 \times 10^{-3} \pm 6.81 \times 10^{-5}$, $5.62 \times 10^{-3} \pm 2.89 \times 10^{-5}$, and $4.87 \times 10^{-3} \pm 9.21 \times 10^{-5}$ $OD \cdot min^{-1} \cdot mL^{-1}$, respectively. These results agree with the information already published on the optimum pH of MTG (Ando et al., 1989), where $A_0$ is higher at pH 6, presenting a lower activity at lower pH values. It is noteworthy to mention MTG's ability to maintain activity at pH 4.

Values obtained for the relative residual enzyme activity ($A_r$) (Eq. 3.1) under the different HPP processing conditions and pH values are shown in Table 3.1. and illustrated in Figure 3.1. It is quite clear from Figure 3.1(A) that the average $A_r$ values decrease with increasing pressure values, for all pH levels. Furthermore, average $A_r$ values are higher for higher pH levels, for all pressure values, except for pH 4 and pressure 600 MPa. With respect to the remaining variables (Figures 3.1(B) and 3.1(C)), the $A_r$ tendency to decrease for increasing temperature or holding time is less evident than that observed for pressure. Overall, Figure 3.1. suggests that the variation of the $A_r$ values are more associated with pH and pressure variations rather than with temperature and holding time variations. The model described in Equation 3.2 was fitted to these experimental data at the four pH values under study. The estimates of the models coefficients are presented in Table 3.2. Constant and linear terms on pressure, temperature, and holding time are statistically significant for all pH models ($p < 0.05$). With respect to quadratic terms, pressure is significant for all models whereas temperature is only significant for pH 4 and holding time is significant for pH 5. Models with pH 6 and 7 excluded quadratic temperature and holding time terms. Finally, 2-way interaction terms are significant in the models except for pH 5 for which all interaction terms were not considered. The negative coefficients on each term reported in Table 3.2. indicate that all linear, quadratic and interaction

terms favor the inactivation since the increase of any of these parameters results in a lower $A_r$. The only exception is observed at pH 4, where the existing quadratic and interaction terms exhibit a positive coefficient.

Table 3.2. Estimated coded coefficients for the four developed models

| **Estimated coded Coefficients** | **pH 4** | **pH 5** | **pH 6** | **pH 7** |
|---|---|---|---|---|
| Constant | 0.285 ± 0.006 | 0.613 ± 0.012 | 0.877 ± 0.005 | 0.946 ± 0.007 |
| x1 | -0.156 ± 0.006 | -0.400 ± 0.011 | -0.374 ± 0.005 | -0.161 ± 0.007 |
| x2 | -0.086 ± 0.006 | -0.048 ± 0.011 | -0.025 ± 0.005 | -0.024 ± 0.007 |
| x3 | -0.037 ± 0.006 | -0.041 ± 0.011 | -0.012 ± 0.005* | -0.041 ± 0.007 |
| x1·x1 | 0.090 ± 0.010 | -0.059 ± 0.019* | -0.301 ± 0.007 | -0.109 ± 0.010 |
| x2·x2 | 0.037 ± 0.010 | - | - | - |
| x3·x3 | - | -0.047 ± 0.019** | - | - |
| x1·x2 | 0.054 ± 0.006 | - | - | -0.026 ± 0.008* |
| x1·x3 | 0.058 ± 0.006 | - | -0.030 ± 0.006 | -0.074 ± 0.008 |
| x2·x3 | - | - | -0.017 ± 0.006** | -0.032 ± 0.008 |

Values are presented as a mean ± standard error; $x_1$, $x_2$, and $x_3$ represent dimensionless coded forms of pressure, temperature, and holding time, respectively. All terms are significant ($p < 0.001$) otherwise marked: *0.001 ≤ p < 0.01; **0.01 ≤ $p$ < 0.05.

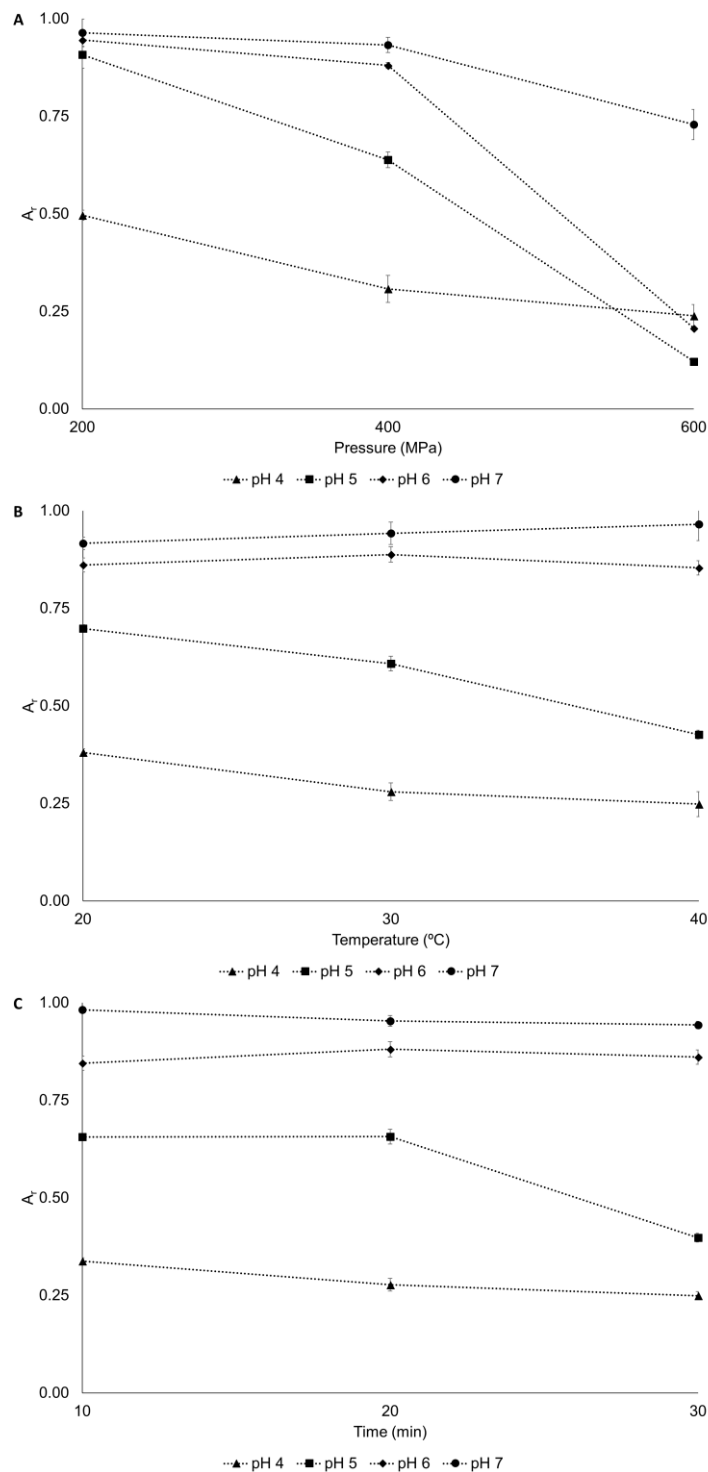



Figure 3.1. Main effects of the independent variables on the residual activity (Ar) of microbial transglutaminase at four different pH. A – Effects of pressure (temperature = 30 ºC, time = 20 min); B – Effects of temperature (pressure = 400 MPa, time = 20 min); C – Effects of time (pressure = 400 MPa, temperature = 30 ºC).

On the overall assessment of the models, Table 3.3. shows the models summary statistics. All models were statistically significant, with the ANOVA showing large values for the F statistics (F>120 for all models) and, concordantly, low values of the corresponding *p*-values. Regarding the goodness of fit and predictive ability of the models, Table 3.3. shows that $R^2$, adjusted $R^2$ and predicted $R^2$ were large for all pH levels, hence supporting that a large percentage of the variability of the data is explained by the constructed models (with values ranging from 0.93 and 0.99, in all cases). Furthermore, these statistics showed to exhibit similar values within each pH thus excluding over parametrization and overfitting of the constructed models. Finally, the significant lack of fit exhibited at all pH levels ($P < 0.001$) suggests that a (statistically) relevant part of the data variability is not explained by the model. It is however worth to refer that lack of fit weights less than 4 % of the total variability to be explained by the model, and exhibits similar values to that of the pure error term. Furthermore, plots of residuals *vs.* the predicted response showed no defined structure and the normal probability plots of residuals exhibited a straight line (*some examples are shown in Figure A1 as Supplementary Material*). Thus, overall, the four models seem to be good representatives of the high-pressure inactivation of MTG at each specified pH level.

Table 3.3. additionally presents the contribution of the data variability explained by the model terms (expressed as a %), distinguishing linear, quadratic and interaction ones. It is evident that the large contribution is due to the linear terms (with values varying between 66 % and 75 % for all models). Furthermore, pressure alone is the term with the largest contribution for all models, exhibiting contribution values larger than 50 % for the linear components and larger than 75 % when also accounting for the quadratic and the interaction terms. Thus, the pressure contribution in these models surpasses the contribution of the remaining variables, including temperature. Contrarily, Lauber et al. (2001) reported that the stability of MTG was more affected by temperature than pressure, probably related to the broader temperature range analyzed by these authors, reaching 60 ºC. MTG is very stable up to 40 ºC, retaining 100 % of its activity for 10 min at this temperature (atmospheric pressure; pH 6) (Ando et al., 1989), thus it is not surprising that, within the temperature range analysed in the present work, the thermal effect is reduced when compared to the pressure effect.

Table 3.3. Model summary.

| Contribution (%) | | | | |
|---|---|---|---|---|
| | **pH 4** | **pH 5** | **pH 6** | **pH 7** |
| Model | 96.57 | 96.38 | 99.31 | 94.31 |
| Linear | 71.21 | 93.84 | 74.72 | 66.56 |
| P | 52.26 | 91.54 | 74.32 | 61.24 |
| T | 15.94 | 1.32 | 0.33 | 1.40 |
| t | 3.01 | 0.98 | 0.07 | 3.92 |
| Square | 14.40 | 2.54 | 24.09 | 14.15 |
| P*P | 13.47 | 2.15 | 24.09 | 14.15 |
| T*T | 0.94 | - | - | - |
| t*t | - | 0.40 | - | - |
| 2-Way Interaction | 10.96 | | 0.50 | 13.60 |
| P*T | 5.13 | - | - | 1.33 |
| P*t | 5.83 | - | 0.38 | 10.29 |
| T*t | - | - | 0.12 | 1.98 |
| Error | 3.43 | 3.62 | 0.69 | 5.69 |
| Lack of fit | 1.73 | 3.31 | 0.38 | 3.11 |
| Pure Error | 1.70 | 0.30 | 0.30 | 2.58 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 |
| **Model Summary** | | | | |
| F-Value | 209.37 | 287.83 | 1275.64 | 123.13 |
| p-value | $< 0.001$ | $< 0.001$ | $< 0.001$ | $< 0.001$ |
| $R^2$ | 0.966 | 0.964 | 0.993 | 0.943 |
| Adjusted $R^2$ | 0.961 | 0.961 | 0.992 | 0.935 |
| Predicted $R^2$ | 0.951 | 0.955 | 0.991 | 0.929 |
| Lack-of-Fit | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |

### 3.3.2. Effects of different factors on MTG's inactivation

At all pH tested it was possible to achieve over 80 % of inactivation, except for pH 7, where the maximum inactivation was around 50 %. Nonetheless, MTG was relatively resistant to pressure, being necessary at least 400 MPa for 30 min to achieve an inactivation greater than 20 % when the enzyme was near its optimal pH (6 - 7) - Figure 3.2. Thus, MTG follows the behaviour of most monomeric proteins that only denature above 400 MPa, when at ambient temperature and neutral pH (Gross & Jaenicke, 1994). Concordantly, Henle and coworkers (Lauber et al., 2001; Menéndez et al., 2006) reported a similar low degree of inactivation for MTG under analogous conditions.

According to the developed models, at these pH (particularly at pH 7), an apparent slight activation of MTG can be observed at 200-300 MPa. This is a common effect that has been reported for several enzymes, particularly monomeric enzymes, usually observed within low pressure range and dependent on several factors such as the type of enzyme, processing conditions (*e.g.* pressure level, time and temperature), and environmental conditions (*e.g.* pH) (Chakraborty et al., 2014; Terefe, Buckow, & Versteeg, 2014). There are a couple of possible explanations for enzyme pressure activation, such as conformational changes promoted by pressure (Eisenmenger & Reyes-De-Corcuera, 2009) or the dissociation of existent aggregates (Silva & Weber, 1993).

The temperature and pressure may have antagonistic or synergetic effects in the inactivation of enzymes, depending on the enzyme and overall conditions. Both may promote enzyme denaturation, or the thermal unfolding (associated with a positive $V_a$) may be counteracted by pressure (Balny & Masson, 1993; Meersman et al., 2006). Pressure-temperature inactivation of enzymes typically displays elliptical contour diagrams as a function of pressure and temperature (Ludikhuyze, Van Loey, Indrawati, Smout, & Hendrickx, 2003). In the present study, no major interaction effects between pressure and temperature were verified for MTG's inactivation. This may be related to the thermal stability of MTG in the temperature range studied, as previously mentioned. The major impact of temperature was verified at lower pressures, in the activation of MTG. At pH 6 the increase in activity only occurred below 30 ºC, whereas at pH 7 it was verified within the whole temperature range tested, although to a lesser extent at higher temperatures – Figure 3.3.

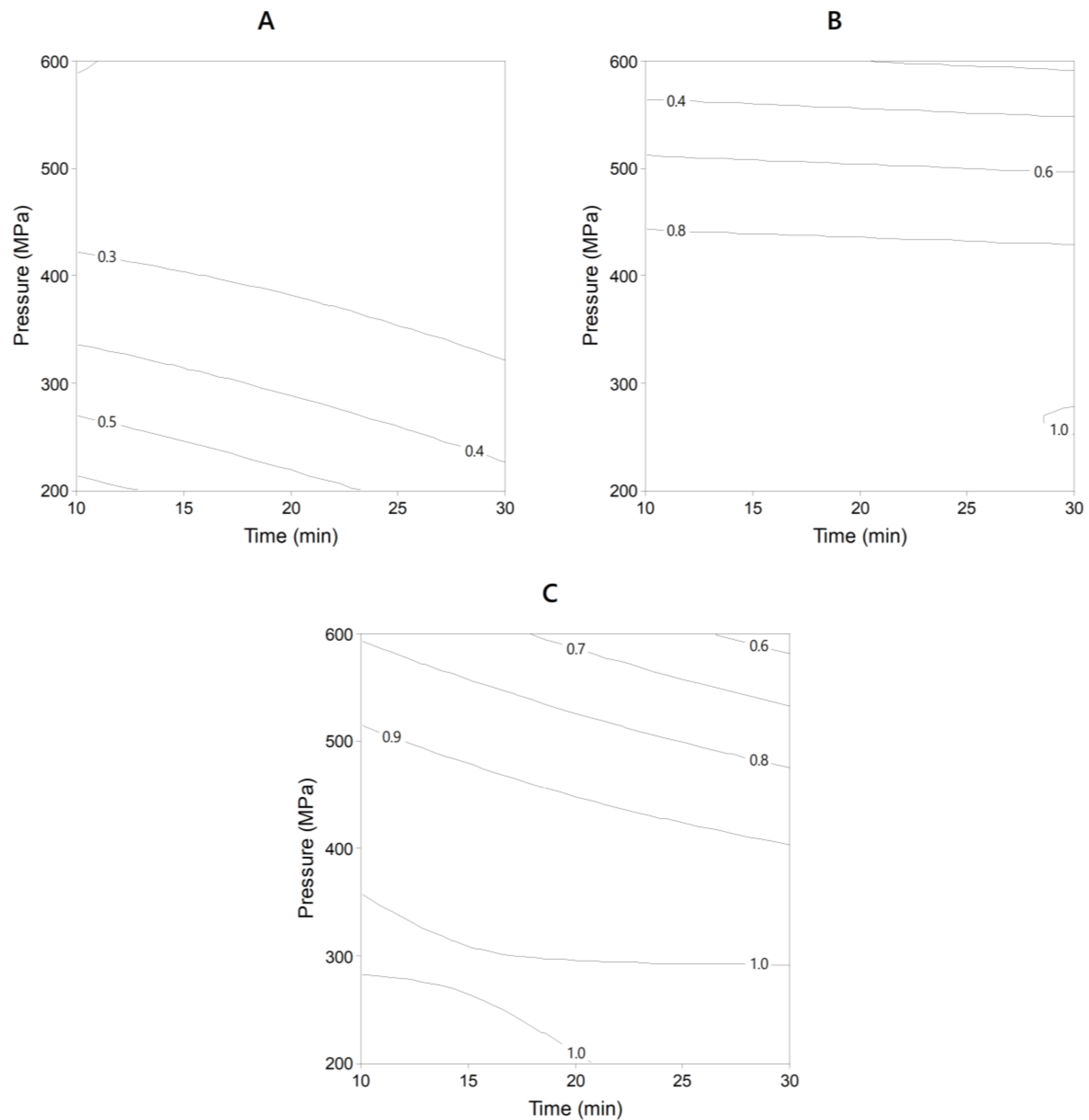



Figure 3.2. Contours of the residual activity (Ar) of microbial transglutaminase as a function of pressure and time at 30 ºC, at different pH. A - pH 4; B - pH 6; C - pH 7.

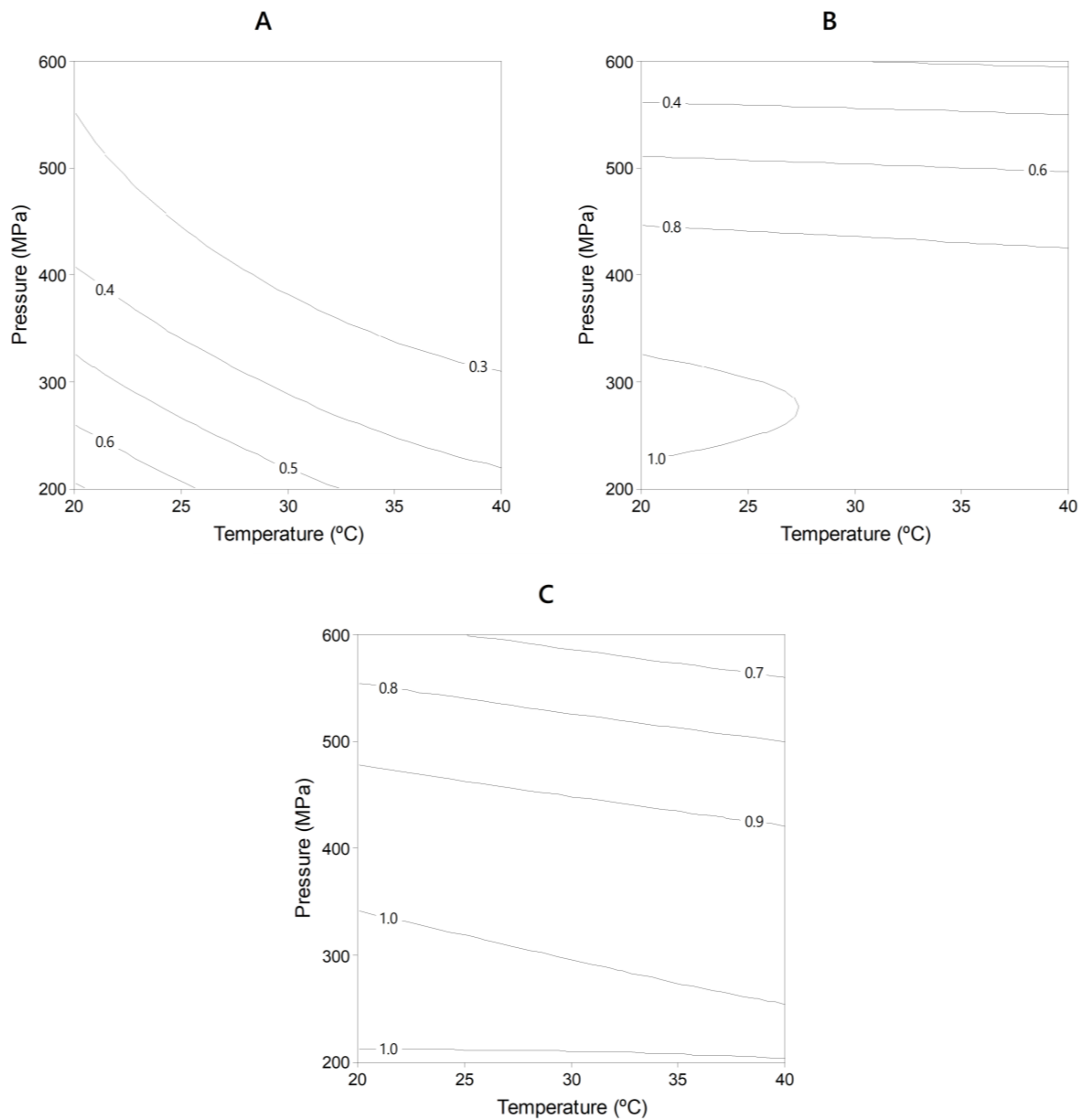



Figure 3.3. Contours of the residual activity (Ar) of microbial transglutaminase as a function of pressure and temperature after a holding time of 20 min, at different pH. A - pH 4; B - pH 6; C - pH 7.

As previously stated, the $A_0$ of MTG at pH 4 was considerably lower than at the others pH. The pH influences the stability of enzymes mainly because of the p$K_a$ values of the different ionizable groups that cause different local electrostatic effects and consequently affect the intramolecular interactions. It is worth to mention that the studied pH range is below MTG's isoelectric point (*c.a.* 9), therefore, at these pH values the enzyme's charge is positive, showing a higher density with a decrease in pH. The active site of MTG contains many residues with negatively charged side chains, namely four aspartic acid and two glutamic acid residues (Kashiwagi et al., 2002). Both residues have a side chain p$K_a$ of around 4, which means that at this pH a high number of these residues begins to protonate rendering a less active enzyme. In fact, a higher inactivation occurred at the lower pH, *i.e.* 4 and 5, with 50 % of inactivation after approximately 15 min at 200 MPa (pH 4) and 20 min at 450 MPa (pH 5) (Figure 3.2.), suggesting that pressure inactivates MTG more easily at low pH, which has already been verified for other enzymes, such as pectin methylesterase, polyphenol oxidase, peroxidase (Kaushik, Nadella, & Rao, 2015), and amylase (Riahi & Ramaswamy, 2004). The increase of pressure usually induces the dissociation of weak acids, resulting in electrostriction that, together with a higher amount of ion pairs, result in further electrostatic effects around the enzyme. The increased exposure of hydrophobic groups and their hydration induced by pressure, associated with these electrostatic effects, may consequently induce higher conformational changes and loss of enzyme activity (Gross & Jaenicke, 1994).

### 3.3.3. ESTIMATED INACTIVATION KINETICS

Inactivation kinetics strongly depends on the experimental conditions (*e.g.* type of buffer, pH, enzyme concentration, etc.), as well the accurateness of the projected parameters. For all the obtained models, MTG inactivation followed first-order kinetics. An example of the obtained MTG's inactivation kinetics, at 500 MPa and pH 6, can be seen in Figure 3.4 In a general way, *k* increased with increasing pressure and temperature within the pH range from 5 to 7 (Figure 3.5). The studies performed by Lauber et al. (2001) and Menéndez et al. (2006), at pH 6, are in line with our findings. For example, at 40 ºC and 600 MPa, we estimated an inactivation rate of 0.034 min$^{-1}$, Lauber and co-workers 0.023 min$^{-1}$ and Menéndez and co-workers 0.047 min$^{-1}$.

On the other hand, at pH 4, *k* decreased with increasing pressure but increased with increasing temperature. These results suggest that, particularly at this pH, pressure and temperature do not work synergistically, most likely because temperature affects mainly heat

labile interactions, such as hydrogen bonds (not much affected by pressure), while pressure affects mainly electrostatic and hydrophobic interactions (not as sensitive to temperature) (Ledward, 2000). It seems that at a more acidic pH, the increase of pressure leads to effects that counteract the effects of pH, probably because high pressure promotes ionization of the acidic groups, whereas the decrease of pH promotes their protonation. Worth to mention that *k* was generally higher at pH 5 and 7, indicating a faster pressure inactivation at these pH values.

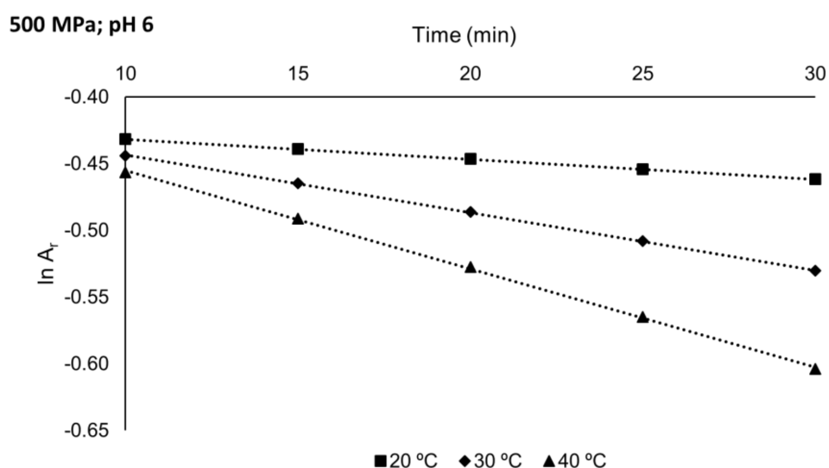



Figure 3.4. Model predicted inactivation kinetics of microbial transglutaminase at 500 MPa and pH 6 (Ar = Residual activity)

Table 3.4 shows the estimated $E_a$ for different pressure values at the different pH. At the lower pH values (*i.e.* 4 and 5), no significant changes with pressure were observed, at each pH, except for 600 MPa at pH 5 where the $E_a$ increased considerably, suggesting a higher temperature sensitivity of *k* at this pressure. On the contrary, at pH 6 and 7 there was a tendency for $E_a$ to decrease with increasing pressure, suggesting a lower temperature sensitivity at higher pressures. The temperature dependence at 200 and 300 MPa, at pH 6 and 7, and at 600 MPa at pH 4, could not be described by the Arrhenius equation. Comparing the estimated $E_a$ by our model with the values reported by Lauber et al. (2001) under analogous conditions, *i.e.* 400 MPa at pH 6, both values were relatively close, namely 51.4 and 43.8 kJ·mol$^{-1}$, respectively. Moreover, a similar decrease of $E_a$ (*c.a.* 7 kJ·mol$^{-}$1) with increasing pressure from 400 to 600 MPa was also verified, in accordance with the previous study of Lauber and co-workers.

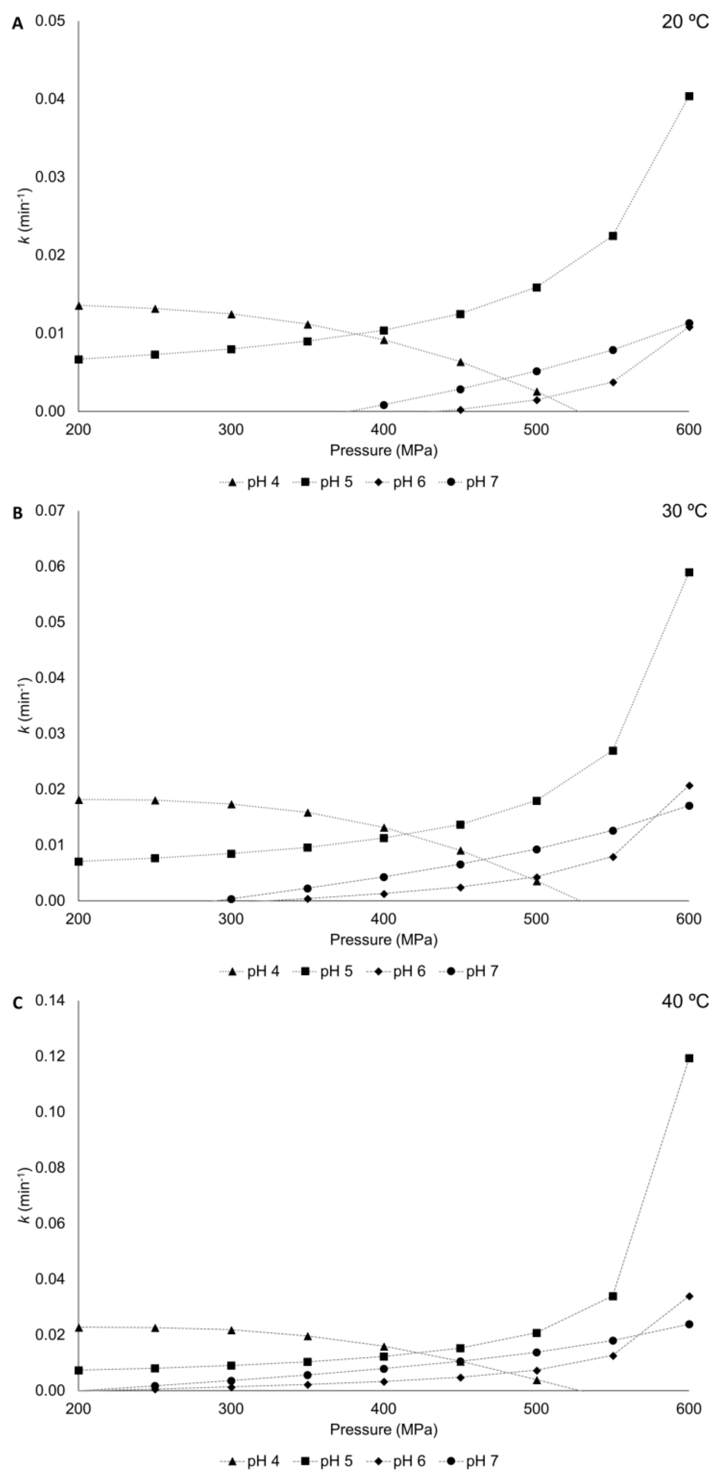



Figure 3.5. Estimated rate constants (k) for isothermal inactivation of microbial transglutaminase.

Table 3.4. Estimated activation energies (kJ·mol$^{-1}$) for inactivation of microbial transglutaminase

| P | pH 4 | | pH 5 | | pH 6 | | pH 7 | |
|---|---|---|---|---|---|---|---|---|
| (MPa) | $E_a$ ± SE | $R^2$ | $E_a$ ± SE | $R^2$ | $E_a$ ± SE | $R^2$ | $E_a$ ± SE | $R^2$ |
| 200 | 19.7 ± 1.1 | 0.99 | 20.6 ± 1.0 | 0.81 | N.E. | | N.E. | |
| 300 | 21.4 ± 1.8 | 0.99 | 22.7 ± 1.1 | 0.80 | N.E. | | N.E. | |
| 400 | 21.2 ± 3.3 | 0.98 | 25.0 ± 1.2 | 0.82 | 51.4 ± 18.5 | 0.89 | 85.4 ± 20.4 | 0.95 |
| 500 | 16.5 ± 4.5 | 0.93 | 29.1 ± 1.2 | 0.85 | 60.9 ± 10.3 | 0.97 | 37.6 ± 3.4 | 0.99 |
| 600 | N.E. | | 58.4 ± 1.9 | 0.91 | 43.6 ± 2.6 | 0.97 | 28.5 ± 1.1 | 1.00 |

$E_a$ = Activation energy; SE = standard error; $R^2$ = regression coefficient; N.E. = not estimated.

It is worth to recall that, in agreement with the Le Chatelier's principle, a $V_a < 0$ favors the pressure denaturation of proteins and subsequent inactivation (in the particular case of enzymes), whereas $V_a > 0$ favors their stability. The Eyring equation was valid within the entire temperature range (Table 3.5). A positive $V_a$ was found at pH 4, not being much influenced by the temperature, which means that increasing pressure retarded the inactivation of MTG, as expected due to the consistent decrease of the $k$ with increasing pressure (Figure 3.4), as previously discussed. Negative $V_a$ values were obtained for the other pH values. At pH 5, $V_a$ was not influenced by temperature.

Table 3.5. Estimated activation volumes (cm$^3$·mol$^{-1}$) for inactivation of microbial transglutaminase at different temperatures

| T | pH 4 | | pH 5 | | pH 6 | | pH 7 | |
|---|---|---|---|---|---|---|---|---|
| (ºC) | $V_a$ ± SE | $R^2$ | $V_a$ ± SE | $R^2$ | $V_a$ ± SE | $R^2$ | $V_a$ ± SE | $R^2$ |
| 20 | 8.6 ± 1.7 | 0.89 | -11.1 ± 1.4 | 0.90 | -58.4 ± 6.0 | 0.98 | -30.1 ± 4.7 | 0.93 |
| 30 | 8.0 ± 1.9 | 0.86 | -9.1 ± 1.0 | 0.93 | -36.5 ± 2.2 | 0.99 | -28.1 ± 5.0 | 0.86 |
| 40 | 9.0 ± 2.1 | 0.86 | -10.6 ± 1.3 | 0.92 | -26.6 ± 1.6 | 0.98 | -18.0 ± 1.4 | 0.96 |

*T = Temperature; Va = activation volume; se = standard error;* $R^2$ *= regression coefficient.*

Considering pH 6 and 7, a decreasing $V_a$ was obtained with increasing temperature. Although Lauber et al. (2001) did not identify a trend in the $V_a$ with increasing pressure in the inactivation of MTG, this trend was identified for other enzymes (Ludikhuyze, Indrawati, Van den Broeck, Weemaes, & Hendrickx, 1998). It is well described in the literature that the temperature and pressure may have antagonistic effects in the denaturation of proteins, depending on the protein and overall conditions. The main reason is that thermal unfolding is associated with a positive $V_a$ that is countered by pressure (Balny & Masson, 1993; Meersman et al., 2006). There are no studies of high-pressure inactivation of MTG at different pH, still, at similar conditions (40 ºC and pH 6), Lauber et al. (2001) and Menéndez et al. (2006) reported activation volumes of -17.4 and -10.1 $cm^3 \cdot mol^{-1}$, respectively, that are comparable to our estimation, -26.6 $cm^3 \cdot mol^{-1}$. It is important to keep in mind that these studies used a different buffer and pressurization/depressurization rates, which may have influenced the inactivation kinetics. The obtained results indicate that MTG is very stable under pressure, at least when near its optimal pH (5 – 7) and temperatures below 40 ºC.

## 3.4. CONCLUSIONS

Combining MTG treatments and HPP may be an interesting tool to tailor food proteins and develop new products and applications. Still, it is necessary to gather knowledge regarding the behaviour not only of the target proteins under pressure but also of the MTG. In our study, we verified that low pH enhances the pressure inactivation of MTG and that higher pressure (> 400 MPa) is required to achieve enzyme's inactivation near its optimal pH. The increase of both pressure and temperature increased $k$ at pH 5, 6 and 7, while the opposite was found at pH 4. It was possible to observe that at pH 4 and 5, pressure and temperature did not significantly affect $E_a$ and $V_a$, respectively. Nonetheless, an antagonistic effect of pressure and temperature on MTG inactivation is suggested to occur at pH 6 and 7, associated to the decrease of $E_a$ and $V_a$ with increasing pressure and temperature, respectively. Considering all kinetic parameters obtained in this study, and also those available in literature, it can be concluded that the developed models (at least at pH 6) accurately describe the high-pressure inactivation of MTG. Still, further research is necessary to better understand the effects of pressure on the inactivation of MTG under alkaline pH, and how the observed effects are influenced by other

food components, having in mind the real food matrices where the combined effects or HPP and MTG may have important applications.

## 3.5. References

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., and Motoki, M. (1989). Purification and characteristics of a novel transglutaminase derived from microorganisms. *Agricultural and Biological Chemistry*, 53(10), 2613-2617.

Balasubramaniam, V.M., Martínez-Monteagudo, S.I. and Gupta, R.,( 2015). Principles and application of high pressure–based technologies in the food industry. *Annual Review of Food Science and Technology*, 6(1), 435–462.

Balny, C., & Masson, P. (1993). Effects of high pressure on proteins. *Food Reviews International*, 9(4), 611–628.

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, 78(3), 836–845.

Boonyaratanakornkit, B. B., Park, C. B., & Clark, D. S. (2002). Pressure effects on intra- and intermolecular interactions within proteins. *Biochimica et Biophysica Acta*, 1595, 235–249.

Chakraborty, S., Kaushik, N., Rao, P. S., & Mishra, H. N. (2014). High-Pressure Inactivation of Enzymes: A Review on Its Recent Applications on Fruit Purees and Juices. *Comprehensive Reviews in Food Science and Food Safety*, 13(4), 578–596.

de Góes-Favoni, S. P., & Bueno, F. R. (2014). Microbial Transglutaminase: General Characteristics and Performance in Food Processing Technology. *Food Biotechnology*, 28(1), 1–24.

Eisenmenger, M. J., & Reyes-De-Corcuera, J. I. (2009). High pressure enhancement of enzymes: A review. *Enzyme and Microbial Technology*, 45(5), 331–347.

Folk, J., & Cole, P. (1966). Mechanism of action of guinea pig liver transglutaminase. I. Purification and properties of the enzyme: identification of a functional cysteine essential for activity. *The Journal of Biological Chemistry*, 241(23), 5518–5525.

Gaspar, A. L. C., & de Góes-Favoni, S. P. (2015). Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review. *Food Chemistry*, 171, 315–322.

Gross, M., & Jaenicke, R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. *European Journal of Biochemistry*, 221(2), 617–630.

Herranz, B., Tovar, C. A., Borderias, A. J., & Moreno, H. M. (2013). Effect of high-pressure and/or microbial transglutaminase on physicochemical, rheological and microstructural properties of flying fish surimi. *Innovative Food Science & Emerging Technologies*, 20, 24–33.

Kashiwagi, T., Yokoyama, K., Ishikawa, K., Ono, K., Ejima, D., Matsui, H., & Suzuki, E. (2002). Crystal Structure of Microbial Transglutaminase from Streptoverticillium mobaraense. *Journal of Biological Chemistry*, 277(46), 44252–44260.

Kaushik, N., Nadella, T., & Rao, P. S. (2015). Impact of pH and total soluble solids on enzyme inactivation kinetics during high pressure processing of mango (Mangifera indica) pulp. *Journal of Food Science*, 80(11), 2459–2470.

Kauzmann, W. (1959). Some Factors in the Interpretation of Protein Denaturation. In *Advances in Protein Chemistry*, 14, 1–63.

Kieliszek, M., & Misiewicz, A. (2014). Microbial transglutaminase and its application in the food industry. A review. *Folia Microbiologica*, 59(3), 241–250.

Lauber, S., Krause, I., Klostermeyer, H., & Henle, T. (2003). Microbial transglutaminase crosslinks β-casein and β-lactoglobulin to heterologous oligomers under high pressure. *European Food Research and Technology*, 216, 15–17.

Lauber, S., Noack, I., Klostermeyer, H., & Henle, T. (2001). Stability of microbial transglutaminase to high pressure treatment. *European Food Research and Technology*, 213(4–5), 273–276.

Ledward, D. A. (2000). Effects of pressure on protein structure. *High Pressure Research*, 19(1–6), 1–10.

Lee, E. Y., & Park, J. (2002). Pressure inactivation kinetics of microbial transglutaminase from Streptoverticillium mobaraense. *Journal of Food Science*, 67(3), 1103–1107.

Ludikhuyze, L., Indrawati, Van den Broeck, I., Weemaes, C., & Hendrickx, M. (1998). Effect of Combined Pressure and Temperature on Soybean Lipoxygenase. 1. Influence of Extrinsic and Intrinsic Factors on Isobaric−Isothermal Inactivation Kinetics. *Journal of Agricultural and Food Chemistry*, 46(10), 4074–4080.

Ludikhuyze, L., Van Loey, A., Indrawati, Smout, C., & Hendrickx, M. (2003). Effects of combined pressure and temperature on enzymes related to quality of fruits and vegetables: From kinetic information to process engineering aspects. *Critical Reviews in Food Science and Nutrition*, 43(5), 527-86.

Ma, X., Lozano-Ojalvo, D., Chen, H., Lopez-Fandiño, R., & Molina, E. (2015). Effect of high pressure-assisted crosslinking of ovalbumin and egg white by transglutaminase on their potential allergenicity. *Innovative Food Science & Emerging Technologies*, 29, 143–150.

Meersman, F., Smeller, L., & Heremans, K. (2006). Protein stability and dynamics in the pressure–temperature plane. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1764(3), 346–354.

Menéndez, O., Rawel, H., Schwarzenbolz, U., & Henle, T. (2006). Structural changes of microbial transglutaminase during thermal and high-pressure treatment. *Journal of Agricultural and Food Chemistry*, 54, 1716–1721.

Menéndez, O., Schwarzenbolz, U., Partschefeld, C., & Henle, T. (2009). Affinity of microbial transglutaminase to $\alpha_{s1}$-, β-, and acid casein under atmospheric and high pressure conditions. *Journal of Agricultural and Food Chemistry*, 57, 4177-4184.

Mozhaev, V. V., Lange, R., Kudryashova, E. V., & Balny, C. (1996). Application of high hydrostatic pressure for increasing activity and stability of enzymes. *Biotechnology and Bioengineering*, 52, 320–331.

Motoki, M., & Seguro, K. (1998). Transglutaminase and its use for food processing. *Trends in Food Science & Technology*, 9(5), 204–210.

Nonaka, M., Ito, R., Sawa, A., Motoki, M., & Nio, N. (1997). Modification of several proteins by using Ca2+-independent microbial transglutaminase with high-pressure treatment. *Food Hydro*colloids, 11(3), 351–353.

Préstamo, G., Arabas, J., Fonberg-Broczek,M. and Arroyo, G. (2001). Reaction of B. cereus bacteria and peroxidase enzymes under pressures >400 MPa. *Journal of Agricultural and Food Chemistry,* 49(6), 2830–2834

Riahi, E., & Ramaswamy, H. S. (2004). High pressure inactivation kinetics of amylase in apple juice. *Journal of Food Engineering*, 64(2), 151–160.

Rodrigo, D., Cortes,C., Clynen, E., Schoofs, L.,VanLoey, A., & Hendrickx, M. (2006). Thermal and high-pressure stability of purified polygalacturonase and pectinmethylesterase from four different tomato processing varieties. *Food Research International,* 39(4), 440–448.

Silva, J. L., & Weber, G. (1993). Pressure stability of proteins. *Annual Review of Physical Chemistry*, 44, 89–113.

Terefe, N. S., Buckow, R., & Versteeg, C. (2014). Quality-related enzymes in fruit and vegetable products: effects of novel food processing technologies, part 1: high-pressure processing. *Critical Reviews in Food Science and Nutrition*, 54(1), 24–63.

Trespalacios, P., & Pla, R. (2007). Simultaneous application of transglutaminase and high pressure to improve functional properties of chicken meat gels. *Food Chemistry*, 100(1), 264–272.

# CHAPTER 4

## High Pressure Effects on Selected Properties of Pea and Soy Protein Isolates

## 4.1. INTRODUCTION

In recent decades, there has been an increase in the pursuit of technologies and products that allow to tailor the technological properties of macromolecules present in foods, to improve nutritional and sensorial quality of food products. In this way, the use of vegetable proteins has been the focus of many research efforts to develop new products and to replace other sources of protein (Day, 2013). In the context of the objectives of this thesis, the main interest was focused on the study of two different types of proteins obtained from legumes, namely soy and peas. One of the most cultivated crops in the world is soybean due to the global growing demand for its oil and protein content (*c.a.* 35%). Soy proteins are one of the most studied legume proteins and have several applications in food manufacture, mainly due to its nutritional and functional properties. There are four major protein fractions composing soy proteins, namely 2S, β-conglycinin (7S), glycinin (11S) and 15S. β-conglycinin and glycinin constitute approximately 70% of the total protein content (Ahmed, Ayad, Ramaswamy, Alli, & Shao, 2007; Day, 2013). Similarly to soy, pea has been increasingly exploited as a source of protein, largely due to its ability to grow worldwide. Peas contain approximately 25 % of proteins that have several applications in the food industry, for instance emulsification, gelation and texture improvement, which is increasing this pulse popularity. Pea proteins are mostly globulins that comprise three distinct major groups, namely legumin (11S), vicilin (7S) and convicilin (7S) (Day, 2013).

The widespread applications of these proteins, particularly in food formulations processed by newer technologies, is restricted due to the limited knowledge concerning adequate processing parameters. Since the structure, conformation and physicochemical properties of proteins can be affected by pressure, high pressure processing (HPP) can be a useful tool to tailor the techno-functional properties of food proteins (Mirmoghtadaie, Shojaee Aliabadi, & Hosseini, 2016; Queirós, Saraiva, & Lopes da Silva, 2018). HPP is a non-thermal technology mainly used in the food industry as a cold pasteurization process. A pressure level ranging from 400 to 600 MPa is generally applied for a few minutes to foods regardless of their shape and size. Generally, HPP does not considerably affect physicochemical properties like color or flavor, and has a small impact on bioactive compounds, while inactivating pathogens and vegetative spoilage microorganisms (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015).

A detailed discussion on the actual knowledge regarding the effects of HPP on proteins' structure, conformation and functionality was already carried out in Chapter 2. In fact, several studies using vegetable proteins have shown HPP advantages to modify the supramolecular structures of proteins, creating new structured systems and textures, and improving their functionality, thus increasing their commercial applications (Queirós, Saraiva, & Lopes da Silva, 2018). However, the effects of HPP on protein technological properties are highly dependent on the type of protein, its environment and also, on the processing conditions. Thus, the main goal of the work described in this chapter was to evaluate the effects of different processing parameters, *i.e.* pressure and holding time, on some technological characteristics, *e.g.* solubility, content of sulfhydryl groups, surface hydrophobicity, and emulsifying properties of pea (PPI) and soy (SPI) protein isolates at three different pH values (6, 7 and 8).

## 4.2. Materials and Methods

### 4.2.1. Materials

Readily dispersible PPI (Pisane® M9, Cosucra) and SPI (Induxtra W, Induxtra) were obtained from Induxtra (Induxtra de Suministros Llorella Portuguesa - Indústria Alimentar, Lda., Moita, Portugal). Protein content was determined by elemental analysis (N x 6.25): PPI = 81.1 ± 0.1 %; SPI = 86.7 ± 0.03 %. The water content of both protein isolates was less than 3%. According to the supplier, ash and fat content were less than 6% and 4%, respectively. All reagents used were of analytical grade.

### 4.2.2. Sample Preparation

The protein isolates were dispersed in distilled water (5 $g \cdot 500\ mL^{-1}$) and stirred for 4 h at room temperature for hydration. The pH was adjusted to 6, 7 or 8 with 0.1 $mol \cdot L^{-1}$ citric acid or 0.01 $mol \cdot L^{-1}$ NaOH and stirred for 40 min at room temperature. The dispersions (approximately 40 mL) were placed in flasks (Thermo Scientific™ Nalgene™ Wide-Mouth Lab Quality HDPE Bottles) for processing.

### 4.2.3. Pressure Treatments

The samples were treated at 200, 400 or 600 MPa for 5, 10 or 15 min, at room temperature (approximately 20 °C) using a hydrostatic press (Hiperbaric 55, Burgos, Spain). This HPP equipment has a pressure vessel of 200 mm inner diameter and 2000 mm length and a maximum operating pressure of 600 MPa. It is connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) that allows controlling the temperature of the input water used as pressurizing fluid. For those tests where the soluble protein fraction was to be studied, the dispersions were centrifuged at 6,000 rpm for 20 min at 4 ºC.

### 4.2.4. ELECTROPHORESIS

Equal volumes of each sample were diluted (1:2) in loading buffer (0.5 M Tris-HCl pH 6.8, 4% (w/v) SDS, 15% (v/v) glycerol, 1 mg·mL$^{-1}$ bromophenol blue and 20% (v/v) β-mercaptoethanol) and incubated at 100 °C for 5 min. Then, the samples were loaded in a 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) prepared according to Laemmli (1970). Electrophoretic separation was performed at 180 V for 45 min in running buffer (250 mM glycine, 25 mM Tris, pH 8.6 and 0.1 % (w/v) SDS). Protein molecular weight markers corresponding to 20, 25, 37, 50, 75, 100, 150 and 250 kDa (Precision Plus Protein Unstained Standards; Bio-Rad) were run under the same electrophoretic conditions. Gels were removed from the cassette and incubated in a fixation solution (40 % (v/v) methanol and 10 % (v/v) glacial acetic acid) for 30 minutes. After being washed with water, gels were stained with Colloidal Coomassie Blue G250 and destained with 25 % (v/v) methanol until an optimal contrast was achieved. The gels were then scanned in the ChemiDoc Imaging System v.2.3.0.07 (Bio-Rad®, Hercules, CA, USA) and analyzed with the ImageLab software (Bio-Rad).

### 4.2.5. SOLUBILITY

Control and pressurized protein dispersions, prepared as described in section 4.2.2, were centrifuged at 6,000 rpm for 20 min at 4 ºC. Soluble protein concentration was determined in the supernatant using the method of Bradford (1976) with a few modifications. 250 µL of Bradford Reagent were added to an aliquot of 50 µL of protein dispersion, mixed for 30 s and then incubated 20 min at room temperature. The absorbance was measured at 595 nm using a spectrophotometer (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA)

and the protein concentration was determined using a calibration curve using BSA standards. Protein solubility was expressed as the ratio of soluble to initial total protein:

$$\text{solubility (\%)} = \frac{\text{protein in supernatant (mg.mL}^{-1}\text{)}}{\text{initial protein (mg.mL}^{-1}\text{)}} \times 100 \qquad (4.1)$$

### 4.2.6. SULFHYDRYL GROUPS

The content of free sulfhydryl groups (SH) was determined according to the method of Beveridge, Toma & Nakai (1974) with some modifications. After centrifugation (6,000 rpm for 20 min at 4 ºC) the control and pressurized protein dispersions were diluted in 0.086 mol·L$^{-1}$ Tris buffer (pH 8.0). Specifically, 500 µL of the dispersions were added to 500 µL Tris buffer and 50 µL Ellman's reagent and kept for 60 min at room temperature (~20 ºC). The mixture's absorbance was measured at 412 nm using a spectrophotometer (Shimadzu UV-1280, Japan). The content of SH was determined by dividing the absorbance value by the molar extinction coefficient of 13600.

### 4.2.7. SURFACE HYDROPHOBICITY

Protein surface hydrophobicity ($H_0$) was determined using the fluorescent probe 1-anilino-8-naphthalene-sulfonate (ANS) according to the method of Kato & Nakai (1980). Protein dispersions prepared as previously described in section 4.2.2 and centrifuged, were diluted to 0.05 - 0.25 mg·mL$^{-1}$ with 0.01 mol·L$^{-1}$ phosphate buffer pH 7. An aliquot of 20 µL of ANS (0.008 mol·L$^{-1}$ in 0.01 mol·L$^{-1}$ phosphate buffer) was added to 4 mL of each protein solution and then the fluorescence intensity was measured (390 nm – excitation; 470 nm – emission) using a fluorescence spectrometer (Hitachi F2000 fluorescence spectrophotometer, Tokyo, Japan). The index of $H_0$ was calculated using the initial slope of fluorescence intensity *vs* protein concentration (mg·mL$^{-1}$) plot (calculated by linear regression analysis).

### 4.2.8. SURFACE TENSION

Measurement of surface tension (air-water interface, γ) was carried out for centrifuged 0.01 g·mL$^{-1}$ protein solutions at pH7. The surface tension was determined by analyzing the shape of a pendant drop using a Dataphysics contact angle system OCA-20. Drop volumes of

(5 ± 1) µL were obtained using a Hamilton DS 500/GT syringe connected to a Teflon coated needle, with a diameter of 0.52 µm, placed inside an aluminum air chamber. The temperature inside the aluminum chamber was measured with a Pt100 within ± 0.1 ºC, placed at approximately 2 cm to the liquid drop. The drop was formed and the measurements were carried out after 10 min stabilization at 20 ºC. The analysis of the drop shape was performed using the SCA 20 software module, with basis on the Young-Laplace equation.

### 4.2.9. Emulsifying Properties

Emulsifying properties were evaluated for both the whole isolate protein dispersions and for the soluble protein fractions obtained after centrifugation of the protein isolate dispersions (6,000 rpm for 20 min at 4 ºC). The emulsions were prepared by mixing 3 mL of 10 $mg \cdot mL^{-1}$ protein dispersion (centrifuged or not) with 1 mL of sunflower oil, and then homogenized using a T25 Ultra-turrax homogenizer (IKA-Werke, Germany) at 10,000 rpm for 30 s. The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined according to the method of Pearce & Kinsella (1978). An aliquot (50 µL) of the emulsion was retrieved from the bottom of the emulsion, immediately (0 min) and after 10 min and diluted (1:500, v/v) in 0.1% (w/v) SDS solution. The absorbance was measured at 500 nm using a spectrophotometer (Perkin-Elmer Instruments Lambda 35, Perkin-Elmer Instruments, USA). The indexes were calculated as follows:

$$\mathrm{EAI}\ (\mathrm{m^2 \cdot g^{-1}}) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \varphi \times (1-\theta)} \tag{4.2}$$

$$\mathrm{ESI}\ (\mathrm{min}) = \frac{A_0}{A_0 - A_{10}} \times \Delta t \tag{4.3}$$

Where $A_0$ and $A_{10}$ are the absorbance (500 nm) of the diluted emulsion at 0 and 10 min, respectively, *DF* is the dilution factor (500), *c* is the initial protein concentration ($g \cdot 100\ mL^{-1}$), $\varphi$ is the optical path (0.01 m), $\theta$ is the oil volumetric fraction (0.25), and $\Delta t$ is 10 min.

### 4.2.10. Statistical Analysis

ANOVA and Tukey's honestly significant difference test was used to determine significant differences between samples with a 5% level of significance.

## 4.3. RESULTS AND DISCUSSION

### 4.3.1. ELECTROPHORESIS

Figure 4.1 shows the results obtained from the SDS-PAGE analysis of the whole isolate protein dispersions, carried out under reducing conditions. For the PPI samples (Figure 4.1(A)), the results show a similar polypeptide composition between untreated and HPP treated samples, *i.e.* the processing of PPI did not induce any relevant changes in protein intermolecular interactions, under the electrophoretic conditions studied, in accordance to previously reported results (Chao, Jung, & Aluko, 2018). Worth to note that whole protein isolates were analyzed, and it is therefore expected that high molecular weight aggregates originally present, with low solubility, may not have even entered the gel - note the high-intensity bands still present in the region of sample application after analysis. Eight bands were identified, being most likely legumin's acid (α = ~39 kDa) and basic (β = ~20 kDa) subunits, the three vicilin's subunits (~46 kDa; ~32 kDa; ~29 kDa), convicilin's globular fraction (~68 kDa), lipoxygenase (LOX = ~93 kDa), and albumin's major subunit (PA2 = ~23 kDa) (Tamm, Herbst, Brodkorb, & Drusch, 2016). According to densitometric analysis, there were no major changes in the volume of the bands with different pH values and HPP conditions analysed.

Applying pressure to SPI samples also led to no major differences in the overall profile of the protein bands (Figure 4.1(B)). Similar observations were reported after the pressurization of soymilk (Lakshmanan, de Lamballerie, & Jung, 2006) and soy-protein gels (Apichartsrangkoon, 2003). Eleven bands were identified, being most likely β-conglycinin subunits (α' = ~74 kDa; α = ~70 kDa; β = ~52 kDa), glycinin's acid ($A_{1-4}$ = ~31 – 45 kDa) and basic (B = ~20 kDa) subunits, glycinin's acid-basic subunits pair (AB = ~62 kDa), lipoxygenase (LOX = ~93 kDa), and agglutinin (~28 kDa) (Molina, Defaye, & Ledward, 2002; Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011). Changes induced by HPP seemed pH dependent. While at pH 6 there were no major changes observed, at pH 7, HPP induced a decrease in glycinin's acid subunit (MW ~41 kDa) relative content and a simultaneous increase of a smaller glycinin's acid subunit (MW ~31 kDa) and agglutinin. On the other hand, at pH 8, both HPP treatments at 400 and 600 MPa led to an increase of the higher molecular weight glycinin's acid subunits, at an expense of those with lower molecular weight, likely resulting from HPP-induced protein aggregation. Overall, the distribution of protein fractions' molecular weight was either unaffected by HPP or the treatment effect was relatively small, and in this case, pH dependent.

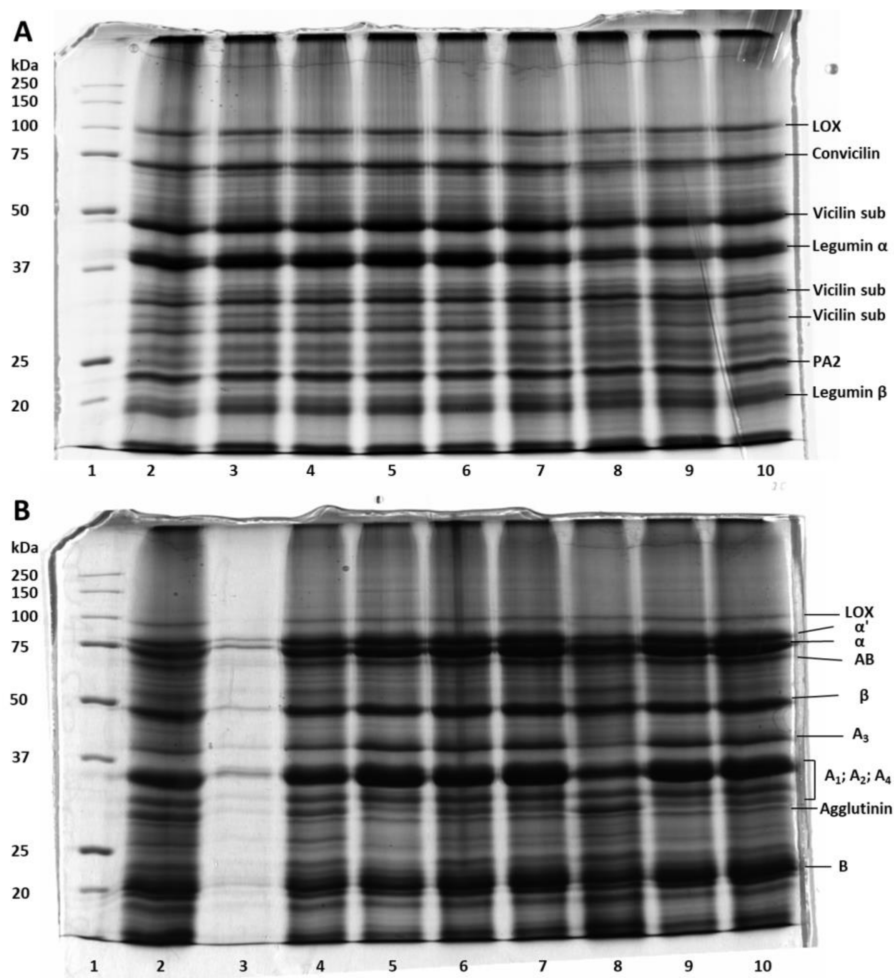



Figure 4.1. SDS-PAGE analysis of high pressure-treated (A) pea and (B) soy protein isolates at 0.1, 400 and 600 MPa for 15 min at pH 6 (lanes 2 – 4), pH 7 (lanes 5 – 7) and pH 8 (lanes 8 – 10). Lane 1 – protein molecular weight markers. To the right are the most likely protein fractions based on the standards' molecular masses – LOX denotes for lipoxygenase, and PA2 for the major component of the albumin fraction; in (B) α', α and β denote for the β-conglycinin subunits, $A_1$, $A_2$, $A_3$, and $A_4$ for the glycinin's acid subunits, B for the glycinin's basic subunit, and AB for acid-basic subunits pair.

### 4.3.2. Solubility

The solubility of proteins strongly affects their techno-functional properties including gelation and interfacial activity and, consequently, their ability to help on the formation/stabilization of disperse systems. The effects of HPP on the solubility of protein isolates are presented in Figure 4.2. Non-processed PPI showed soluble protein values of 25.5 ± 0.6, 25.4 ± 0.3 and 28.0 ± 0.3 % at pH 6, 7 and 8, respectively. This variation is in agreement with the literature, where it is described that, in general, pea protein isolates have the lowest

solubility between pH 4 and 6. The increase of solubility at pH 8 can be attributed to electrostatic repulsion brought on by negative net charges on the protein surface, as it was also reported in other commercial pea protein isolates (Adebiyi & Aluko, 2011; Lam, Can Karaca, Tyler, & Nickerson, 2018). SPI presented higher protein solubility than PPI, namely 33.6 ± 1.3, 43.8 ± 1.7 and 34.3 ± 0.3 % at pH 6, 7 and 8, respectively. A solubility variation similar to that of the PPI with the pH increase from 6 to 8 was expected, *i.e.* an increase of solubility as the pH moves away from the isoelectric point (~4 – 6). However, a drop in the solubility of SPI was verified from pH 7 to 8. Molina and co-workers (Molina, Papadopoulou, & Ledward, 2001) also reported a decrease in the solubility of SPI with the increase of the pH, from 6.5 to 7.5, particularly in the glycinin fraction. This effect can be due to the relative content of acidic and basic sub-units of glycinin. Glycinin's basic sub-units have an isoelectric point of 8.0 – 8.5 (Derbyshire, Wright, & Boulter, 1976), therefore at pH 8 their net charge is zero, promoting their precipitation. That is to say, soy protein isolates with a high content of glycinin's basic sub-units may have a lower solubility at pH 8.

The relatively low protein solubility is a general characteristic of commercial protein isolates and has been related to a high degree of protein denaturation and the presence of varying amounts of soluble and insoluble proteins (aggregates) within the protein isolates (Lee, Ryu, & Rhee, 2003; Monteiro & Lopes da Silva, 2019). Overall, the soluble protein increased with increasing pressure and processing time, regardless of the pH value and protein isolate, although in a few cases the differences were not statistically significant ($p > 0.05$). Still, the increase in protein solubility was considerably higher for SPI than for PPI samples.

Regarding PPI, at pH 6, for 200 and 400 MPa and a holding time of 5 and at 600 MPa and a holding time of 15 min, no significant ($p > 0.05$) changes of protein solubility were observed. Slight increases in protein solubility (2-5%) were observed for other HPP conditions. At higher pH values, *i.e.* 7 and 8, the increase in solubility with pressure was more pronounced, although not always being noticeable an increase with increasing holding time, reaching maximums of 32.8 ± 0.7 % at 600 MPa/15 min at pH 7 and 39.4 ± 0.4 % at 600 MPa/10 min at pH 8.

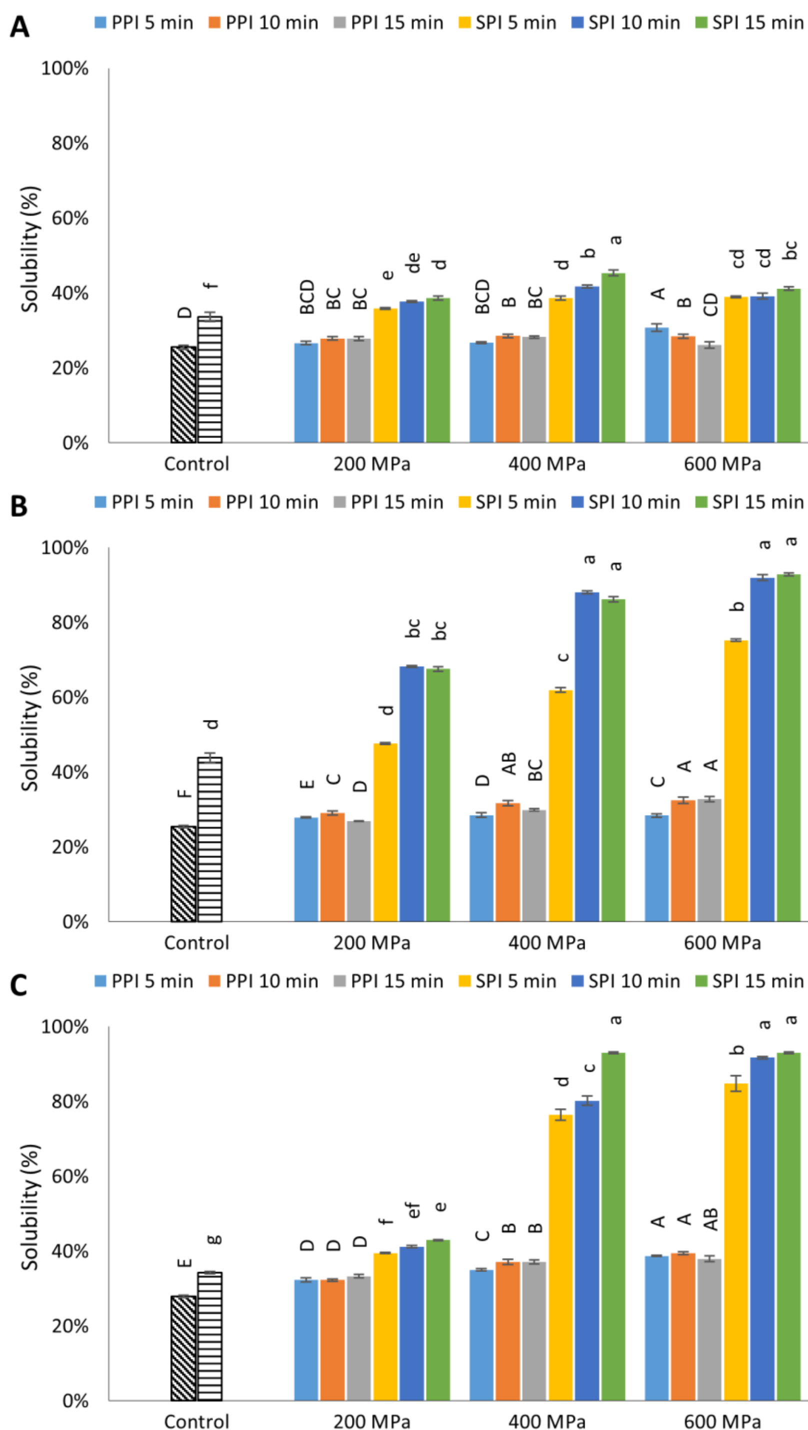



Figure 4.2. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the solubility of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent non-pressurized pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

SPI presented a similar behaviour, with smaller increases after processing at pH 6 and higher increases at pH 7 and 8, doubling the amount of soluble protein in many cases. At pH 6, HPP slightly increased the solubility, ranging between 35.9 ± 0.2 % at 200 MPa/5 min and 45.3 ± 0.7 % at 400 MPa/15 min. Overall, at pH 7 and 8 the solubility increased with increasing pressure and holding time up to 400 MPa/10 min, reaching *c.a.* 90 % of soluble protein. More intensive conditions (*i.e.* higher pressure or holding time) did not further increase the solubility significantly ($p > 0.05$).

The solubility of vegetable proteins treated with HPP depends, among other factors, on the type of protein. As it has been seen for proteins from amaranth (Condés, Añón, & Mauri, 2015), cowpea (Peyrano, Speroni, & Avanza, 2016), potato (Baier & Knorr, 2015) or rice (Zhu, Lin, Ramaswamy, Yu, & Zhang, 2016), the solubility of vegetable proteins may be reduced when pressure is applied, mostly due to the formation of insoluble aggregates (Condés et al., 2015). Chao et al. (2018) reported little to no effects of HPP on the solubility of pea proteins at pH below 7, and minor decreases at higher pH values. The difference between that work and our results may be due to either the source of proteins or the utilized extraction methods since each extraction method leads to different extraction yields and different protein functionality (Lam, Can Karaca, Tyler, & Nickerson, 2018). Still, there are studies suggesting that solubility of proteins from other vegetable sources increase with the application of pressure, soy being the most notorious case (Molina, Papadopoulou, & Ledward, 2001; Puppo et al., 2004), follow by kidney bean (Yin, Tang, Wen, Yang, & Li, 2008) and white sesame seeds (Achouri & Boye, 2013). This increase occurs probably due to the promotion of protein unfolding and dissociation of preformed aggregates (Achouri & Boye, 2013; Yin et al., 2008), which is likely to be the case with the commercial samples analyzed in this study.

### 4.3.3. Sulfhydryl Groups

SH groups and disulfide bonds play important roles in the technological properties of proteins. They are weak secondary bonds and help maintain proteins' tertiary structure, therefore their changes are essential to manipulate the functional properties of proteins (Bulaj, 2005). Figure 4.3 shows the effects of HPP (200 - 600 MPa/5 - 15 min) on the free SH of PPI and SPI at different pH values. The initial content of free SH of PPI was not significantly different ($p>0.05$) at pH 6 and 8, being 3.77 ± 0.18 and 3.88 ± 0.13 $\mu mol \cdot g^{-1}$ protein, respectively. However, was higher at pH 7, *i.e.* 4.64 ± 0.23 $\mu mol \cdot g^{-1}$ protein. At pH 6, processing at 200 MPa

did not let to significant changes ($p > 0.05$), regardless of the holding time. At higher processing pressures, 400 and 600 MPa, the effects depended on the holding time, *i.e.* a shorter holding time (5 min) increased the content of free SH approximately 50 %, whereas a larger holding time (15 min) considerably decrease the free SH content. Still, no significant changes ($p > 0.05$) comparatively to the control were verified when a holding time of 10 min was used. At this pH, all processing conditions either did not significantly ($p > 0.05$) affected the content of SH or decreased it. The holding time appeared to have a greater impact on the content of free SH than the pressure level, as the higher decreases were verified for 15 min holding time under all the pressure values tested. The lowest SH content was verified at 400 MPa/15 min that was about 40 % of the control. A similar trend was shown for pH 8, with the SH content decreasing with increasing holding time, regardless of the pressure value.

Regarding SPI, the initial SH content increased with pH, being 6.05 ± 0.21, 6.78 ± 0.15 and 7.37 ± 0.25 $\mu mol \cdot g^{-1}$ protein at pH 6, 7 and 8 respectively. All HPP conditions decreased the SH content between 15 and 30 %, not following any particular trend, but in general with a lower effect of the holding time than that observed for the PPI samples. It is worth pointing out that the quantification of free SH was assessed on the soluble proteins present in the dispersions, which precludes the physical meaning of any possible correlation with the solubility.

In general, the few studies concerning plant-based proteins agree with the results here described. Overall, pressure below 300 MPa preserve or may even improve the content of free SH, whereas more intensive processing conditions, *i.e.* higher pressure values or longer holding times seem to decrease the content of SH (Queirós et al., 2018). For instance, He, He, Chao, Ju, & Aluko (2013) studied the effect of HPP (200 – 600 MPa/15 min) on the content of SH of rapeseed proteins at pH 7. The authors described an increase in the content of SH after treatment at 200 MPa. Still, higher pressure (*i.e.* 400 and 600 MPa) led to a decrease in this parameter. Similar results were obtained for soy protein isolates at pH 6.8, *i.e.* an increase of the content of SH with pressure up to 300 MPa and a decrease at pressure values above that (Li, Zhu, Zhou, & Peng, 2012). Taking into consideration the effect of pH, studies with soy protein isolates also revealed the influence of this parameter on the effects of pressure on plant-based proteins. It was reported that 200 MPa at a pH 3 increase the content of SH and 400 and 600 MPa decrease it. However, at pH 8 none of the applied pressure values led to the decrease of the content of SH (Puppo et al., 2004).

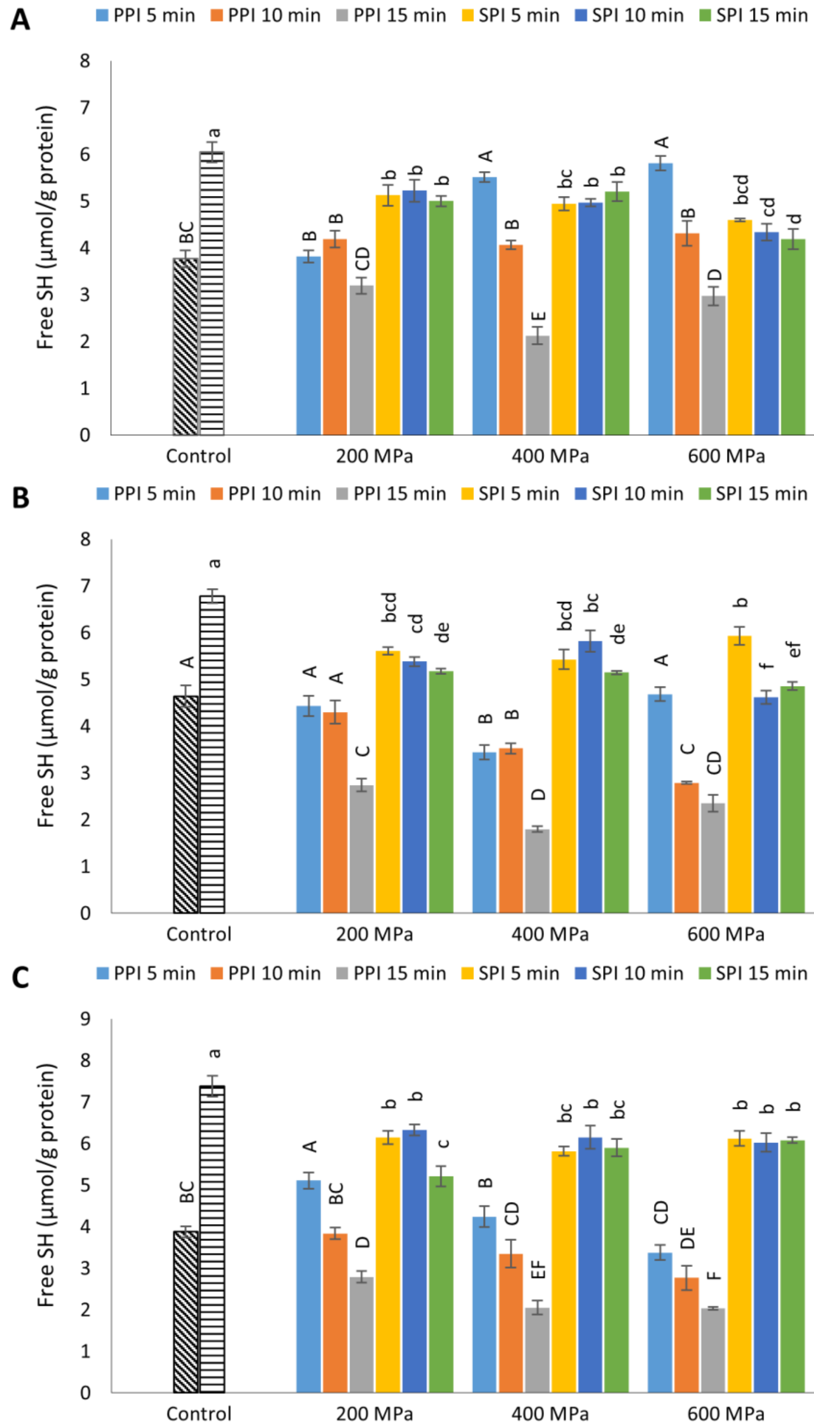



Figure 4.3. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the content of free sulfhydryl groups of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

The results suggest, in the case of PPI, that the lower pressure applied led to the dissociation/unfolding of the proteins exposing SH groups. However, on proteins from both sources, higher pressure values may have promoted hydrophobic interactions that led to sulfhydryl/disulfide interchange and/or formation of new disulfide bonds (Li et al., 2012; Yin et al., 2008), even among those proteins that make up the soluble fraction of these isolates.

### 4.3.4. Surface Hydrophobicity

Figure 4.4. presents the effects of HPP, 200 – 600 MPa for 5 – 15 min, on the $H_0$ of PPI and SPI solubilized proteins, at different pH values (6 – 8). For unprocessed PPI, the highest value of $H_0$ was found at pH 7, followed by pH 8 and 6. Worth to note that the higher amount of free SH groups was also observed at pH 7, probably related to a net balance favoring more expanded protein conformations at this pH, thus exposing more hydrophobic zones and free SH groups. Unprocessed SPI samples showed higher $H_0$ values than PPI, but a much lower pH effect.

PPI samples at pH 6 showed a maximum $H_0$ value for P = 600 MPa and t = 15 min, about 145% higher than the control value ($H_0 = 906$). Additionally, shorter treatments (5 min) at 200, 400 and 600 MPa led to no significant differences ($p > 0.05$). At pH 7, the moderate conditions (200 MPa/5 and 10 min) had no significant effects on $H_0$ ($p > 0.05$) when compared with the control, whereas 400 MPa/5 min increased $H_0$ 29 % and all other conditions increased $H_0$ between 40 and 51 % without any statistical difference among them. Under slightly alkaline conditions (pH 8), all processing conditions significantly increased ($p < 0.05$) $H_0$ comparatively to the control samples, with the exception of 200 MPa/5 min, where the increase was not statistically significant ($p > 0.05$). Furthermore, $H_0$ increased with increasing pressure although in some cases, this increase was not statistically significant ($p > 0.05$). Under 400 and 600 MPa, $H_0$ increased by increasing the holding time from 5 to 10 min, however, further increasing the holding time to 15 min resulted in $H_0$ values similar to those obtained with 5 min holding time.

Regarding SPI samples, all tested HPP conditions increased $H_0$ but the pH did not have much impact on it. $H_0$ increased up to 50 % with increasing pressure, particularly with 15 min holding time at pH 6 and 7, and with 10 min holding time at pH 8.

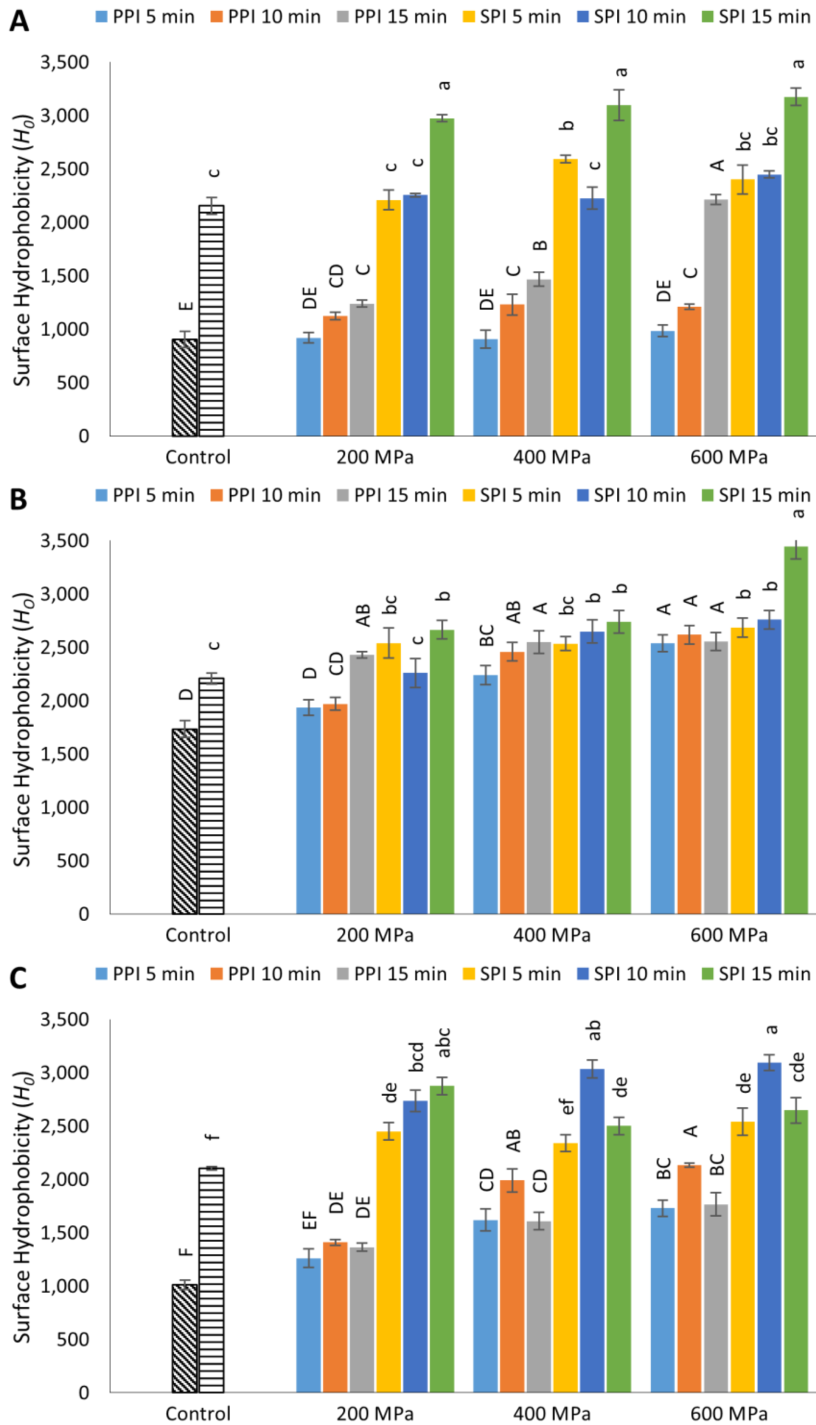



Figure 4.4. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the surface hydrophobicity of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent non-pressurized pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

These results suggest that the increase in pressure leads to a gradual exposure of hydrophobic regions within the proteins. Similar results have been reported in the literature, where relatively high pressure values (400-600 MPa) increased $H_0$ of vegetable proteins. Both Zhao, Zhou, Zhang, Ni, & Li (2015) and Yin et al. (2008) observed an increase of the $H_0$ of proteins from peanut and red kidney bean seeds, respectively, with increasing pressure (up to 600 MPa), with holding times of 20 min. However, a further increase in pressure does not always translate into an increase of $H_0$. He et al. (2013) described no differences between the $H_0$ of proteins from rapeseed submitted to 400 and 600 MPa for 15 min. The $H_0$ can even be reduced above a certain pressure, for instance, increasing pressure led to an increase of $H_0$ of walnut protein isolate (up to 500 MPa), although 600 MPa led to a decrease of this parameter (still being higher than the control). This data suggest that higher pressure may promote interactions between protein and solvent or between proteins, decreasing $H_0$ (Qin et al., 2013). Along with the pressure, the holding time also has an impact on $H_0$, as demonstrated by the results discussed above, in accordance with Li et al. (2012) that reported an increase in $H_0$ for soy proteins by increasing the holding time at 300 MPa, at least up to 15 min, followed by a slight decrease in $H_0$ after a further increase in holding time to 20 min. Similar results were reported by Wang et al. (2015) on rapeseed proteins at 400 MPa. Overall, the results reported in this work agree with those reported in the literature, *i.e.* an increase in pressure and holding time lead to an increase of exposed hydrophobic regions. However, more intensive processing conditions may promote the interaction between these hydrophobic regions contributing to negligible variations, or even a decrease in $H_0$. These effects were observed particularly at pH 7 and 8.

### 4.3.5. Surface Tension

Figure 4.5 shows the results obtained for the surface tension measured at the air-water interface for (A) pea and (B) soy protein solutions (0.01 $g\cdot mL^{-1}$, pH7), for different pressure and holding time values. As expected from the more than probable adsorption of proteins at the air-water interface, all samples analyzed showed a surface tension significantly lower than that of pure water, at the temperature of the tests. The $\gamma$ of the unprocessed samples was 47.4 ± 0.7 $mN\cdot m^{-1}$ and 46.6 ± 0.4 $mN\cdot m^{-1}$ for pea and soy proteins, respectively, with no significant differences ($p > 0.05$) between them.

In general, the effect of HPP on the surface tension of protein solutions was slight and, in most cases, not statistically significant, either in relation to the control sample or in relation to the different processing conditions analyzed. For pea proteins, the most significant influence of HPP and worth highlighting is the decrease in surface tension with increasing pressure, for samples processed for a reduced holding time (5 min, $p < 0.05$) (Figure 4.5(A)). The decrease of $\gamma$ with pressure was also observed for 15 min holding time, but apparently higher values of $\gamma$ were obtained by increasing the holding time. For soy proteins (Figure 4.5(B)), the treatment by HPP seems to originate a more pronounced increase of $\gamma$ in relation to the control. For these samples, it is noteworthy to mention the significant decrease ($p < 0.05$) of $\gamma$ with the increase of the holding time for those samples treated at 600 MPa.

Thus, the influence of HPP upon the ability of the proteins to decrease the air-water surface tension is dependent on the processing conditions (pressure and holding time), but also on the type of protein, what can help to better understand the diversity of results regarding the emulsifying properties of the pea and soy proteins, as affected by HPP, as will be discussed further below.

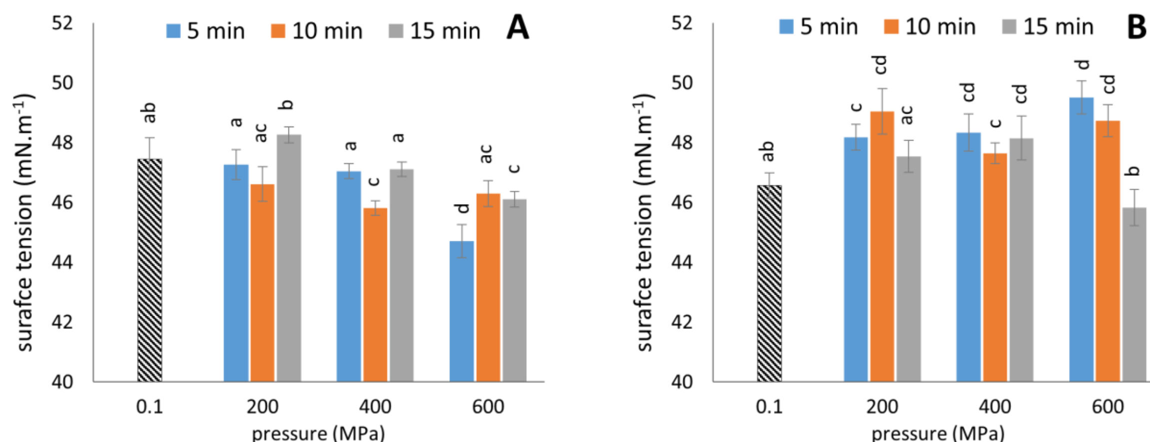



Figure 4.5. Effects of high-pressure processing on the surface tension of (A) pea and (B) soy proteins (soluble fractions at 0.01 g·mL$^{-1}$, pH7), for different pressure and holding time values. The non-pressurised control sample is indicated by the blank column with stripes. Different letters indicate significant differences ($p < 0.05$) among the different samples.

Studies with 11S globulin from Vicia faba, treated at 200 MPa for 20 min resulted in a decrease of γ (Galazka, Dickinson, & Ledward, 2001). Along with the results obtained here, especially for the pea proteins, both results suggest that certain pressure/time combinations may alter protein conformation in a way that increases the availability of hydrophobic groups,

ergo decreasing the free energy at the air-water interface. On the other hand, the possible changes in the molecular organization of proteins due to HPP may also promote greater interaction between proteins and formation of aggregates that will hinder the migration of the protein to the interface and subsequent adsorption, resulting in higher values of surface tension, compared to non-pressurized samples, as was observed for soy proteins.

### 4.3.6. Emulsifying Properties

Proteins are generally used as stabilizers for food emulsions due to their amphiphilic nature (Day, 2013). As mentioned previously, since HPP can alter protein solubility, conformation and hydrophobicity, it is expected to affect proteins' influence on emulsion formation and stability, and thus the EAI and ESI values. Figure 4.6 shows the effects of the total fraction of PPI and SPI treated by HPP on the EAI, at different pH values. Worth to note that for the emulsions prepared with non-HPP treated protein isolates, the effect of pH on EAI followed the general trend observed for its effect on the amount of SH groups or on the surface hydrophobicity, *i.e.* pH values leading to a higher expansion of the protein chains also lead to an increase of EAI. Similar effects of pH on proteins' emulsion activity have been reported for other plant protein isolates (Khalid, Babiker, & El Tinay, 2003).

EAI values obtained for the emulsions prepared with the total fraction of PPI at pH 6 and 7 were 44 ± 1 and 54 ± 3 $m^2 \cdot g^{-1}$, respectively, and suffered no significant differences ($p > 0.05$) with the pressure treatments. However, at pH 8, HPP seems to slightly decrease the EAI. The EAI at pH 8 was 52 ± 4 $m^2 \cdot g^{-1}$ and decreased by approximately 17% for pressures below 600 MPa, regardless of the holding time, although no significant changes ($p > 0.05$) were observed at 600 MPa. Since the EAI is calculated in relation to the protein concentration and considering the relatively low solubility of these proteins, one can expect that the amount of protein in the total PPI fraction that effectively will contribute to the interfacial activity will be relatively low. Therefore, not unexpected, the soluble PPI fraction showed higher EAI values (Figure 4.7).

The EAI of soluble unprocessed PPI samples was similar, specifically 180 ± 11, 193 ± 7 and 179 ± 5 $m^2 \cdot g^{-1}$ at pH 6, 7 and 8, respectively. When considering the effects of pressure, the EAI of soluble PPI samples at pH 6 submitted to HPP did not show significant ($p > 0.05$) differences compared to the control. Similarly, HPP did not significantly affect the EAI of soluble PPI at pH 7, with the exception of 200 MPa/5 min that originated an increase of about 19 %. In general, at pH 8, a decrease of EAI was promoted with increasing pressure, although this decrease was

only significant ($p < 0.05$) at 400 MPa for 10 and 15min and 600 MPa/10 min, with a decrease of approximately 17 %.

The EAI of the total fraction of unprocessed SPI increased with increasing pH, from 55 ± 2 $m^2 \cdot g^{-1}$ at pH 6 to 87 ± 4 $m^2 \cdot g^{-1}$ at pH 8. Similarly to PPI, in general, HPP did not have a great impact on EAI of SPI, other than a reduction of up to 25% (200 and 600 MPa/5 and 15 min) and a slight increase of 13 % when processed at 400 MPa/15 min at pH 8.

In general, the EAI of soluble SPI samples was lower than for PPI samples, with the exception of some samples at pH 8, namely unprocessed or processed samples at 200 MPa. The EAI of untreated SPI at pH 6 and 7 was similar (~155 $m^2 \cdot g^{-1}$) and changing the pH to 8 increased this value to 201 ± 7 $m^2 \cdot g^{-1}$. Still, HPP affected the EAI of SPI samples differently. At pH 6, a pressure of 600 MPa did not significantly ($p > 0.05$) affect the EAI, however, lower pressure levels (*i.e.* 200 and 400 MPa) resulted in a reduction of EAI of around 15%. At pH 7, with the exception of the less intensive HPP condition (200 MPa/5 min), HPP led to a decrease of EAI for the soluble soy protein fraction. At pH 8, although no significant ($p > 0.05$) differences were verified at 200 MPa, a considerable reduction of EAI occurred for higher pressures, in certain cases decreasing more than 50%, especially for the longer holding times (*i.e.* ≥10 min).

There are not many studies available regarding the effects of HPP on emulsifying properties of plant-based proteins. Still, the general trend is the increase of EAI with pressure (Queirós et al., 2018). This trend was observed, for instance, in proteins of kidney beans (Yin et al., 2008), peanut arachin (Zhao et al., 2015), soy (Wang et al., 2008) and sweet potato (Khan, Mu, Sun, Zhang, & Chen, 2015b). In these studies, the protein isolates were submitted to 200 – 600 MPa, at pH levels within the range of the current study. The described results suggested that HPP induced protein unfolding, which led to the exposure of hydrophobic groups thus improving emulsifying properties (Li, Zhu, Zhou, & Peng, 2011). Chao et al. (2018) reported a decrease in the oil droplet size for pea protein emulsions, treated with increasing pressures (200 – 600 MPa, 5 min) at pH 3 and 5, particularly at pH 3, and a small increase at pH 7.

The ability of the proteins to decrease the water surface tension is expected to be correlated to the ability of those proteins to also adsorb and decrease the interfacial tension at the water-oil interface.

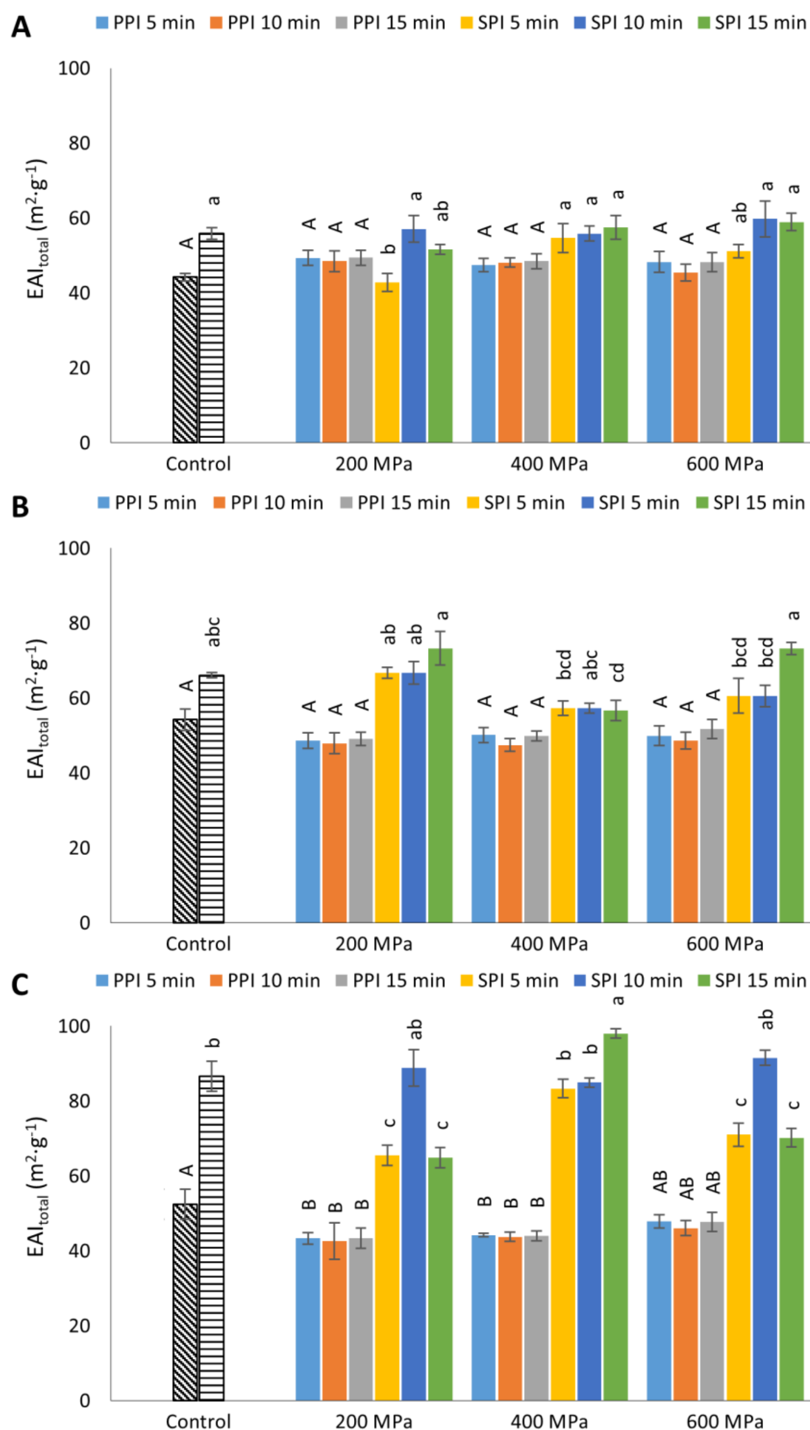



Figure 4.6. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the emulsifying activity index (EAI) of the total fraction of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

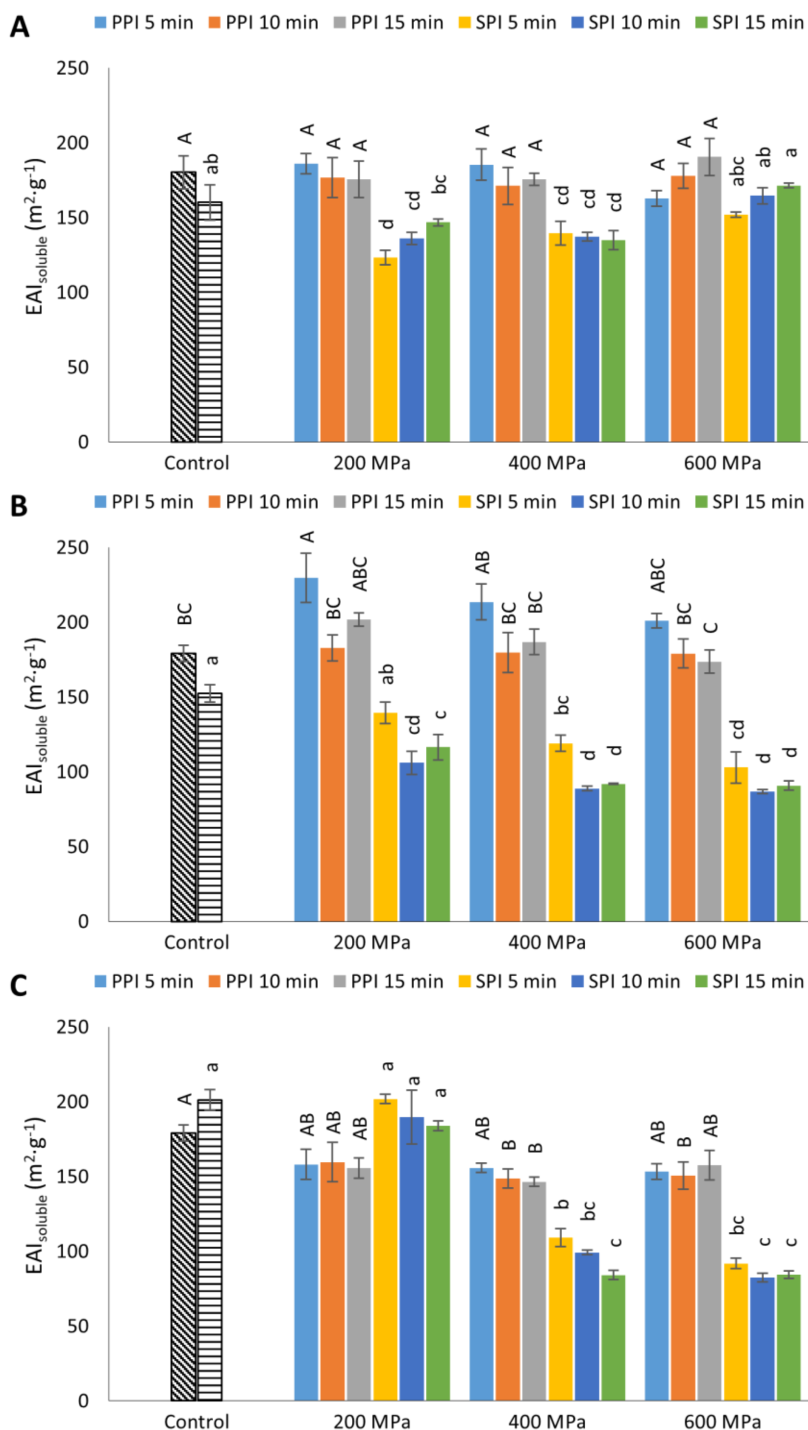



Figure 4.7. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the emulsifying activity index (EAI) of the soluble fraction of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

No clear correlations could be established between the surface tension values previously discussed at pH 7 (§4.3.5) and the emulsifying activity of those proteins at the same pH. However, at least for soy proteins, for which there was, at pH 7, a more pronounced effect of HPP on EAI, compared to that observed for pea proteins, and considering the protein soluble fraction, we can assume that the overall decrease observed for the EAI of soy proteins may be related to an increase in surface air-water tension caused by HPP, as previously discussed (Figure 4.5 (B)).

It is expected that a complex set of factors, interacting with each other, influence the interfacial activity of these proteins, including protein charge, solubility, surface hydrophobicity, and the overall distribution of hydrophilic and hydrophobic and regions on the protein chains. In fact, in the present study, no significant correlations were observed between the changes of $H_0$ and EAI with pressure, both in the total and soluble fractions at the different pHs. However, in general, for both proteins and under the analysed pH conditions, a strong negative correlation was observed between the effect of HPP on solubility and EAI: for example, under pH 7, $r = -0.702$ ($p<0.001$) for PPI and $r = -0.953$ ($p<0.001$) for SPI. For these samples and under the conditions analysed, possible positive effects of HPP on higher protein solubility and on the development of more open and extended protein conformations, which at first would be advantageous to obtain an increase in the proteins' emulsifying capacity, may be compromised by an increase in intermolecular protein interactions, even though mostly forming soluble aggregates, thus compromising their interfacial activity and emulsifying activity.

The effects of HPP on the ESI of the total fraction of PPI, at different pH values, are presented in Figure 4.8. pH did not show any major effect on ESI for the untreated samples. Likewise, HPP had no significant ($p > 0.05$) effects on ESI of PPI at any pH, with the exception of an increase of ~30 % verified in the samples processed at 600 MPa/5 and 15 min.

The ESI of the total fraction of SPI was in general higher than the ones verified for PPI. Unprocessed samples had an ESI of 59 ± 3, 47 ± 5 and 65 ± 3 min at pH 6, 7 and 8 respectively. Contrary to PPI, the ESI of the total fraction of SPI at pH 6 decreased with all HPP conditions, particularly for longer holding times (15 min) at 200 and 600 MPa, with a reduction of more than 40 %. At pH 7, most HPP conditions did not significantly ($p > 0.05$) affected the ESI, except for 200 MPa/10 min and 400 MPa/5 min that reduced it 21 and 32 %, respectively. At pH 8, HPP treatments slightly increase ESI or lead to a non-significant effect. The highest increases (*c.a.* 20%) were observed at 400 MPa, regardless of the holding time, and 600 MPa/15 min.

Considering only the soluble fraction of PPI (Figure 4.9), the ESI was 36 ± 5, 28 ± 2 and 34 ± 3 min at pH 6, 7 and 8, respectively. At pH 7, ESI increased with pressure at some particular conditions, namely 200 MPa/10 min, 400 MPa for 5 and 15 min, and 600 MPa/10 min with increases of 37, 56, 46, and 65 %, respectively. The other HPP conditions did not led to significant differences ($p > 0.05$). No major significant differences ($p > 0.05$) were verified at the other pH values.

Considering only the soluble fraction of SPI, unprocessed values of ESI were 37 ± 2, 35 ± 1 and 45 ± 4 at pH 6, 7 and 8 respectively. At pH 6, the ESI increased up to 36 % with increasing pressure and a holding time of 5 min. When longer holding times ≥10 min were used, no significant ($p > 0.05$) effects were observed. At pH 7, most HPP conditions did not significantly affect ESI. The exception was 200 MPa/5 min that decreased ESI 20 %. On the contrary, at pH 8 all the HPP conditions increased the ESI of the soluble fraction of SPI, which increased with increasing holding time, almost doubling the ESI with 15 min.

The ESI of plant-based proteins is variable since there are reports showing both increase and diminishment with HPP (Queirós et al., 2018). For example, the application of 200 or 400 MPa for 20 min increased ESI of ginkgo seeds proteins (Yin et al., 2008), whereas similar processing conditions decreased the ESI of soy proteins (X. S. Wang et al., 2008) and had no significant effects on sweet potato proteins (Khan, Mu, Sun, Zhang, & Chen, 2015a). Since the pressure affects different proteins in different ways and affects the exposition of hydrophobic groups and/or changes in the molecular flexibility, it can either increase or decrease the interactions between the proteins and the oil droplets, altering the ESI in different ways.

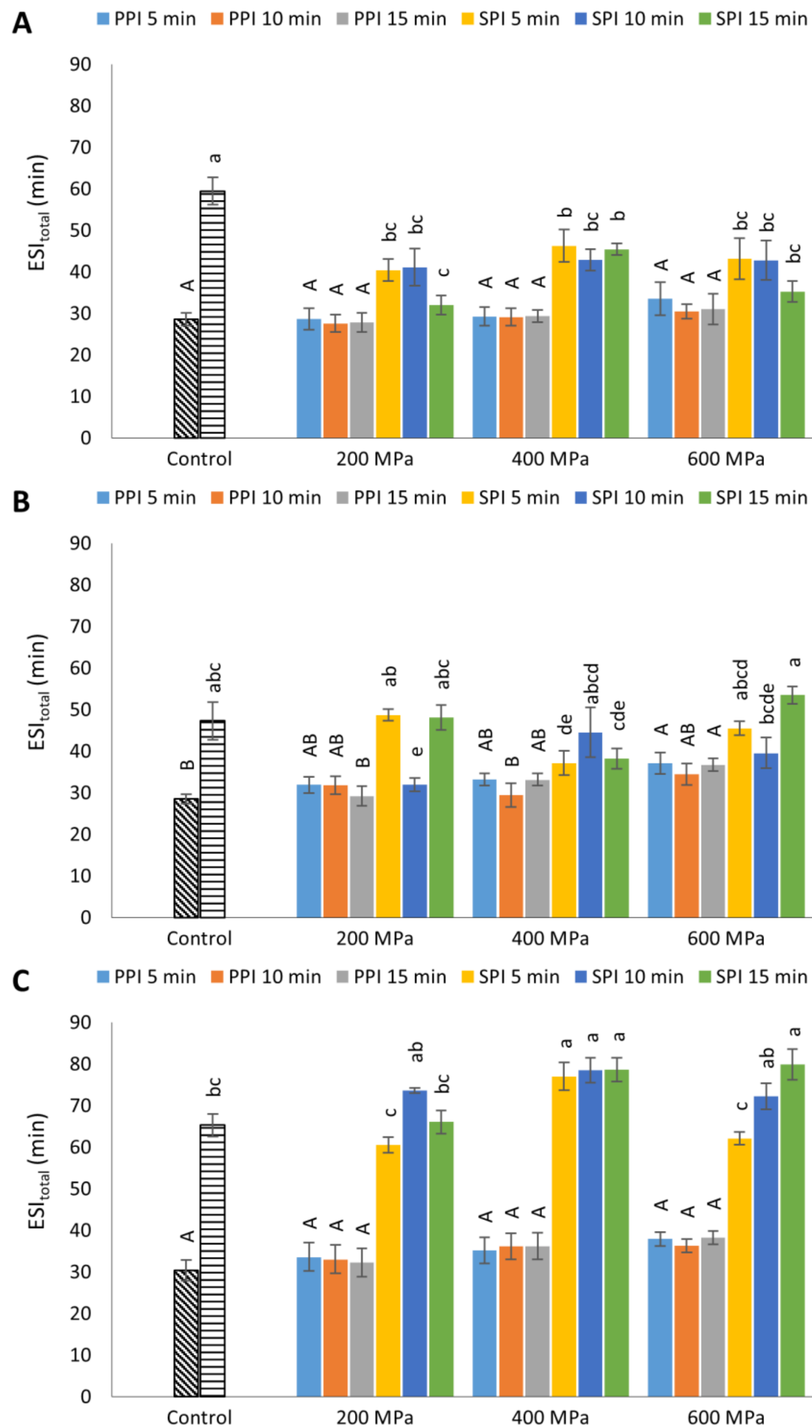



Figure 4.8. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the emulsifying stability index of the total fraction of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

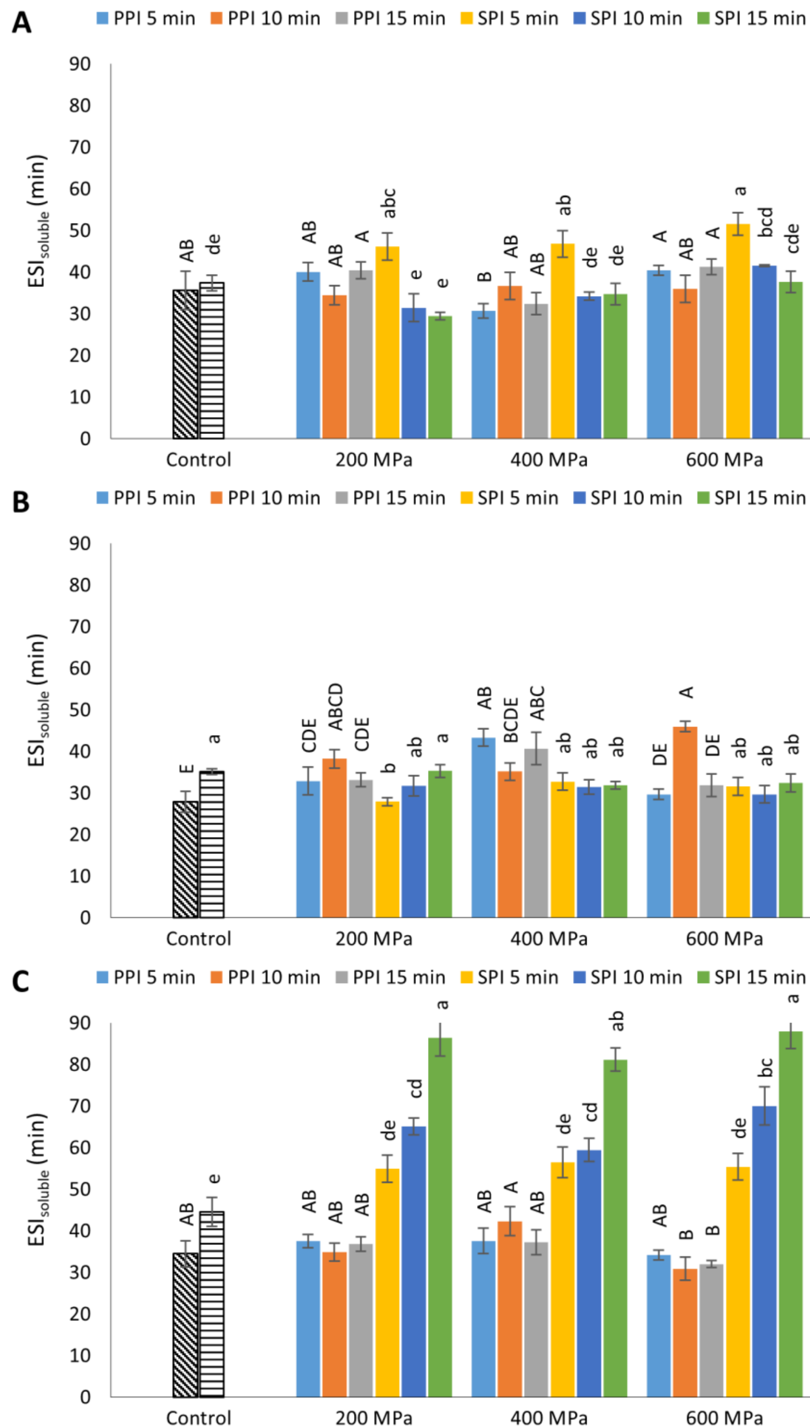



Figure 4.9. Effects of high-pressure processing, 200, 400 and 600 MPa for 5, 10 and 15 minutes on the emulsifying stability index of the soluble fraction of pea and soy protein isolates at different pH values: pH 6 (A), pH 7 (B) and pH 8 (C). The columns with diagonal and horizontal strips represent pea and soy control samples, respectively. Different capital letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different lowercase letters indicate significant differences ($p < 0.05$) between soy protein isolates.

## 4.4. Conclusion

This work has shown that HPP can lead to substantial changes in the structure of pea and soy proteins that may improve some of its techno-functional properties. Therefore, HPP increases, for instance, the solubility and surface hydrophobicity of protein isolates from both pea and soy, although the consequence on interfacial and emulsifying protein properties is strongly dependent on the selected processing conditions, and often with a compromised positive effect due to further protein aggregation. Overall, the presented results reveal that HPP can be effectively used to improve and tailor pea or soy proteins to be used as technological ingredients in the formulation of food products.

## 4.5. References

Achouri, A., & Boye, J. I. (2013). Thermal processing, salt and high pressure treatment effects on molecular structure and antigenicity of sesame protein isolate. *Food Research International*, *53*(1), 240–251.

Adebiyi, A. P., & Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (Pisum sativum L.) seed protein isolate. *Food Chemistry*, *128*(4), 902–908.

Ahmed, J., Ayad, A., Ramaswamy, H. S., Alli, I., & Shao, Y. (2007). Dynamic Viscoelastic Behavior of High Pressure Treated Soybean Protein Isolate Dispersions. *International Journal of Food Properties*, *10*(2), 397–411.

Baier, A. K., & Knorr, D. (2015). Influence of high isostatic pressure on structural and functional characteristics of potato protein. *Food Research International*, *77*, 753–761.

Balasubramaniam, V. M., Martínez-Monteagudo, S. I., & Gupta, R. (2015). Principles and application of high pressure–based technologies in the food industry. *Annual Review of Food Science and Technology*, *6*(1), 435–462.

Beveridge, T., Toma, S. J., & Nakai, S. (1974). Determination of SH- and SS-groups in some food proteins using ellman's reagent. *Journal of Food Science*, *39*(1), 49–51.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Bulaj, G. (2005). Formation of disulfide bonds in proteins and peptides. *Biotechnology Advances*, *23*(1), 87–92.

Burger, T. G., & Zhang, Y. (2019). Recent progress in the utilization of pea protein as an emulsifier for food applications. *Trends in Food Science and Technology*, 86(July 2018), 25–33.

Chao, D., Jung, S., & Aluko, R. E. (2018). Physicochemical and functional properties of high pressure-treated isolated pea protein. *Innovative Food Science & Emerging Technologies*, *45*, 179–185.

Condés, M. C., Añón, M. C., & Mauri, A. N. (2015). Amaranth protein films prepared with high-pressure treated proteins. *Journal of Food Engineering*, *166*, 38–44.

Day, L. (2013). Proteins from land plants – Potential resources for human nutrition and food security. *Trends in Food Science & Technology*, *32*(1), 25–42.

Derbyshire, E., Wright, D. J., & Boulter, D. (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry*, *15*(1), 3–24.

Galazka, V. B., Dickinson, E., & Ledward, D. (2001). Effect of high-pressure on surface behaviour of adsorbed films formed from mixtures of sulfated polysaccharides with various proteins. *Innovative Food Science & Emerging Technologies*, *1*(3), 177–185.

He, R., He, H.-Y., Chao, D., Ju, X., & Aluko, R. (2013). Effects of high pressure and heat treatments on physicochemical and gelation properties of rapeseed protein isolate. *Food and Bioprocess Technology*, *7*(5), 1344–1353.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA) - Protein Structure*, *624*(1), 13–20.

Khalid, E. K., Babiker, E. E., & El Tinay, A. H. (2003). Solubility and functional properties of sesame seed proteins as influenced by pH and/or salt concentration. *Food Chemistry, 82*,

361-366.

Khan, N. M., Mu, T. H., Sun, H. N., Zhang, M., & Chen, J. W. (2015a). Effects of high hydrostatic pressure on emulsifying properties of sweet potato protein in model protein – hydrocolloids system. *Food Chemistry*, *169*, 448–454.

Khan, N. M., Mu, T. H., Sun, H. N., Zhang, M., & Chen, J. W. (2015b). Effects of high hydrostatic pressure on secondary structure and emulsifying behavior of sweet potato protein. *High Pressure Research*, *35*(2), 189–202.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, *227*(5259), 680–685.

Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2018). Pea protein isolates: structure, extraction, and functionality. *Food Reviews International*, *34*(2), 126–147.

Lee, K. H., Ryu, H. S., & Rhee, K. C. (2003). Protein solubility characteristics of commercial soy protein products. *Journal of the American Oil Chemists' Society*, *80*, 85-90.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2011). Effects of high hydrostatic pressure on some functional and nutritional properties of soy protein isolate for infant formula. *Journal of Agricultural and Food Chemistry*, *59*, 12028–12036.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2012). Effects of high hydrostatic pressure treatment on allergenicity and structural properties of soybean protein isolate for infant formula. *Food Chemistry*, *132*(2), 808–814.

Liang, H. N., & Tang, C. H. (2013). PH-dependent emulsifying properties of pea [Pisum sativum (L.)] proteins. *Food Hydrocolloids*, 33(2), 309–319.

Mirmoghtadaie, L., Shojaee Aliabadi, S., & Hosseini, S. M. (2016). Recent approaches in physical modification of protein functionality. *Food Chemistry*, *199*, 619–627.

Molina, E., Papadopoulou, A., & Ledward, D. . (2001). Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. *Food Hydrocolloids*, *15*(3), 263–269.

Monteiro, S. R., & Lopes da Silva, J. A. (2019). Critical evaluation of the functionality of soy protein isolates obtained from different raw materials. *European Food Research and*

*Technology*, *245*, 199-212.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, *26*(3), 716–723.

Peyrano, F., Speroni, F., & Avanza, M. V. (2016). Physicochemical and functional properties of cowpea protein isolates treated with temperature or high hydrostatic pressure. *Innovative Food Science & Emerging Technologies*, *33*, 38–46.

Puppo, C., Chapleau, N., Speroni, F., De Lamballerie-Anton, M., Michel, F., Añón, C., & Anton, M. (2004). Physicochemical modifications of high-pressure-treated soybean protein isolates. *Journal of Agricultural and Food Chemistry*, *52*(6), 1564–1571.

Qin, Z., Guo, X., Lin, Y., Chen, J., Liao, X., Hu, X., & Wu, J. (2013). Effects of high hydrostatic pressure on physicochemical and functional properties of walnut (juglans regia l.) protein isolate. *Journal of the Science of Food and Agriculture*, *93*(5), 1105–1111.

Queirós, R. P., Saraiva, J. A., & Lopes da Silva, J. A. (2018). Tailoring structure and technological properties of plant proteins using high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition*, *58*(9), 1538–1556.

Tamm, F., Herbst, S., Brodkorb, A., & Drusch, S. (2016). Functional properties of pea protein hydrolysates in emulsions and spray-dried microcapsules. *Food Hydrocolloids*, *58*, 204–214.

Wang, X. S., Tang, C. H., Li, B. S., Yang, X. Q., Li, L., & Ma, C. Y. (2008). Effects of high-pressure treatment on some physicochemical and functional properties of soy protein isolates. *Food Hydrocolloids*, *22*(4), 560–567.

Wang, Z., Ju, X., He, R., Yuan, J., & Aluko, R. E. (2015). Effect of high pressure treatment on rapeseed protein microparticle properties and gastrointestinal release behavior of the encapsulated peptides. *Food Research International*, *77*, 549–555.

Yin, S. W., Tang, C. H., Wen, Q. B., Yang, X. Q., & Li, L. (2008). Functional properties and in vitro trypsin digestibility of red kidney bean (Phaseolus vulgaris L.) protein isolate: Effect of high-pressure treatment. *Food Chemistry*, *110*(4), 938–945.

Zhang, T., Jiang, B., Mu, W., Wang, Z. (2009). Emulsifying properties of chickpea protein

isolates: Influence of pH and NaCl. *Food Hydrocolloids, 23,* 146–152.

Zhao, J., Zhou, T., Zhang, Y., Ni, Y., & Li, Q. (2015). Optimization of arachin extraction from defatted peanut (Arachis hypogaea) cakes and effects of ultra-high pressure (UHP) treatment on physiochemical properties of arachin. *Food and Bioproducts Processing*, *95*(17), 38–46.

Zhu, S. M., Lin, S. L., Ramaswamy, H. S., Yu, Y., & Zhang, Q. T. (2017). Enhancement of functional properties of rice bran proteins by high pressure treatment and their correlation with surface hydrophobicity. *Food and Bioprocess Technology*, *10*, 317-327.

# CHAPTER 5

## Single and Simultaneously Combined Effects of Transglutaminase and High Pressure Treatments on Pea and Soy Protein Isolates

## 5.1. INTRODUCTION

Enzymatic crosslinking of food proteins is an attractive 'green' approach to manipulate food structure. Among potential enzymes for protein crosslinking is the transglutaminase, particularly microbial transglutaminase (MTG), which has been broadly studied and is commercially available. As previously discussed (§2.6), transglutaminase (E.C 2.3.2.13) is an enzyme that catalyses the acyl transfer reaction between the γ-carboxyamide group of protein-bound glutamine residues and primary amines, preferentially the ε–amino group of lysine residues. This reaction may lead to the formation of intra- and/or intermolecular crosslinks between proteins (Partschefeld, Richter, Schwarzenbolz, & Henle, 2007). Although most studies have been directed to meat, fish, seafood, and dairy proteins, the influence of MTG crosslinking on some technical and physiological functionality of soy and other plant proteins was already reported (Babiker, 2000; Dube, Schäfer, Neidhart, & Carle, 2007; Schäfer, Zacherl, Engel, Neidhart, & Carle, 2007; Tang, Li, & Yang, 2006).

The extent of the crosslinking reaction is dependent on environmental conditions (pH, temperature and enzyme inhibitors' absence) and on the structure and conformation of the target protein(s). Several studies have shown that non-globular proteins are more easily accessible to MTG crosslinking activity than globular proteins (Lorenzen, Schlimme, & Roos, 1998; Sharma, Lorenzen, & Qvist, 2001). Also, different vegetable proteins have shown different susceptibility to the MTG crosslinking activity (Schäfer et al., 2007). Therefore, despite the ease of MTG to crosslink various proteins, many of them, particularly globular proteins, are not affected by MTG in their native state due to the inaccessibility of the glutamine residues buried within the protein tertiary structure.

High pressure processing (HPP) may induce structural changes to proteins that could expose the mentioned residues, making them accessible to the MTG's acyl active site (Menéndez et al., 2006). With this in mind, research studies were already performed (Lauber et al., 2001; Lauber et al., 2003; Lee & Park, 2002; Nonaka et al., 1997) regarding MTG stability under high pressure, both in buffer solutions and food products, and how the enzyme's crosslinking activity is affected by pressure, as reviewed in Chapter 2 (§2.6.1). As reported and discussed in Chapter 3 of this thesis, MTG is stable under pressure, particularly at pH 6 and 7, where the enzyme retained more than 40 % of its activity even after being submitted to 600 MPa for 30 min. Under the tested conditions, MTG inactivation followed first-order kinetics.

The crosslinking obtained by MTG and HPP combined treatments may be a suitable tool for enhanced proteins' modification, allowing the improvement of functional properties without requiring a pre-treatment or the use of reducing agents (Gharibzahedi et al., 2018; Partschefeld et al., 2007). Hence, it is possible to infer that the combination of MTG and HPP may offer new perspectives for proteins' modification and may allow to tailor-make desirable functional and technological changes in the protein matrix. Despite what is already known regarding HPP effects on protein functionality and the MTG effects on protein crosslinking and structure development, knowledge concerning the influence of combined physical and enzymatic treatments on vegetable proteins' techno-functional properties is still needed, as most studies were performed on fish (Cardoso, Mendes, Saraiva, Vaz-Pires, & Nunes, 2010), meat (Trespalacios & Pla, 2007) and dairy (Sevdou, Eleftheriou, & Taoukis, 2013; Tsevdou et al., 2013). Therefore, the objective of this part of the work was to evaluate the combined effects of MTG and HPP on protein solubility, content of free sulfhydryl groups (SH) and surface hydrophobicity ($H_0$), and viscosity of pea (PPI) and soy (SPI) protein isolates, and how these properties are affected by protein concentration. Initial tests were performed based on a factorial experimental design to analyse the effect of HPP conditions, pressure (200, 400 and 600 MPa) and holding time (5, 10 and 15 min), and transglutaminase concentration (0, 15 and 30 $U{\cdot}g^{-1}$). The analysis of the influence of protein concentration, on the effect of HPP and MTG combined treatments, including on the viscosity of the protein isolate dispersions, was performed for selected HPP conditions (600 MPa, 15 min) and at 30 U MTG / (g protein).

## 5.2. MATERIALS AND METHODS

### 5.2.1. MATERIALS

Commercial PPI (Pisane® M9, Cosucra) and SPI (Induxtra W, Induxtra) used in the work described in this chapter were from the same source than those described in Chapter 4, but from a different production batch. Protein content was 80.9 ± 0.2 % for PPI and 86.3 ± 0.4 % for SPI. The water content of both protein isolates was ~ 10 %. According to the supplier, ash was lower than 6% and fat content lower than 4%. All reagents used in this part of the work were also of analytical grade. Activa® Transglutaminase (100 $U{\cdot}g^{-1}$) was a kind gift from

Ajinomoto Foods Europe SAS (Hamburg, Germany), and was the same enzyme sample used in Chapter 3 (§3.2.1).

### 5.2.2. Experimental design and modelling

A Box–Behnken design was the experimental design adopted to analyse the effect of HPP conditions, pressure and holding time, and transglutaminase concentration on some properties of PPI and SPI, namely solubility, SH and $H_0$. A set of 45 experiments, including 9 replicates at the central point, were performed in a randomized order.

For the description of the response, a quadratic polynomial equation (Eq. 5.1) and its subsets were evaluated. The general formulation of the model was as follows:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i<j} \sum \beta_{ij} x_i x_j \qquad (5.1)$$

where $Y$ is the response (dependent variable), $n$ is the number of independent variables, $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are model coefficients, and $x_i$ and $x_j$ are the independent variables (Baş & Boyacı, 2007). In this work, a regression model was constructed for each protein property, by setting $Y$ = solubility or SH or $H_0$ for the corresponding protein, considering $n$ = 3 with $x_1$, $x_2$, and $x_3$ representing dimensionless coded forms of pressure, holding time, and MTG concentration, respectively (Table 5.1). Therefore, six models were constructed: two for soluble protein - pea protein isolate ($PPI_{SOL}$) and soy protein isolate ($SPI_{SOL}$); two for free sulfhydryl groups - pea protein isolate ($PPI_{SH}$) and soy protein isolate ($SPI_{SH}$); and two for surface hydrophobicity - pea protein isolate ($PPI_{H0}$) and soy protein isolate ($SPI_{H0}$).

### 5.2.3. Sample preparation

The protein isolates were dispersed in distilled water (1 %, w/v) and stirred for 4 h at room temperature for hydration. The pH was adjusted to 7 with 0.1 $mol{\cdot}L^{-1}$ citric acid and the dispersions stirred for 40 min at room temperature. The dispersions (40 mL) were placed in flasks (Thermo Scientific™ Nalgene™ Wide-Mouth Lab Quality HDPE Bottles) for processing.

To evaluate the processing effects on the proteins' properties for different protein concentrations, dispersions at 15 % (w/v) of each protein isolate were first prepared in distilled water and stirred for 20 h at room temperature for hydration. After adjusting the pH to 7, the dispersions were diluted in distilled water to obtain dispersions at 1, 3, 5, 7, 9, 11 and 13 %

(w/v), and then stirred for 4 additional hours (11 and 13 % protein dispersion were only used for viscosity analysis). As above, these dispersions (40 mL) were also placed in flasks (Thermo Scientific™ Nalgene™ Wide-Mouth Lab Quality HDPE Bottles) for processing.

### 5.2.4. TRANSGLUTAMINASE REACTION

To assess the isolated effect of MTG on the protein dispersions, a solution of MTG was prepared in distilled water and diluted to have a final concentration of 10, 20 or 30 U·(g protein)$^{-1}$ when added to the dispersions. After adding the MTG solution to the protein dispersions the samples were incubated at 37 ºC for 60, 120 and 180 min.

To evaluate the combined effects of HPP and MTG, a solution of MTG was prepared in distilled water and diluted to have a final concentration of 15 or 30 U·(g protein)$^{-1}$ when added to the dispersions. The MTG was added to the dispersions immediately before HPP. After processing the samples were kept at 37 ºC for 60 min. At the end of the reaction time of both pressure treated and untreated dispersions, MTG was inactivated by adding N-ethylmaleimide (0.1 ml; 0.1%) (Kato, Wada, Kobayashi, Seguro, & Motoki, 1991). All samples were kept at 4 ºC overnight and were centrifuged at 6000 rpm for 20 min at 4 ºC before analysis.

### 5.2.5. PRESSURE TREATMENTS

For the samples to be studied accordingly to the experimental design described above, HPP conditions and MTG concentrations were those shown in Table 5.1. HPP was performed at room temperature, approximately 20 °C, using a hydrostatic press (Hiperbaric 55, Burgos, Spain), the same equipment used for the studies described in Chapter 4.

Table 5.1. Independent variables and their levels

| | Level (-1) | Level (0) | Level (+1) |
|---|---|---|---|
| Pressure, $P$ (MPa) ($x_1$) | 200 | 400 | 600 |
| Holding time, $t$ (min) ($x_2$) | 5 | 10 | 15 |
| MTG (U·(g protein)$^{-1}$) ($x_3$) | 0 | 15 | 30 |

For the tests aiming to investigate the effect of the combined treatments, HPP and MTG, on the select protein properties, for different initial protein concentration, protein dispersions, with and without added MTG, were treated at 600 MPa for 15 min at room temperature.

### 5.2.6. Soluble protein, free sulfhydryl groups and protein surface hydrophobicity

Determination of protein concentration in the supernatant of the centrifuged dispersions was performed based on the method of Bradford (1976), according to what was previously described in Chapter 4 (§4.2.5). The content of free sulfhydryl groups and protein surface hydrophobicity were also determined according to the procedures described before (§4.2.6 and §4.2.7, respectively), after keeping them at 4 ºC overnight and centrifuging the protein dispersions at 6000 rpm for 20 min at 4 ºC before analysis.

### 5.2.7. Shear viscosity

Shear flow measurements were performed in an AR-1000 controlled stress rheometer (TA Instruments, UK) equipped with a cone and plate measuring system (6 cm diameter acrylic cone, 2° angle). The flow curves were obtained after equilibrating the sample on the rheometer geometry, at 20 ºC, for 10 min, by an up-down step program applying a different shear stress range to each sample. Apparent viscosity measured at a shear rate of 50 $s^{-1}$ was considered to compare among different samples. Deviation from the Newtonian behaviour was quantified by applying a power law model (Eq. 5.2) to the shear-thinning region of the flow curves and considering the flow index:

$$\eta_a = K\,\dot{\gamma}^{n-1} \tag{5.2}$$

Where $\eta_a$ is the apparent viscosity (Pa.s), $\dot{\gamma}$ is the shear rate ($s^{-1}$), $K$ is the consistency coefficient (Pa.$s^n$), and $n$ is the flow behaviour index (dimensionless). This analysis was only performed for pea protein isolates.

### 5.2.8. Statistical analysis

A stepwise method was used to construct the regression models where the variables were included or excluded from the model based on its *p*-value with a significance of $p = 0.05$. When no more variables were eligible for inclusion or removal the iterative method ended. The coefficients of the model were estimated by maximum likelihood. Their standard errors and *p*-values were used to inspect the statistical significance of the coefficients.

The model summary statistics include model significance, goodness-of-fit and predictive ability. Analysis of variance (ANOVA) and an evaluation of the F statistics and its significance were used to assess the statistical significance of the regression models. The goodness-of-fit was evaluated by the coefficient of determination ($0\% \leq R^2 \leq 100\%$) and the $R^2$ adjusted for the number of terms in the model (adjusted $R^2$). Additionally, the model predictive ability was quantified from predicted $R^2$ obtained as the $R^2$ evaluated from residuals of observations not considered in the construction of the model (*i.e.*, each observation was removed from the dataset, the regression model was estimated, and the corresponding residual was evaluated). It is worth to mention that predicted $R^2$ is expected to always be lower than $R^2$ and that a value distinctly lower than $R^2$ suggests that the model is overfitting the data. The variance of the data was decomposed into the contribution of the model terms, discerning between the contribution of linear, quadratic and 2-way interaction terms, which sums up to $R^2$, and error terms, distinguishing lack-of-fit and pure error. The lack-of-fit of the models was also investigated as experimental data contained replicate measures, where differences between replicate measures are assumed to represent the pure error in the analysis.

All statistical analyses were performed with Minitab v19 (PA, USA) and Microsoft Excel 2010 (Microsoft Office System, USA), considering a statistical significance of $p = 0.05$.

## 5.3. RESULTS AND DISCUSSION

### 5.3.1. COMBINED EFFECTS OF TRANSGLUTAMINASE AND HIGH PRESSURE TREATMENTS AT A LOW PROTEIN CONCENTRATION

#### 5.3.1.1. REGRESSION MODELLING

The model described in Equation 5.1 was fitted to the experimental data in Table 5.2. and the estimates of the coefficients of models are presented in Table 5.3. Constant terms are

statistically significant in all models ($p < 0.001$). Regarding the linear terms, pressure, holding time and transglutaminase concentration are statistically significant in the models $PPI_{SOL}$, $PPI_{SH}$, and $SPI_{H0}$.

Still regarding the linear terms, in $SPI_{SOL}$ the holding time was not statistically significant, and in the $PPI_{H0}$ and $SPI_{SOL}$ only the MTG's concentration was statistically significant ($p < 0.001$). With respect to quadratic terms, the pressure was statistically significant only in $PPI_{SH}$ and $SPI_{SOL}$, whereas the holding time was statistically significant in $PPI_{SOL}$, $PPI_{H0}$, $SPI_{SOL}$, and $SPI_{H0}$. MTG's concentration was not statistically significant only in $SPI_{SH}$. Finally, there are 2-way interaction terms statistically significant in all the models, with emphasis on pressure and holding time, which were significant in all models.

On the overall assessment of the models, Table 5.4. shows the model's summary statistics. All models were statistically significant, with the ANOVA, low values of the corresponding *p*-values. Regarding the goodness-of-fit and predictive ability of the models, Table 5.4. shows that $R^2$, adjusted $R^2$ and predicted $R^2$ were large for all models, hence supporting that a large percentage of the variability of the data is explained by the constructed models. Finally, lack-of-fit was not significant ($p > 0.01$) in all models. Furthermore, plots of residuals *vs.* the predicted response showed no defined structure and the normal probability plots of residuals exhibited a straight line (*Annex B; Figures B1 – 6*). Thus, overall, the six models seem to be good representatives of the combined effects that HPP and MTG have on the referred properties of PPI and SPI.

Table 5.4. also presents the contribution of the data variability explained by the model terms (expressed as a %), distinguishing linear, quadratic and interaction ones. In the soluble protein models, linear components have the largest contribution to the respective models (*c.a.* 50 %), whereas, in the SH models, 2-way interactions have the largest contribution (*c.a.* 50 %). For soy proteins, a large contribution of the linear components was also observed for the surface hydrophobicity model.

Table 5.2. Levels of independent variables per the experimental design and resultant solubility, free SH groups and surface hydrophobicity for pea and soy protein isolates (protein dispersions at 1% (w/v), pH 7).

| | | | Pea protein isolates | | | Soy Protein Isolates | | |
|---|---|---|---|---|---|---|---|---|
| **Pressure (MPa)** | **Time (min)** | **MTG ($U{\cdot}g^{-1}$)** | **Soluble protein ($mg{\cdot}mL^{-1}$)** | **SH groups ($\mu mol{\cdot}mg\ prot^{-1}$)** | **Surface hydrophobicity** | **Soluble protein ($mg{\cdot}mL^{-1}$)** | **SH groups ($\mu mol{\cdot}mg\ prot^{-1}$)** | **Surface hydrophobicity** |
| 200 | 5 | 15 | 0.67 ± 0.02 | 2.47 ± 0.26 | 1939 ± 108 | 2.78 ± 0.04 | 3.12 ± 0.09 | 3487 ± 104 |
| 200 | 10 | 0 | 2.02 ± 0.15 | 2.59 ± 0.19 | 1896 ± 66 | 2.79 ± 0.07 | 3.57 ± 0.16 | 3467 ± 108 |
| 200 | 10 | 30 | 1.19 ± 0.07 | 1.45 ± 0.17 | 1951 ± 151 | 2.33 ± 0.10 | 2.07 ± 0.22 | 3083 ± 86 |
| 200 | 15 | 15 | 1.40 ± 0.06 | 0.63 ± 0.09 | 1678 ± 87 | 2.57 ± 0.09 | 2.11 ± 0.20 | 2036 ± 105 |
| 400 | 5 | 0 | 1.55 ± 0.09 | 1.11 ± 0.14 | 2853 ± 112 | 3.00 ± 0.02 | 2.90 ± 0.13 | 3035 ± 110 |
| 400 | 5 | 30 | 1.34 ± 0.11 | 1.41 ± 0.21 | 1649 ± 111 | 2.46 ± 0.08 | 2.12 ± 0.09 | 2387 ± 51 |
| 400 | 10 | 15 | 1.67 ± 0.07 | 0.68 ± 0.22 | 1470 ± 57 | 2.26 ± 0.04 | 2.48 ± 0.23 | 2080 ± 91 |
| 400 | 10 | 15 | 1.80 ± 0.05 | 0.54 ± 0.10 | 1550 ± 123 | 2.27 ± 0.09 | 2.61 ± 0.09 | 1960 ± 71 |
| 400 | 10 | 15 | 1.74 ± 0.04 | 0.70 ± 0.21 | 1474 ± 83 | 2.29 ± 0.09 | 2.64 ± 0.16 | 2013 ± 54 |
| 400 | 15 | 0 | 2.02 ± 0.09 | 0.54 ± 0.20 | 2021 ± 45 | 3.21 ± 0.09 | 2.86 ± 0.03 | 2813 ± 112 |
| 400 | 15 | 30 | 1.61 ± 0.05 | 0.64 ± 0.09 | 2057 ± 127 | 2.57 ± 0.07 | 2.14 ± 0.20 | 2182 ± 102 |
| 600 | 5 | 15 | 1.67 ± 0.07 | 0.55 ± 0.20 | 1587 ± 64 | 2.70 ± 0.08 | 2.07 ± 0.06 | 1491 ± 106 |
| 600 | 10 | 0 | 2.10 ± 0.07 | 0.40 ± 0.18 | 2563 ± 103 | 2.86 ± 0.10 | 2.11 ± 0.22 | 2675 ± 99 |
| 600 | 10 | 30 | 2.49 ± 0.11 | 2.12 ± 0.24 | 1367 ± 108 | 2.27 ± 0.10 | 2.43 ± 0.16 | 2269 ± 64 |
| 600 | 15 | 15 | 1.76 ± 0.14 | 1.76 ± 0.20 | 1926 ± 58 | 2.90 ± 0.13 | 2.87 ± 0.23 | 2352 ± 16 |

Values are presented as a mean ± standard deviation (n=3).

Table 5.3. Estimated coded coefficients for the developed models.

| | Pea protein isolates | | | Soy protein isolates | | |
|---|---|---|---|---|---|---|
| **Estimated coded coefficients** | **Soluble protein** | **SH groups** | **Surface hydrophobicity** | **Soluble protein** | **SH groups** | **Surface hydrophobicity** |
| Constant | 1.72 ± 0.03 | 0.64 ± 0.06 | 1524 ± 35 | 2.27 ± 0.04 | 2.54 ± 0.03 | 2035 ± 32 |
| $x_1$ | 0.34 ± 0.02 | -0.29 ± 0.05 | - | - | -0.17 ± 0.04 | -411 ± 23 |
| $x_2$ | 0.19 ± 0.02 | -0.25 ± 0.05 | - | - | - | -127 ± 23 |
| $x_3$ | -0.13 ± 0.02 | 0.12 ± 0.05** | -289 ± 26 | -0.28 ± 0.02 | -0.34 ± 0.04 | -259 ± 23 |
| $x_1 \cdot x_1$ | - | 0.71 ± 0.07 | - | 0.11 ± 0.03* | - | - |
| $x_2 \cdot x_2$ | -0.34 ± 0.03 | - | 239 ± 38 | 0.35 ± 0.03 | - | 294 ± 34 |
| $x_3 \cdot x_3$ | 0.24 ± 0.03 | 0.28 ± 0.07 | 401 ± 38 | 0.18 ± 0.03 | - | 557 ± 34 |
| $x_1 \cdot x_2$ | -0.16 ± 0.03 | 0.76 ± 0.07 | 150 ± 36 | 0.11 ± 0.03* | 0.45 ± 0.06 | 578 ± 33 |
| $x_1 \cdot x_3$ | 0.30 ± 0.03 | 0.71 ± 0.07 | -313 ± 36 | - | 0.45 ± 0.06 | - |
| $x_2 \cdot x_3$ | - | - | 310 ± 36 | - | - | - |

Values are presented as a mean ± standard error; $x_1$, $x_2$, and $x_3$ represent dimensionless coded forms of pressure, holding time, and transglutaminase concentration, respectively. All terms are significant ($p < 0.001$) otherwise marked: *$0.001 \leq p < 0.01$; **$0.01 \leq p < 0.05$

Table 5.4. Summary of statistics for the developed models.

| | | Pea protein isolates | | | Soy protein isolates | | |
|---|---|---|---|---|---|---|---|
| | | **Soluble protein** | **SH Groups** | **Surface Hydrophobicity** | **Soluble protein** | **SH Groups** | **Surface Hydrophobicity** |
| **Contribution** | Model | 94.76% | 91.97% | 92.37% | 90.23% | 84.07% | 96.65% |
| | Linear | 51.89% | 15.14% | 27.24% | 46.32% | 34.43% | 41.03% |
| | *P* | 35.28% | 7.98% | - | - | 7.08% | 27.50% |
| | *t* | 11.30% | 5.75% | - | - | - | 2.64% |
| | MTG | 5.30% | 1.41% | 27.24% | 46.32% | 27.16% | 10.90% |
| | Square | 25.21% | 24.89% | 30.52% | 40.73% | - | 28.41% |
| | *P***P* | - | 21.34% | - | 1.39% | - | 4.92% |
| | *t***t* | 17.54% | - | 6.62% | 30.64% | - | - |
| | MTG*MTG | 7.68% | 3.56% | 23.90% | 8.70% | - | 23.49% |
| | 2-Way Interaction | 17.66% | 51.93% | 34.61% | 3.18% | 49.64% | 27.22% |
| | *P***t* | 3.80% | 27.70% | 3.61% | 3.18% | 24.77% | 27.22% |
| | *P**MTG | 13.86% | 24.24% | 15.64% | - | 24.86% | - |
| | *t**MTG | - | - | 15.36% | - | - | - |
| | Error | 5.24% | 8.03% | 7.63% | 9.77% | 15.93% | 3.35% |
| | Lack-of-fit | 0.61% | 1.67% | 1.71% | 2.20% | 3.34% | 0.75% |
| | Pure Error | 4.63% | 6.36% | 5.93% | 7.58% | 12.59% | 2.60% |
| **Model summary** | *p*-value | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | $R^2$ | 0.948 | 0.920 | 0.924 | 0.902 | 0.841 | 0.967 |
| | Adjusted $R^2$ | 0.938 | 0.905 | 0.907 | 0.884 | 0.820 | 0.961 |
| | Predicted $R^2$ | 0.920 | 0.877 | 0.877 | 0.851 | 0.779 | 0.953 |
| | $p$-value $_{\text{Lack-of-fit}}$ | 0.527 | 0.168 | 0.080 | 0.130 | 0.325 | 0.198 |

*P* = pressure (MPa); *t* = holding time (min); MTG = concentration of transglutaminase (U/g protein).

#### 5.3.1.2. EFFECTS OF HPP AND MTG ON PROTEIN'S SOLUBILITY

Protein's solubility is greatly associated with their techno-functional properties, being decisive for their stabilizing, thickening, and gelling capabilities (Baier & Knorr, 2015). From the initial amount of PPI dispersed in water, 10 mg/mL, the amount of solubilized pea protein for the unprocessed sample was 0.70 ± 0.04 $mg{\cdot}mL^{-1}$, less than half of the soluble protein amount obtained for the unprocessed SPI (1.90 ± 0.06 $mg{\cdot}mL^{-1}$). The low amount of soluble protein is a common characteristic of commercial protein isolates, already reported in previous studies with PPI (Adebiyi & Aluko, 2011) and SPI (Lee, Ryu, & Rhee, 2003), and is generally attributed to a high degree of protein's denaturation and the presence of insoluble aggregates formed during isoelectric precipitation.

Overall, the addition of MTG to non-HPP protein isolates (up to 30 $U{\cdot}(g\ protein)^{-1}$ and to a reaction time of 180 min) resulted in no significant differences ($p > 0.05$) in the concentration of soluble proteins relative to the control samples – Table 5.5 and Annex C. The lower solubility of the protein isolates, particularly in the case of globular proteins that have a compact structure, may limit the accessibility of MTG to glutamine and lysine residues, limiting the enzyme effects (Jong & Koppelman, 2002). An exception was verified for PPI, where a MTG's concentration of 30 $U{\cdot}(g\ protein)^{-1}$ and a reaction time above 120 minutes led to an increase in the concentration of soluble protein of approximately 31 %. MTG catalyses the acyl transfer reaction between glutamine and lysine residues, however, in the absence of lysine or other primary amines, water will react as a nucleophile, resulting in deamidation (Jong & Koppelman, 2002).

Pea proteins are rich in glutamine and asparagine, which can be converted through MTG into glutamic acid and aspartic acid, respectively. In the absence of conditions that could lead to a pronounced crosslinking and formation of large protein aggregates that would decrease protein solubility, the resulting increased electrostatic repulsion between deamidated proteins may increase their solubility (Babiker, 2000; Gaspar & de Góes-Favoni, 2015).

Analysing Table 5.2. it is quite clear that for the pressurized samples, all tested processing conditions increased the concentration of soluble proteins for PPI, except for 200 MPa/5 min/15 $U{\cdot}g^{-1}$ that did not have a significant effect, and also for SPI.

Table 5.5. Effects of microbial transglutaminase on the concentration of soluble protein, free sulfhydryl groups and surface hydrophobicity of pea and soy protein isolates (protein dispersions at 1% (w/v), pH 7).

| | | Pea protein isolates | | | Soy protein isolates | | |
|---|---|---|---|---|---|---|---|
| **MTG (U·(g protein)$^{-1}$)** | **Time (min)** | **Soluble protein (mg·mL$^{-1}$)** | **SH Groups (µmol·mg prot$^{-1}$)** | **Surface hydrophobicity** | **Soluble protein (mg·mL$^{-1}$)** | **SH Groups (µmol·mg prot$^{-1}$)** | **Surface hydrophobicity** |
| 10 | 0 | 0.79 ± 0.08 a | 2.48 ± 0.18 a | 2517 ± 63 a | 2.09 ± 0.20 a | 1.98 ± 0.22 a | 2595 ± 89 a |
| | 60 | 0.81 ± 0.13 a | 2.48 ± 0.09 a | 2586 ± 78 a | 1.93 ± 0.08 a | 2.01 ± 0.07 a | 2573 ± 31 a |
| | 120 | 0.82 ± 0.13 a | 2.46 ± 0.05 a | 2535 ± 85 a | 1.92 ± 0.07 a | 2.04 ± 0.06 a | 2555 ± 86 a |
| | 180 | 0.93 ± 0.04 a | 2.43 ± 0.18 a | 2510 ± 57 a | 2.10 ± 0.13 a | 1.88 ± 0.09 a | 2622 ± 96 a |
| 20 | 0 | 0.86 ± 0.05 a | 2.34 ± 0.15 a | 2528 ± 72 a | 2.05 ± 0.10 a | 1.93 ± 0.06 a | 2630 ± 86 a |
| | 60 | 0.93 ± 0.07 a | 2.42 ± 0.12 a | 2509 ± 60 a | 1.94 ± 0.12 a | 2.11 ± 0.12 a | 2643 ± 107 a |
| | 120 | 0.90 ± 0.02 a | 2.39 ± 0.11 a | 2519 ± 51 a | 2.00 ± 0.08 a | 1.90 ± 0.05 a | 2590 ± 62 a |
| | 180 | 0.91 ± 0.04 a | 2.38 ± 0.06 a | 2499 ± 86 a | 1.99 ± 0.10 a | 1.92 ± 0.10 a | 2484 ± 103 a |
| 30 | 0 | 0.80 ± 0.07 a | 2.33 ± 0.16 a | 2571 ± 74 a | 1.99 ± 0.20 a | 1.97 ± 0.17 a | 2524 ± 76 a |
| | 60 | 0.91 ± 0.03 a | 2.36 ± 0.09 a | 2504 ± 60 a | 1.99 ± 0.14 a | 1.94 ± 0.14 a | 2557 ± 44 a |
| | 120 | 1.09 ± 0.08 b | 2.40 ± 0.14 a | 2558 ± 57 a | 1.94 ± 0.13 a | 1.98 ± 0.16 a | 2490 ± 92 a |
| | 180 | 1.05 ± 0.09 b | 2.51 ± 0.13 a | 2629 ± 51 a | 2.00 ± 0.22 a | 2.00 ± 0.08 a | 2538 ± 120 a |

Values are presented as a mean ± standard deviation (n=3). Different letters indicate significant differences ($p < 0.05$).

Figure 5.1 illustrates the individual main effects of each one of the parameters on the concentration of soluble proteins present in the PPI (Fig. 5.1(A)), as predicted by the model $PPI_{SOL}$, and in SPI (Fig. 5.1(B)), as predicted by the $PPI_{SOL}$ model.

For pea protein samples, it is evident that raising pressure increases the amount of soluble proteins of PPI. Similarly, increasing holding time up to approximately 13 min also increased proteins' solubility, however, a further increase in time does not further increase the concentration of soluble proteins. Figure 5.2(A) shows the interaction effects of HPP parameters (pressure and holding time) on the concentration of soluble proteins without the addition of MTG. The smallest increase of the concentration of soluble proteins (an increase of 91 %) was verified at the lowest HPP conditions (*i.e.* 200 MPa/5 min). Overall, increasing pressure and increasing holding time, up to *c.a.* 13 min, led to an increase of up to approximately 200 % in the concentration of soluble protein comparatively to control samples. When a pressure above 400 MPa is considered, a longer holding time did not further increase the proteins' solubility.

In what concerns SPI, every tested condition involving pressure increased proteins' solubility (Table 5.2). Considering only the individual parameters, although pressure increased the concentration of soluble protein, the pressure level seemed to not have much influence (Fig. 5.1(B)). On the other hand, varying the holding time impacted the proteins' solubility, as intermediate holding times (around 10 min) resulted in a smaller increase than at 5 or 15 min.

Although there are some interaction effects of pressure and holding time, these are not very large. All combinations of pressure and holding time led to an increase of soluble proteins and a synergetic effect was observable at the higher pressures and longer times, peaking at 600 MPa/15 min where the concentration of soluble proteins increased 77 % relatively to unprocessed samples (Figure 5.3(A)). The smaller increase of the concentration of soluble proteins, around 44 %, was verified at intermediate HPP conditions (375 MPa/9 min).

As previously discussed in Chapter 4 (§4.3.2), in general HPP originates an increase in protein solubility, although with a magnitude dependent on type of protein and HPP conditions, what is in accordance with what is described in literature, especially for soy proteins (Manassero et al., 2016; Puppo et al., 2004; Speroni et al., 2009; Yang, Yang, Gao, & Chen, 2014).

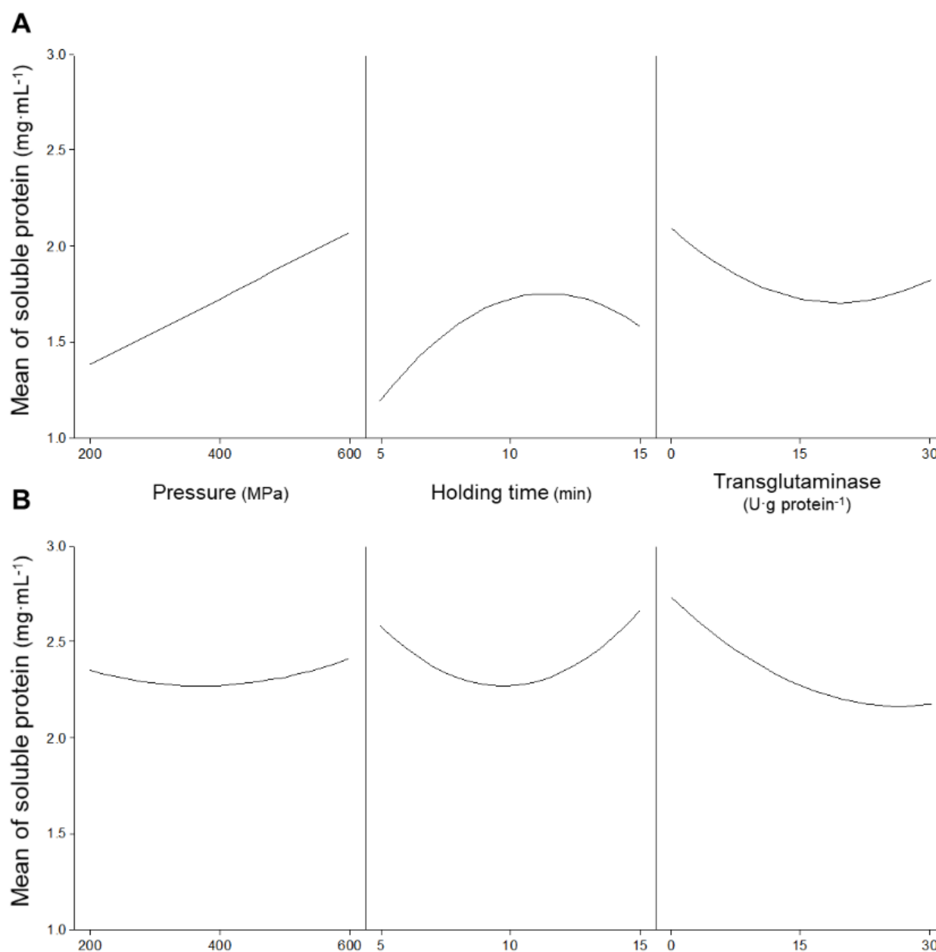



Figure 5.1. Main effects of the independent variables (pressure, holding time, and concentration of transglutaminase) on the concentration of soluble protein present in (A) pea and (B) soy protein isolates (initial protein dispersions at 1% (w/v), pH 7).

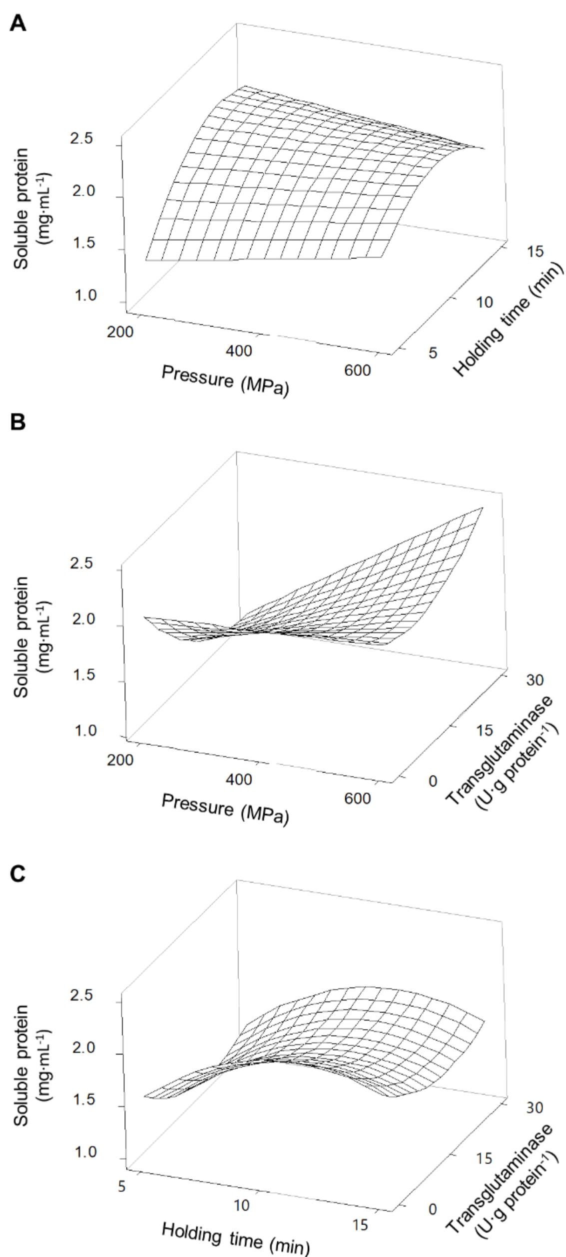



Figure 5.2. Response surface of the concentration of soluble protein present in pea protein isolates as a function of (A) pressure and holding time without MTG; (B) pressure and MTG concentration with a holding time of 10 min; and (C) holding time and transglutaminase' concentration while pressurized at 400 MPa.

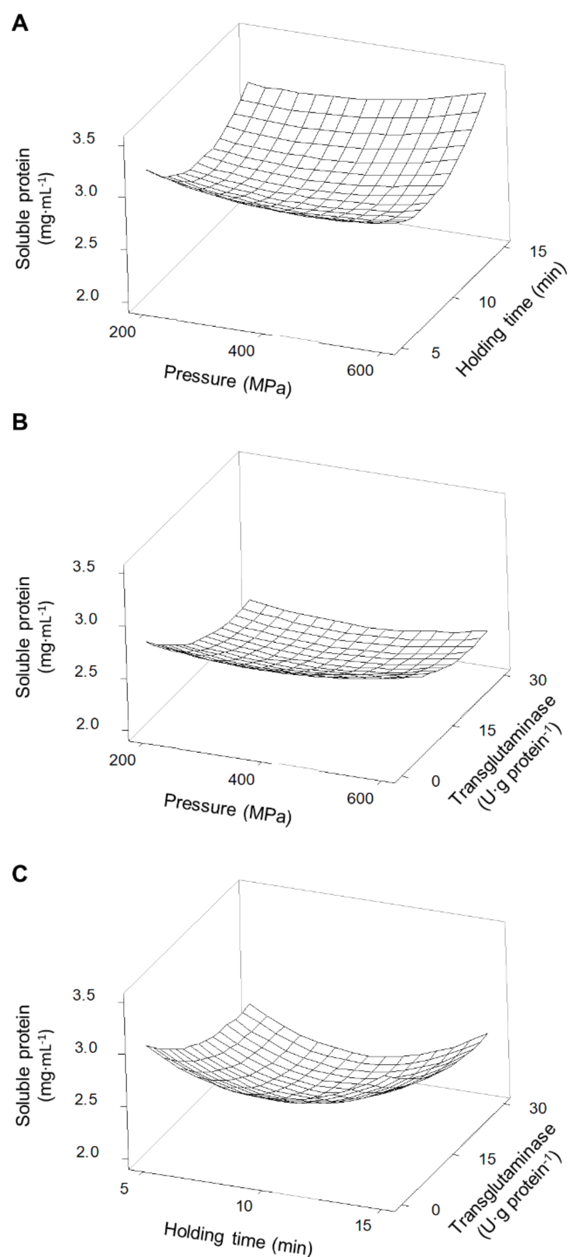



Figure 5.3. Response surface of the concentration of soluble protein present in soy protein isolates as a function of (A) pressure and holding time without MTG; (B) pressure and MTG concentration with a holding time of 10 min; and (C) holding time and transglutaminase' concentration while pressurized at 400 MPa.

This increase is most likely due to some unfolding of the proteins and the dissociation of aggregates promoted by pressure (Achouri & Boye, 2013; Yin, Tang, Wen, Yang, & Li, 2008). Slight discrepancies obtained here and in chapter 4, for the effect of HPP on protein solubility, namely for PPI, that if we compare with some already available reports (*e.g.*, Chao et al, 2018) may be related to the different extraction, isolation and drying procedures utilized. Worth to mention that pressure may enhance interactions between the protein and solvent, thus increasing solubility, however, it also may expose hydrophobic residues increasing intermolecular interactions and the formation of insoluble aggregates, therefore, reducing solubility (Li, Zhu, Zhou, & Peng, 2011; Queirós, Saraiva, & da Silva, 2018). These phenomena may explain why longer holding times did not further increase the pea proteins' solubility, or a smaller increase in SPI's solubility at intermediate holding times compared to 5 or 15 min.

MTG had the contrary effect of the HPP parameters, since the presence of the enzyme during the HPP treatments lead to a general decrease of protein solubility, for both PPI (Fig. 5.1(A)) and SPI (Fig. 5.1(B)). However, when interactions are considered, the addition of MTG resulted in different effects depending on the pressure level and type of protein.

For PPI, at low-pressure levels, *i.e.* < 400 MPa, increasing MTG's concentration decreased proteins' solubility, reaching levels near the control samples at 200 MPa, counteracting the effects of pressure (Fig. 5.2(B)). On the other hand, at pressure levels above 400 MPa and 10 min holding time, increasing MTG's concentration increased the concentration of soluble proteins up to 2.48 ± 0.05 mg·mL$^{-1}$ (~250 % increase compared to control), showing a synergetic effect between pressure and MTG concentration. In a general way increasing holding time, at moderate pressure (400 MPa), increased the concentration of soluble proteins, however, this parameter seems to have no interaction effect with the concentration of MTG – Figure 5.2.C and Table 5.2.

The increase in the concentration of MTG progressively reduced the proteins' solubility present in the SPI – Figure 5.1(B). Overall, the higher the concentration of MTG the more pronounced was the reduction of protein solubility, counteracting, to some extent, the increase promoted by pressure (Fig. 5.3(B)). Overall, there were no synergetic or antagonistic effects between holding time and MTG concentration – Figure 5.3(C) and Table 5.2.

Information about the combined effects of HPP and MTG on the solubility of proteins from legumes is scarce. Still, when considering other types of protein (*e.g.* β-lactoglobulin, casein, bovine serum albumin, ovalbumin, *etc.*), one can expect that HPP facilitates the crosslinking of protein catalysed by MTG (Gharibzahedi et al., 2018; Lauber et al., 2003). HPP might induce

structural changes to proteins that may expose glutamine and lysine residues, making them accessible to the MTG's acyl active site (Menéndez et al., 2006). Studies on the effects of MTG on SPI indicate that β-conglycinin (subunits α, α'and β) and the acidic subunits of glycinin can be crosslinked by MTG, forming high molecular weight biopolymers, whereas the basic subunits of glycinin remain intact. As a result, the solubility of the protein isolates decreased due to MTG activity. In addition, since the glycinin's basic subunits may remain intact, they can form aggregates that add to the reduction of solubility (Tang et al., 2006; Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007). A similar reduction of solubility of crosslinked PPI due to the formation of large molecular weight compounds was also reported (Ribotta, Colombo, & Rosell, 2012).

Therefore, the results suggest that HPP may dissociate proteins' aggregates present in PPI and SPI increasing their solubility. In doing so, and by altering the structure of proteins, pressure can make them more accessible to the action of MTG, which can result in the formation of high molecular weight biopolymers. Still, for PPI, a pressure >400 MPa and an increase in MTG's concentration from 15 to 30 U·(g protein)$^{-1}$ led to an increase in proteins' solubility, suggesting these conditions may have promoted the deamidation of glutamine and asparagine. How these complex effects may be dependent on the amount of protein present in the solution will be further analysed and discussed below (§5.3.2.1).

#### 5.3.1.3. Effects of HPP and MTG on the free sulfhydryl groups

Free sulfhydryl groups and disulfide bonds can influence the technological properties of proteins. These weak secondary bonds help to maintain the tertiary structure of proteins, and its manipulation is important to influence proteins' functional properties (§2.2.4). The addition of MTG up to 30 U·(g protein)$^{-1}$ with a reaction time up to 180 min, without pressure treatment, did not significantly ($p > 0.05$) affect the concentration of the free sulfhydryl groups in any of the protein isolates (Table 5.5). As previously discussed, the lack of effects of MTG on non-HPP protein isolates is most likely due to the inaccessibility of the enzyme to glutamine and lysine residues.

The content of free sulfhydryl groups for unprocessed PPI was 2.2 ± 0.4 μmol·(g protein)$^{-1}$, whereas that measured for the unprocessed SPI was 1.9 ± 0.1 μmol·(g protein)$^{-1}$. Most treatments involving pressure decreased the concentration of free sulfhydryl groups for PPI samples, whereas an opposite effect, although less pronounced, was observed for the SPI

samples, especially at the lowest tested pressure (Table 5.2). As discussed before, HPP alone, at different pHs, has shown a more pronounced effect on decreasing the amount of free SH for pea proteins than for soy proteins (§4.3.3).

Figure 5.4(A) illustrates the individual main effects of each one of the parameters on the concentration of free sulfhydryl groups present in PPI as predicted by the model $PPI_{SH}$. When taking into account the pressure individually, this parameter decreases the concentration of free SH groups, particularly at intermediated pressures, within the range analysed. At 400 MPa, the holding time decreases the free SH content linearly, but the effect seems to be dependent on the applied pressure (Figure 5.5(A)). A maximum SH content was predicted at 200 MPa/5 min, and represents a 91 % increase relatively to non-processed samples (Figure 5.5(A)). However, increasing the severity of HPP conditions led to a progressive reduction of the concentration of free SH groups, reaching a predicted minimum at 600 MPa/5 min. At more severe pressure conditions, *i.e.* 500 – 600 MPa, longer holding times appear to have a less negative effect on free SH than short times.

When MTG was added to PPI, and only considering its main effect, it had a contrary effect to the other processing parameters, as concentrations above 15 U·(g protein)$^{-1}$ increase the content of free sulfhydryl groups (Figure 5.4(A)). The effects of combined pressure and MTG are complex (Figure 5.5(B)), and it seems that these parameters had an antagonistic effect, with the presence of MTG counteracting the reduction of free SH groups caused by HPP. No significant interaction effect was observed between MTG's concentration and the holding time (Table 5.3).

With respect to SPI, Figure 5.4B illustrates the individual main effects predicted by the model $SPI_{SH}$. Although HPP can cause a slight increase in free SH concentration, comparing to the non-pressurized sample, the increase in pressure also caused a decrease in the free SH groups for the soy proteins, whereas the holding time seemed to have no impact (Fig. 5.4(B)). When considering the combined effects of HPP parameters (pressure and time), and without the addition of MTG, the decrease in free SH groups promoted by increasing pressure is clearly dependent on the holding time, and somewhat unexpected, the higher the holding time the less pronounced was the effect of increasing pressure (Figure 5.6(A)), but nevertheless, presenting a behaviour similar to that observed for PPI. Worth mentioning that even so all pressurized samples showed higher free SH concentration than the SPI unprocessed samples (Table 5.2), with the maximum increase predicted to be approximately 110 % at 200 MPa/5 min. The

minimum predicted concentration of free sulfhydryl groups of processed SPI was verified at 600 MPa/5 min, comparable to the value of unprocessed samples.

Overall, the results suggest that applying pressure resulted in the dissociation/unfolding of the proteins exposing buried SH groups, particularly at lower pressure values (<400 MPa). However, higher pressure values may have promoted hydrophobic interactions that led to S-S exchange and/or formation of new disulfide bonds resulting in a decrease of free SH (Li, Zhu, Zhou, & Peng, 2012; Yin et al., 2008).

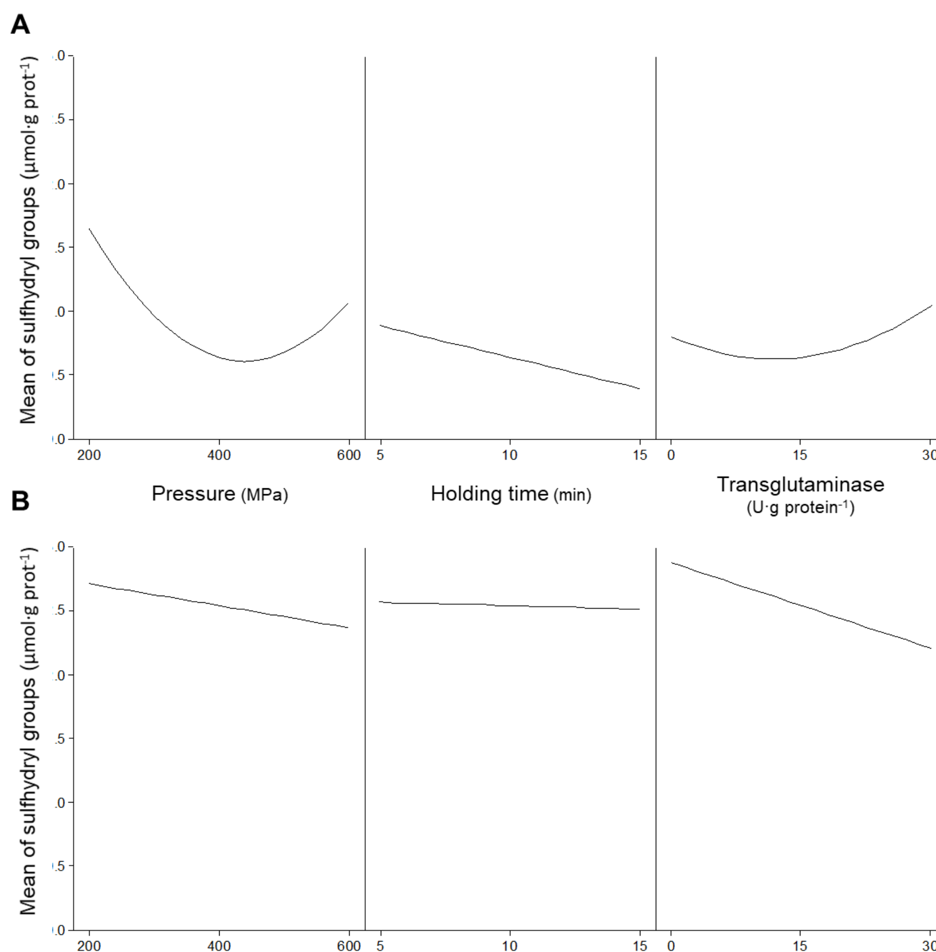



Figure 5.4. Main effects of the independent variables (pressure, holding time, and concentration of transglutaminase) on the concentration of sulfhydryl groups present in (A) pea and (B) soy protein isolates (initial protein dispersions at 1% (w/v), pH 7).

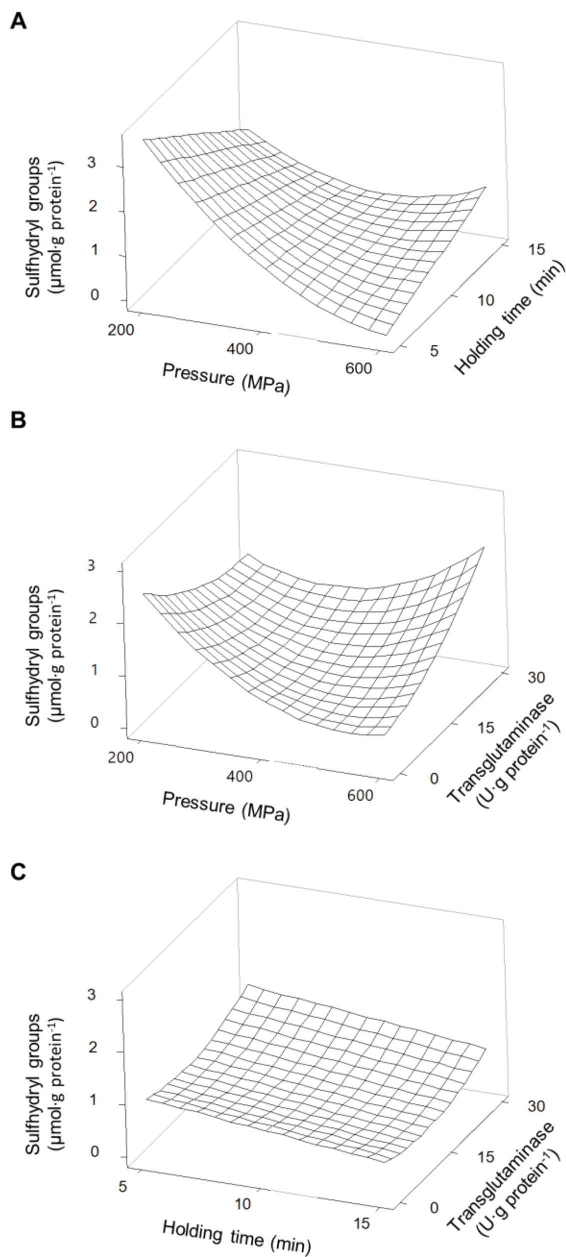



Figure 5.5. Response surface of the concentration of sulfhydryl groups present in pea protein isolates as a function of (A) pressure and holding time without MTG; (B) pressure and MTG concentration with a holding time of 10 min; and (C) holding time and MTG concentration while pressurized at 400 MPa.

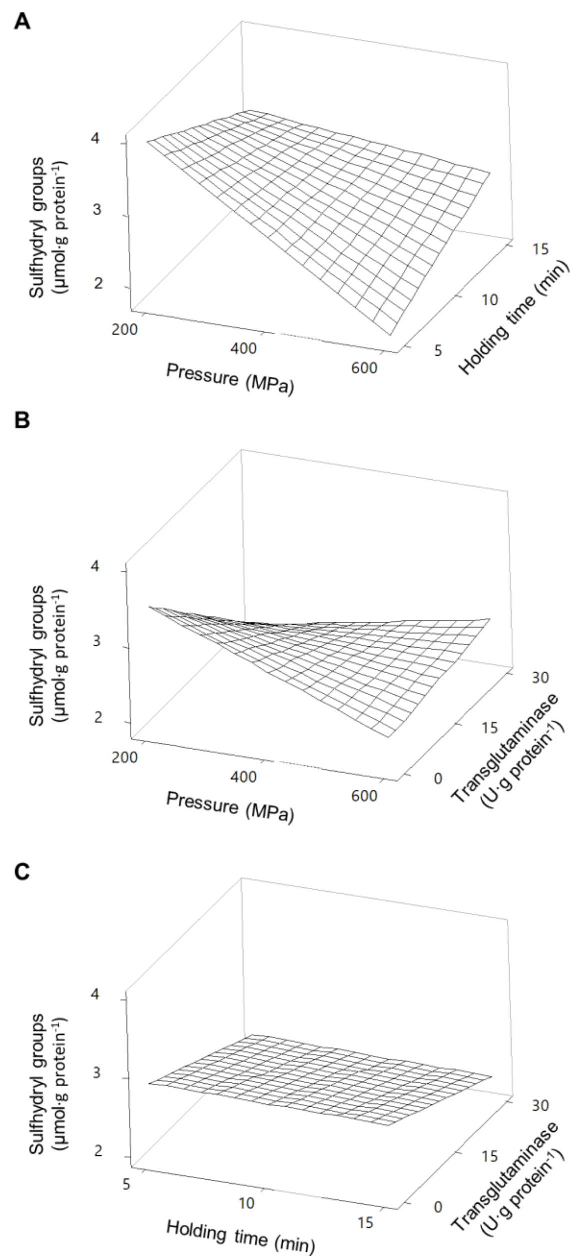



Figure 5.6. Response surface of the concentration of sulfhydryl groups present in soy protein isolates as a function of (A) pressure and holding time without MTG; (B) pressure and MTG concentration with a holding time of 10 min; and (C) holding time and MTG concentration while pressurized at 400 MPa.

As discussed before in Chapter 4 regarding the effect of HPP conditions on the number of free SH groups (§4.3.3), the results here described agree with the available literature concerning soy proteins and proteins from other sources, where it is reported that pressure below 300 MPa may preserve or improve the content of free SH, whereas higher pressure values or longer holding times seem to decrease it (He et al., 2013; Li et al., 2012; Queirós et al., 2018).

The individual main effect of MTG was the decrease in the amount of free SH groups for soy proteins, as can be seen in Figure 5.4B. If fact, the higher the MTG's concentration, the higher the observed decrease in the free sulfhydryl groups. However, when taking into account its combined effect with pressure, this reduction seemed to be much more accentuated at lower pressure values (*e.g.* 200 MPa) than at 600 MPa, where the effect of MTG is barely noticeable (Figure 5.6(B)). Similarly to PPI, it was not verified an interaction effect between MTG's concentration and the holding time.

Therefore, most treatments with MTG led to a decrease in available SH groups. This decrease after MTG catalytic action was already reported for SPI ( Zhang et al., 2016) and vicilin-rich kidney protein isolate (Tang, Sun, Yin, & Ma, 2008). This is most likely due to the crosslinking between proteins, which may have promoted some changes in the proteins' conformation and consequent formation of new disulfide bonds, and/or buried the free SH groups into the resulting high molecular weight aggregates becoming no longer measurable as free SH groups (Tang et al., 2008; Zhang et al., 2016). Still, some conditions have led to the increase of free sulfhydryl groups present in PPI after MTG, namely when pressure >300 MPa was applied. Similar results were reported for sweet potato protein isolate and peanut protein isolate treated with HPP and micro fluidization, respectively, and subsequent MTG crosslink (Hu, Zhao, Sun, Zhao, & Ren, 2011; Zhao, Mu, Zhang, & Richel, 2019). The mechanism by which this happens is not yet fully understood, still, is it suggested that the conformational changes mentioned above promoted by the crosslink may expose buried free sulfhydryl groups (Zhao et al., 2019).

#### 5.3.1.4. Effects of HPP and MTG on the surface hydrophobicity

Another important factor affecting proteins' technological properties is their surface hydrophobicity. Increases in the $H_0$ are related to the exposure of the side chain of aromatic

amino acids, that is to say, the higher the $H_0$ the higher the number of hydrophobic groups exposed to the outside of the protein (Chapter 2, §2.2.3). For the samples here analysed, the $H_0$ of both unprocessed protein isolates presented similar values, *c.a.* 2600. This value was not significantly ($p > 0.05$) affected by the addition of MTG up to 30 U·(g protein)$^{-1}$ and a reaction time up to 180 min without pressure treatment (Table 5.5). Similarly to solubility and content free sulfhydryl groups, $H_0$ was not affected when MTG was added to non-HPP protein isolates most likely due to the inaccessibility of the enzyme to its substrates.

Figure 5.7A illustrates the individual main effects of each one of the studied parameters on the surface hydrophobicity of PPI as predicted by the model $PPI_{H0}$. It seems that the effect of pressure on the surface hydrophobicity is not dependent on the pressure level. On the other hand, intermediate holding times seem to originate lower surface hydrophobicity values than low or high holding time values. When considering the combination of the HPP parameters (pressure and time – Figure 5.8(A)) it was observed that the holding time and pressure values had interaction effects. For instance, the $H_0$ decreased 38 % when the holding time increased from 5 to 15 min at 200 MPa, whereas a reduction of 14 % with the same time increase was observed at 600 MPa. An increase of $H_0$ occurred with increasing pressure, more markedly at longer times. For instance, for a holding time of 5 min, increasing pressure from 200 to 600 MPa resulted in a small increase (12 %), while the same increase pressure with a 15 min holding time led to an increase of 56 %. Still, most HPP conditions with higher holding times decrease the $H_0$ when compared with untreated samples, particularly at lower 200 MPa and 15 min.

In what concerns SPI, considering the individual main effects of HPP (*i.e.* pressure and holding time), the increase of both led to lower values of $H_0$, still, an additional increase of pressure from *c.a.* 500 MPa did not further decrease $H_0$ – Figure 5.7(B). Considering the combined effect of HPP parameters (Figure 5.9(A)) it was possible to observe distinct behaviours at low and high pressure and at shorter and longer holding times. The predicted higher value of $H_0$ was 4261 ± 60 at 200 MPa/5 min, which represents an increase of 67 % relative to the unprocessed samples. From this point, increasing pressure up to 600 MPa resulted in a decrease of 46 % of the $H_0$ and increasing holding time to 15 min caused in a 33 % decrease. On the contrary, increasing the holding time, from 5 to 15 min, at 600 MPa increased $H_0$ by 40 %. Overall, it seems that pressure variations had more impact on the $H_0$ at shorter than longer holding times, with most pressure conditions resulting in an increase in $H_0$ compared to the untreated SPI.

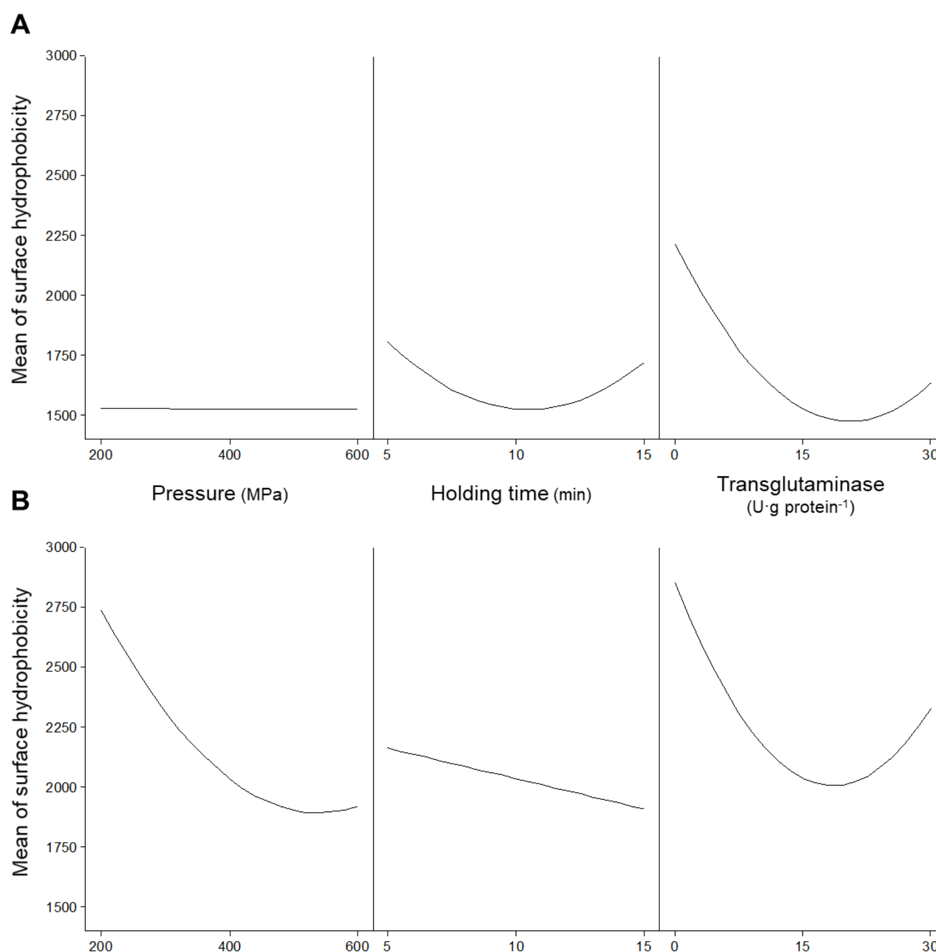



Figure 5.7. Main effects of the independent variables (pressure, holding time, and concentration of transglutaminase) on the surface hydrophobicity of (A) pea and (B) soy protein isolates (initial protein dispersions at 1% (w/v), pH 7).

Applying pressure may result in the unfolding of the proteins, thus exposing the number of hydrophobic groups on its surface. As a result, in general, HPP increases proteins' surface hydrophobicity due to the resulting conformational changes (Queirós et al., 2018). Still, as here described, the combination of low pressure (*i.e.* 200 MPa) and high holding time (15 min) have led to a decrease of $H_0$ of PPI, whereas more intensive HPP conditions did not majorly impact this parameter. Pressure may promote interactions between the proteins' hydrophobic regions and solvent or between proteins, and change the equilibrium between aggregation and dissociation processes decreasing $H_0$ (Qin et al., 2013).

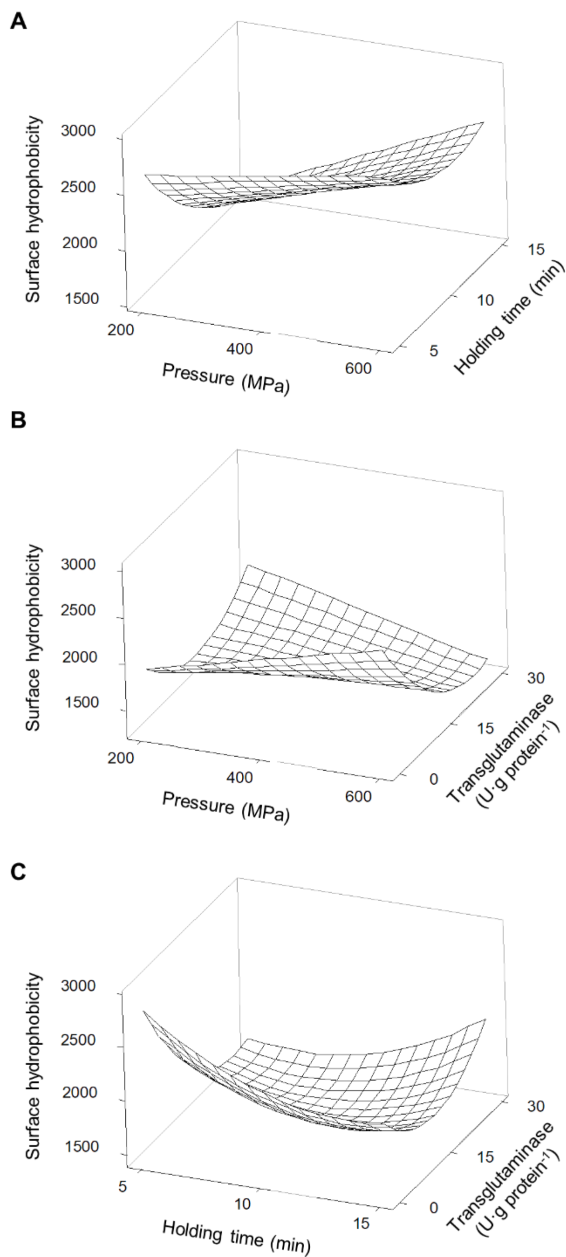



Figure 5.8. Response surface of the surface hydrophobicity of pea protein isolates as a function of (A) pressure and holding time without transglutaminase; (B) pressure and transglutaminase' concentration with a holding time of 10 min; and (C) holding time and transglutaminase' concentration at 400 MPa.

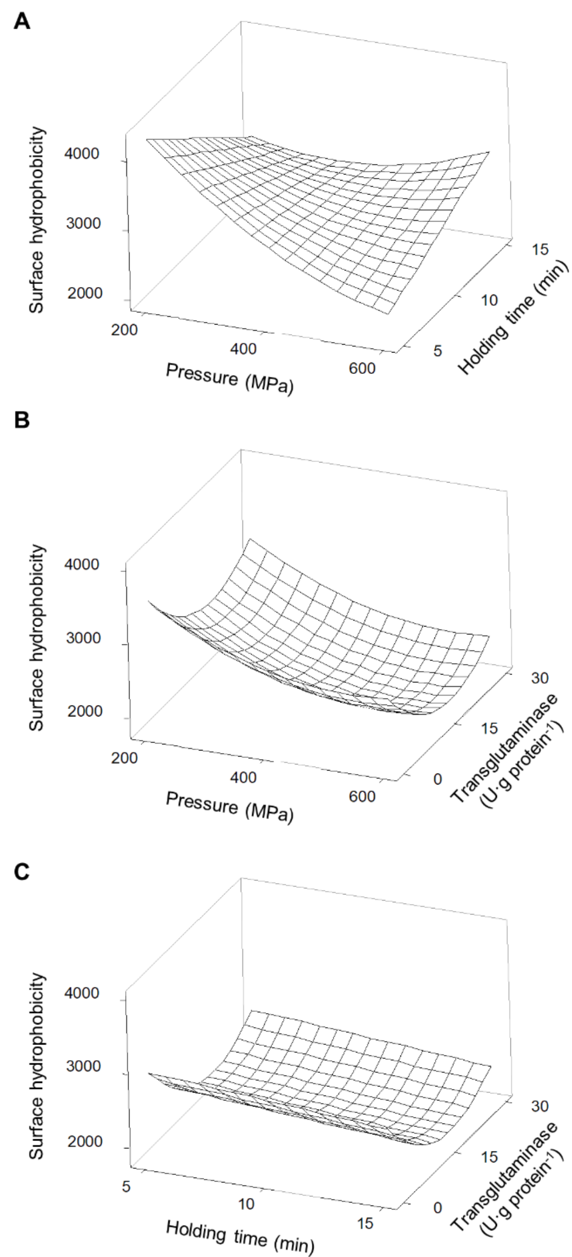



Figure 5.9. Response surface of the surface hydrophobicity of soy protein isolates as a function of (A) pressure and holding time without transglutaminase; (B) pressure and transglutaminase' concentration with a holding time of 10 min; and (C) holding time and transglutaminase' concentration at 400 MPa.

In this particular case, it is possible that these low HPP conditions may have promoted protein–water interactions of PPI, reducing $H_0$ and consequently increasing protein solubility, as the solubility of PPI was higher at these conditions – Figure 5.2(A) The described results for SPI are in agreement with what is reported in the literature, for instance, applying low pressure to soy's glycinin and β-conglycinin increased their $H_0$ (Wang et al., 2011; Zhang, Li, Tatsumi, & Kotwal, 2003). Still, increasing pressure may lead to a smaller increase in $H_0$ or even decrease it. This lower $H_0$ at more severe HPP conditions compared to mild conditions was already reported for SPI (Li et al., 2012; Yang et al., 2014), what is likely due to an increased aggregation accompanied by conformational changes (dissociation of protein subunits, changes in the tertiary and secondary structures, increasing exposure of hydrophobic groups, etc.) (Queirós et al., 2018).

The addition of MTG to PPI changed the effect of pressure on $H_0$ – Figure 5.8(B) While without MTG the increase in pressure from 200 to 600 MPa led to an increase of approximately 30 % of $H_0$ when in combination with MTG, the same increase in pressure led to a decrease of also *c.a.* 30 % with 30 U·(g protein)$^{-1}$. An interaction effect was also observed between holding time and MTG's concentration – Figure 5.8(C) While, at 400 MPa and no MTG, an increase in holding time from 5 to 15 min led to a 25 % reduction of $H_0$, while the combination with 30 U·(g protein)$^{-1}$ of MTG had the contrary effect, *i.e.* the same increase in holding time increased $H_0$ by approximately 30 %. A lower concentration of MTG (*i.e.* around 15 U·(g protein)$^{-1}$) seemed to stabilize $H_0$ regarding HPP parameters variations, as $H_0$ did not vary considerably with pressure or holding time changes when this concentration of MTG was used – Figures 5.8(B) and 4.8(C).

Considering SPI, the addition of MTG at a lower concentration (15 U·(g protein)$^{-1}$) decreased $H_0$, however, it did not seem to have an antagonistic or synergetic effect with any of the other studied parameters, as it can be seen in Table 5.3. and Figures 5.9(B) and 5.9(C) Still, increasing the concentration of MTG to 30 U·(g protein)$^{-1}$ appeared to have a smaller impact on the $H_0$.

Broadly, adding MTG during the HPP yielded a lower $H_0$, however, in most cases, the reduction was higher with an MTG concentration of 15 than 30 U·(g protein)$^{-1}$. There are reports were MTG both increases ( Jiang & Zhao, 2010; Song & Zhao, 2014) and decreases (Agyare, Xiong, & Addo, 2008; Jiang, Tang, Wen, Li, & Yang, 2007) the $H_0$ of different proteins. The crosslink promoted by MTG may occlude hydrophobic residues inside the structure of the

higher molecular polymers formed, decreasing $H_0$ (Agyare et al., 2008). Additionally, the deamination of glutamic and aspartic acids may increase the overall negative charge of the proteins, leading to a decrease in $H_0$ (Jong & Koppelman, 2002). Contrary, the crosslink of proteins may also change their structure and consequently resulting in unfolding, thus exposing hydrophobic regions and increasing the $H_0$ (Hu et al., 2011). Therefore, the concentration of MTG may lead to different degrees of crosslinking and deamination reaction rates, differently affecting the $H_0$ of proteins.

### 5.3.2. Combined effects of transglutaminase and high pressure treatments as a function of protein concentration

#### 5.3.2.1. Effects of HPP and MTG on protein's solubility

Under the particular dispersion conditions used in this part of the work, and described above (§5.2.3), and for a protein dispersion at 1 % (w/v) and pH 7, as those previously described in Chapter 4 and above, the PPI showed a solubility of 14.7 ± 0.5 % and SPI 25.6 ± 2.0 %.

The observed relative variation of each parameter ($x_{ps}$) under study ($\Delta x$ %, Equation 5.3), comparing to that same parameter measured for the control solutions (protein solution without any treatment, $x_c$), was used to analyse the effects of the isolated processing treatments, HPP and MTG, and their combined effects, on the selected protein properties in solution, as a function of the protein concentration.

$$\text{Relative variation, } \Delta x\text{, \%} = (x_{ps} - x_c) \times 100 / x_c \qquad (5.3)$$

Figure 5.10 shows the results obtained, regarding protein solubility, for (A) PPI and (B) SPI dispersions. For the pea proteins, the presence of MTG had only a slight effect on protein solubility within the protein concentration analysed. In general, the tendency was to reduce protein's solubility, although not always statistically significant ($p > 0.05$).

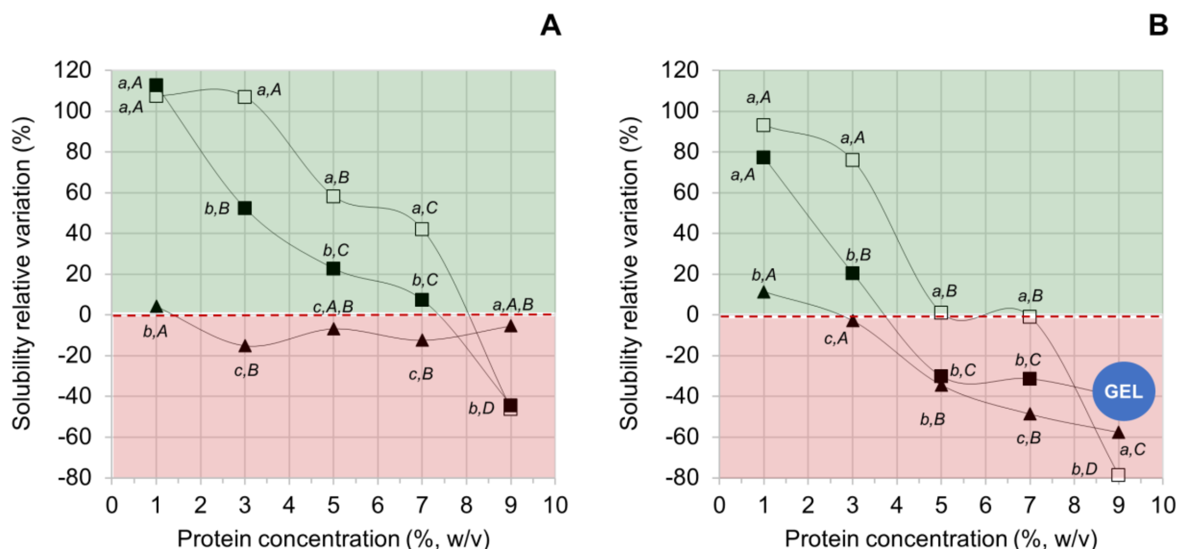



Figure 5.10. Relative variation of protein solubility for (**A**) PPI and (**B**) SPI dispersions, calculated accordingly to equation 5.3, as a function of protein concentration, for protein dispersions (pH 7) under different processing conditions: (▲) in the presence of 30 U/g protein MTG; (□) treated by HPP at 600 MPa, 15 min; (■) processed by a combination of both treatments (MTG+HPP). Different capital letters indicate significant differences ($p < 0.05$) between the same treatment at different protein concentrations. Different lowercase letters indicate significant differences ($p < 0.05$) between different treatments at the same protein concentration.

For the soy proteins (Figure 5.10(B)), there was a more pronounced decrease in protein solubility with the addition of the enzyme for initial protein concentrations ≥ 5%, reaching values lower by around 40-60% of the protein solubility value measured for the non-processed samples. Most likely, higher protein concentrations correspond to a greater availability and amount of substrate for the catalytic action of MTG, promoting the formation of a larger number and/or larger size of protein aggregates and, consequently, a reduction in the solubility of the proteins. This effect was more evident for SPI dispersions, due to a better accessibility of the enzyme to the protein reactive groups and/or to a higher affinity of the enzyme for some of the soy protein fractions, considering the already reported formation of high-molecular polymers, with low solubility, resulting from the crosslinking catalysed by MTG involving β-conglycinin (subunits α, α'and β) and the acidic subunits of glycinin (Yasir et al., 2007).

Considering the effect of HPP alone (600 MPa, 15 min), clearly the increase in protein solubility observed for low protein concentrations, and already discussed before in this Chapter and in Chapter 4, is strongly dependent on protein concentration, with a significant decrease in solubility with an increase in protein concentration. At the higher concentration analysed, the solubility for the pressurized samples, for PPI and SPI, was even lower than the non-pressurized samples. As previously discussed, the possible increase in protein solubility promoted by HPP is probably due to the dissociation of protein aggregates as well as some protein unfolding, enhancing their interactions with the solvent (water) (Achouri & Boye, 2013; Manassero et al., 2015; Yin et al., 2008). On the other hand, the pressure might expose hydrophobic residues and so increase intermolecular interactions that subsequently form insoluble aggregates, particularly at higher protein concentrations. The data here reported suggests that the high protein concentration can favour the formation of these insoluble aggregates as reported elsewhere for amaranth protein isolates (Condés et al. 2012).

The effect of the combined treatments, MTG+HPP, on the solubility of the pea proteins (Figure 5.10A), followed a qualitatively similar profile, as a function of protein concentration, as that observed for the HPP treated samples, what could be expected considering the small effect observed for MTG. In general, also, the combined treatments originated protein samples with lower solubility than those resulting from the HPP alone. For the SPI samples and for protein concentrations ≥ 5%, the combined treatments decreased protein solubility to significant lower values than the non-processed samples. Worth to note that for 9% protein the combined MTG+HPP treatment originate a soy protein gel, reinforcing the hypothesis that a synergistic effect between the presence of MTG and the HPP occurred, both favouring the existence of significant intermolecular interactions between proteins and aggregation. Exposure of glutamine and lysine residues promoted by HPP, otherwise buried within the protein tertiary structure, making them accessible to the action of MTG (Menéndez et al, 2006), is expected to be, at least, an important contribution to the mechanism behind the observed effects. The ability of HPP to enable proteins' crosslinking catalysed by MTG has already been demonstrated for several proteins, although of animal origin (*e.g.* β-lactoglobulin, casein, bovine serum albumin, ovalbumin, *etc.*) (Gharibzahedi et al., 2018; Lauber et al., 2003). Ergo, it is likely that HPP may dissociate protein aggregates and induce conformational changes promoting a solubility increase and facilitating the access of MTG. As previously discussed, MTG can then catalyse a crosslink reaction that produces large molecular weight compounds reducing the solubility.

#### 5.3.2.2. EFFECTS OF HPP AND MTG ON THE AMOUNT OF FREE SULFHYDRYL GROUPS

For the solubilized proteins obtained by the dispersion procedure described above (§5.2.3), and for a protein dispersion at 1 % (w/v) and pH 7, as those previously described in Chapter 4 and above, the concentration of free SH groups obtained for the non-processed samples was 3.0 ± 0.5 and 2.1 ± 0.4 μmol·(g protein)$^{-1}$ for PPI and SPI, respectively.

The addition of MTG to both PPI and SPI, for 1% protein concentration, had no significant effects ($p > 0.05$) (for PPI) or only a slight effect (for SPI) on the concentration of free SH (Figure 5.11), when compared with the control samples, in accordance to what was previously discussed (§5.3.1.3, Table 5.5). On the other hand, the content of free SH groups increased significantly for higher protein concentrations for both PPI and SPI, in the presence of MTG, being the effect more pronounced for the last one (Figure 5.11(B)). For the pea proteins, increasing the protein concentration above 3% did not show any further significant effect on the free SH groups (Figure 5.11(A)). An increase in free SH of peanut protein was also reported as a result of the MTG enzymatic activity (Hu et al., 2011). The authors suggested that MTG, by catalysing the crosslink, may induce conformational changes (unfolding) of the proteins thus exposing free SH, what may also help to explain the observed results. The more pronounced effect observed at higher protein concentrations, namely for the soy protein samples, may be due to a higher degree of crosslinking due to the higher amount of substrate available for the enzymatic reaction.

As expected from the previous results discussed in Chapter 4 and above in this Chapter, when HPP was applied individually to PPI, caused a decrease in the free SH content, when compared to the non-processed samples (control) (Figure 5.11(A)). However, as the protein concentration increased, the effect of the decrease was less pronounced, observing similar levels of free SH groups for the unprocessed sample and for the processed one, for a protein concentration of 9%. Somehow surprising, when HPP was applied to SPI for the different protein samples, the contrary effect was observed, *i.e.* there was a significant increase in SH groups compared to the control samples, also in this case, with a more pronounced effect for samples with protein concentration above 5% (Figure 5.11(B)). The results here obtained suggest that the pressure effects on the content of free SH are dependent on the type and concentration of protein, which can differently affect unfolding or the aggregation processes that consequently influence the content of exposed SH groups. Accordingly, both decreases and increases of free SH groups in protein isolates submitted to pressure have been reported

(Queirós, Saraiva, and da Silva 2018). As previously discussed, HPP can decrease the content of free SH by promoting hydrophobic interactions that led to S-S exchange and/or formation of new disulfide bonds (He et al., 2013; Zhao et al., 2015). On the other hand, pressure can also lead to an increase in free SH by dissociation/unfolding of the proteins, consequently exposing buried SH groups (Zhou et al. 2016).

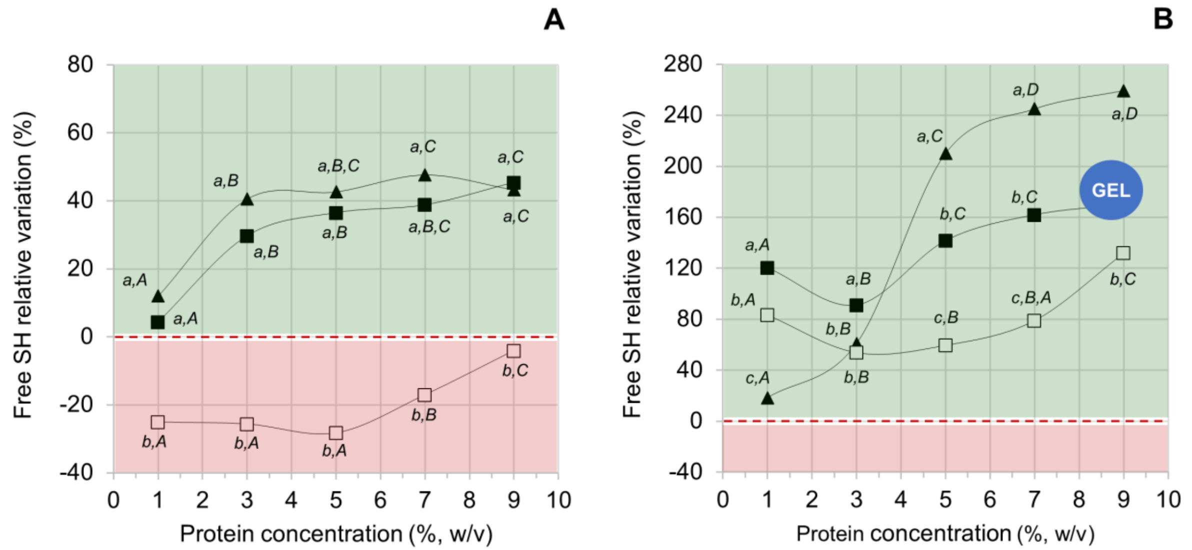



**Figure 5.11.** Relative variation of the amount of free sulfhydryl groups for (**A**) PPI and (**B**) SPI dispersions, calculated accordingly to equation 5.3, as a function of protein concentration, for protein dispersions (pH 7) under different processing conditions: (▲) in the presence of 30 U/g protein MTG; (□) treated by HPP at 600 MPa, 15 min; (■) processed by a combination of both treatments (MTG+HPP). Different capital letters indicate significant differences ($p < 0.05$) between the same treatment at different protein concentrations. Different lowercase letters indicate significant differences ($p < 0.05$) between different treatments at the same protein concentration.

In general, the simultaneous application of HPP and MTG on pea protein samples resulted in a similar effect on the content of free SH as that observed for the MTG alone (Figure 5.11(A)), although indicating a trend for lower free SH values, probably as a result of the negative effect observed for HPP alone, but no significant differences were found. Nonetheless, the combination of HPP and MTG resulted in higher free SH than the controls in all of the protein concentrations studied, except for the lowest concentration where no significant differences were observed.

For SPI at concentrations of 1 and 3 %, the simultaneous HPP and MTG treatments resulted in a higher content of free SH than those treatments applied individually. However, for higher protein concentrations, this combination seemed to result in lower quantities of free SH than those obtained with MTG, but higher than those obtained for HPP, when compared to the non-processed samples. Overall, combined HPP and MTG increased the content of exposed SH when compared to the control or only HPP treated. An increase in the content of SH was also reported for sweet potato protein submitted to HPP with posterior addition of MTG, comparatively to only HPP and controls (Zhao et al., 2019). As previously discussed, the pressure-induced unfolding allowed buried SH groups to be exposed, and most likely added to the crosslinking of protein catalysed by MTG. The reason why the combined treatments led to an increase in free SH groups intermediate of that observed for treatment by HPP or MTG individually cannot be clearly explained on the basis of the available data. However, speculating, we can consider the hypothesis of the catalytic activity of MTG on proteins that have undergone greater conformational expansion due to the effect of pressure, leading to a greater degree of crosslinking, contributes to a reduction in the number of free or reactively available SH groups, in relation to the effect caused by the enzyme activity when not under treatment by HPP.

#### 5.3.2.3. Effects of HPP and MTG on the proteins' surface hydrophobicity

Figure 5.12 shows the variation of protein surface hydrophobicity ($H_0$) for pea and soy protein samples subject to the different processing treatments under study, relatively to what was obtained for the non-processed protein samples. Non-processed samples exhibited a protein surface hydrophobicity of 2489 ± 69 and 2555 ± 80, for 1% pea and soy protein solutions, respectively.

Considering the effect of MTG with no HPP treatment on $H_0$, for the protein solutions at 1%, there was not a significant effect ($p > 0.05$) for both PPI (Figure 5.12(A)) and SPI (Figure 5.12(B)), what could be expected from the results previously obtained (§5.3.1.4, Table 5.5).

For PPI and for protein concentrations above 1%, the effect of MTG was more pronounced, with a first increase in $H_0$, for protein concentrations in the range 3-5%, followed by a significant decrease, for protein concentrations 7-9%, with $H_0$ reaching values significantly lower than for the control samples. For SPI samples, within the concentration range analysed, the magnitude of the decrease in $H_0$, relative to non-enzymatically treated samples, increased

with increasing protein concentration. In some conditions the crosslinking may promote conformational changes that result in protein unfold, exposing hydrophobic regions thus increasing the $H_0$ (Hu et al. 2011), as here described for lower concentrations of PPI. However, in most cases, the crosslink catalysed by MTG may close off hydrophobic residues inside the structure of the higher molecular polymers formed (Agyare, Xiong, and Addo 2008), consequently decreasing $H_0$, as here reported for SPI and higher concentrations of PPI. A higher degree of crosslinking, as likely occurs as the protein concentration increases, probably promotes the occurrence of this second mechanism.

Considering the HPP alone, it was observed an increase in $H_0$ for protein concentrations up to 5%, followed by a significant decrease within the protein high concentration range (Figure 5.12). Worth to recall that it was shown already that HPP processing using similar conditions of pressure and time, and for similar protein solutions, resulted in an increase of protein surface hydrophobicity, more pronounced for soy proteins (Chapter 4, (§4.3.4) (Figure 4.4(B)). As previously discussed, pressure may unfold proteins, thus exposing the number of hydrophobic groups on its surface due to the resulting conformational changes, explaining the higher $H_0$ observed in the lower protein concentration range. Still, at higher concentrations, pressure may promote interactions between proteins, and change the equilibrium between aggregation and dissociation processes, thus decreasing $H_0$ (Khan et al. 2013; Wang et al. 2008).

The MTG+HPP combined treatments originated a significant decrease in $H_0$ for the pea protein samples, comparing to the non-processed samples, within the whole protein's concentration range, especially for higher concentrations (Figure 5.12(A)). For SPI, the combination of methodologies resulted in a $H_0$ similar to the unprocessed SPI for protein concentrations ≤ 5 % (Figure 5.12(B)). However, a further increase in protein's concentration significantly ($p < 0.05$) decreased the $H_0$. At the highest concentration analysed, the soy protein dispersion gelled after the combined treatment. As discussed above, the combination of HPP and MTG may change the equilibrium between aggregation/dissociation processes, thus decreasing/increasing $H_0$. In the case of PPI, the results suggest that enhanced crosslinking and/or aggregation processes seem to prevail as there is a decrease in $H_0$.

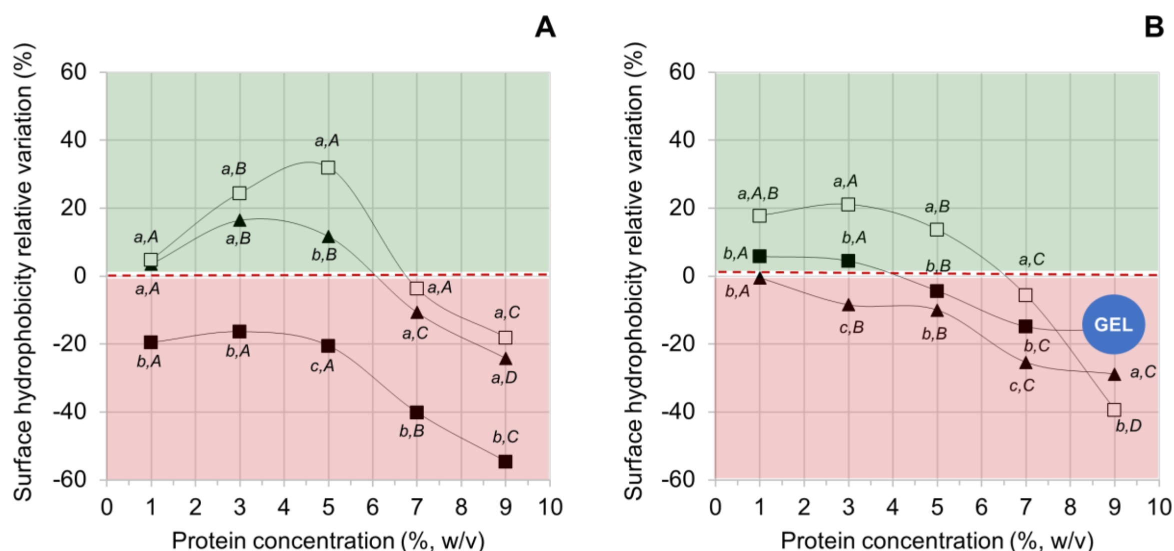



**Figure 5.12.** Relative variation of protein surface hydrophobicity for (**A**) PPI and (**B**) SPI dispersions, calculated accordingly to equation 5.3, as a function of protein concentration, for protein dispersions (pH 7) under different processing conditions: (▲) in the presence of 30 U/g protein MTG; (□) treated by HPP at 600 MPa, 15 min; (■) processed by a combination of both treatments (MTG+HPP). Different capital letters indicate significant differences ($p < 0.05$) between the same treatment at different protein concentrations. Different lowercase letters indicate significant differences ($p < 0.05$) between different treatments at the same protein concentration.

#### 5.3.2.4. Effects of HPP and MTG on the viscosity of pea protein dispersions

Figure 5.13 shows representative flow curves obtained for non-processed pea protein dispersions at different protein concentrations. Within the range of protein concentrations between 1 and 7% the measured values of viscosity were very low, and the measurable shear rate range was limited due to the resolution limit of the rheometer. The apparent shear thickening behaviour observed for the lower concentrations at high shear rates is an artefact due to the usual turbulence observed for cone−plate geometry. Anyway, the observed flow behaviour was essentially Newtonian, with the apparent viscosity showing a very low dependence on the shear rate. Increasing the protein concentration clearly increased the apparent viscosity and the degree of shear-thinning flow behaviour, with the apparent viscosity showing a much more pronounced dependence on shear rate.

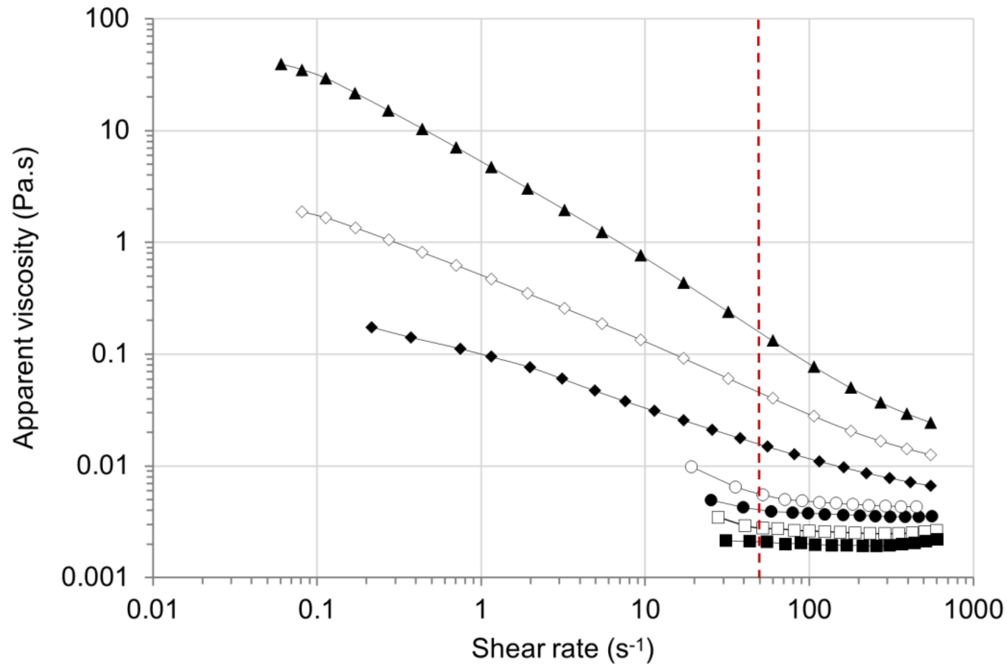



**Figure 5.13.** Representative flow curves obtained from the applied up stress ramp (increasing shear stresses), at 20 ºC, for unprocessed PPI dispersions at different protein concentrations: (■) 1 %, (□) 3 %, (●) 5 %, (○) 7 %, (◆) 9 %, (◇) 11 %, and (▲) 13 % (w/v).

The viscosity of protein dispersions processed only by HPP did not vary significantly for concentrations equal to or less than 5%, presenting a flow behaviour very close to Newtonian, similar to unprocessed dispersions. Figure 5.14 shows the flow curves obtained for 7 % PPI dispersions. At this protein concentration, there was already a significant increase in viscosity with HPP treatments, more pronounced for 600 MPa, with the flow curves also showing a more pronounced shear-thinning behaviour and a greater structural hysteresis between the up and down curves.

The influence of HPP on the flow characteristics of the PPI dispersions is further compared in Figure 5.15. Apparent viscosity for the PPI dispersions treated by HPP was not significantly different from that of unprocessed dispersions for protein concentrations up to 5%.

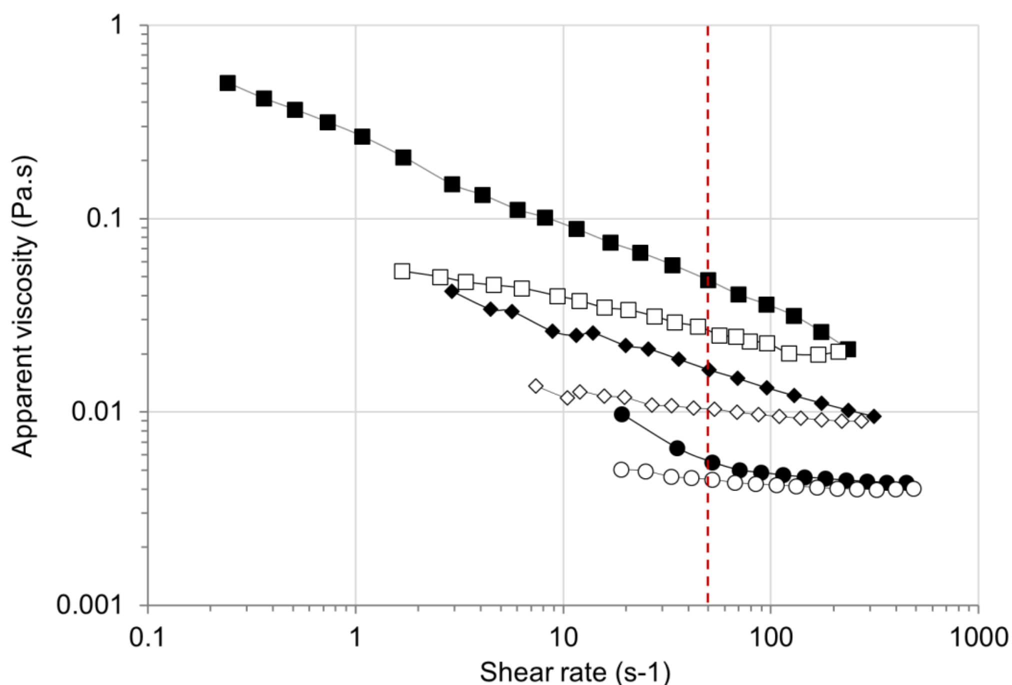



**Figure 5.14.** Representative flow curves obtained from the applied up (filled) and down (open symbols) stress ramps, at 20 ºC, for 7% PPI dispersions subjected to different treatments: unprocessed samples (circles); HPP 400 MPa, 15 min (diamonds); and HPP 600 MPa, 15 min (squares).

However, for concentrations equal to or greater than 7%, HPP clearly leads to higher apparent viscosities (Figure 5.15(A)). There is not much information regarding the effects of HPP on the viscosity of protein solutions, still, an increase in apparent viscosity with pressure, particularly at higher protein concentrations, was also reported for HPP treated soymilk (Lakshmanan, de Lamballerie, and Jung 2006) and amaranth protein isolates (Condés et al. 2012). Similar to the results obtained here, the apparent viscosity of low concentration amaranth protein dispersions (1 %) was not affected by HPP. Still, for protein concentrations ≥ 5 %, increasing pressure increased the apparent viscosity of the dispersions. This increase was larger for a protein concentration of 10 % than 5 % (Condés et al. 2012). The increase in apparent viscosity may be associated with the formation of aggregates promoted by HPP, as previously discussed, which is favoured at higher protein concentrations. The deviation from the Newtonian flow behaviour was also markedly increased by HPP as shown by the more pronounced decrease in the flow behaviour index *n* (Figure 5.15(B)).

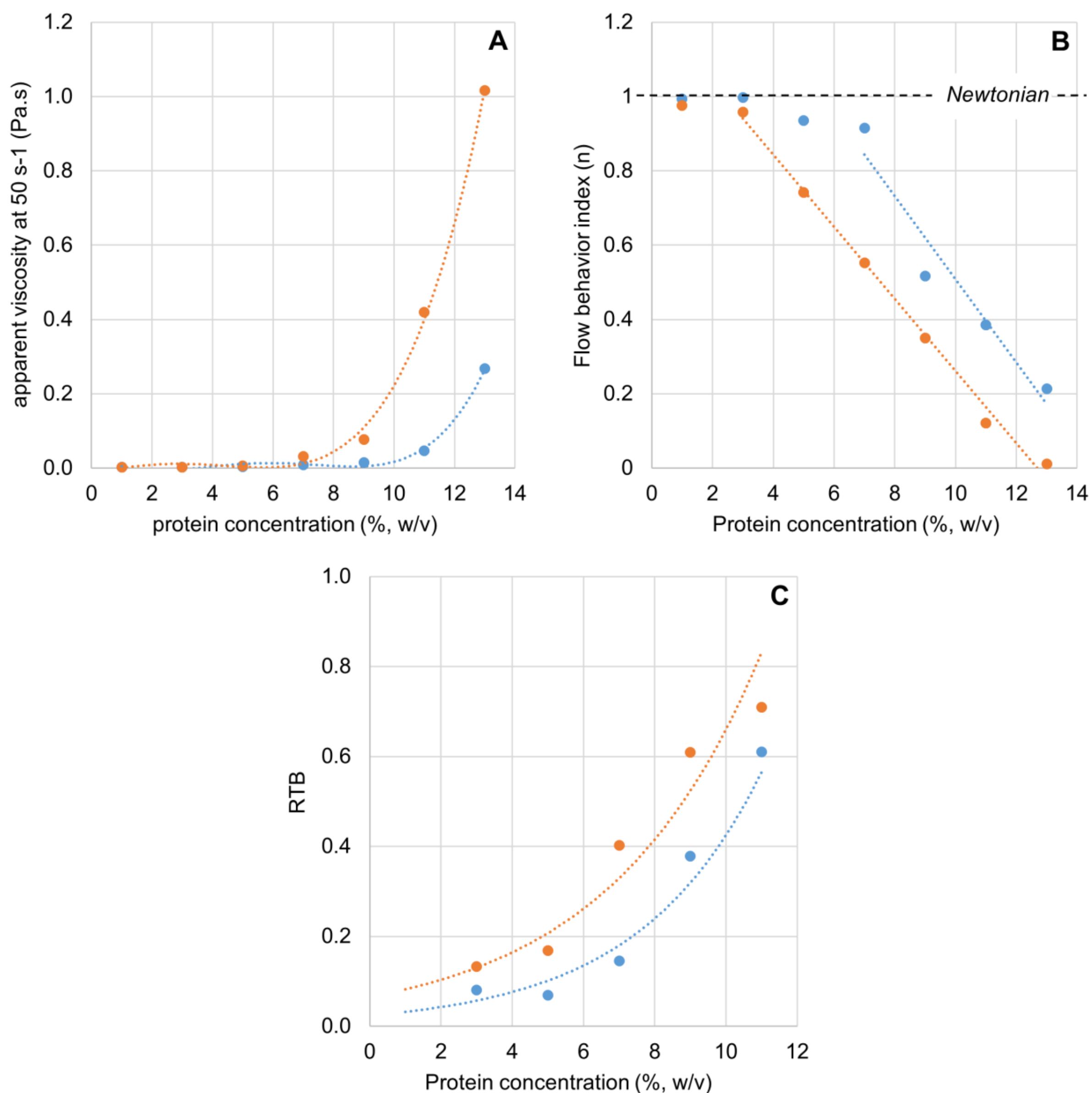



**Figure 5.15.** Comparison between flow behaviour characteristics obtained for (●) unprocessed and (●) HPP treated PPI dispersions: (**A**) Dependence of apparent viscosity (50 $s^{-1}$) on pea protein concentration; (**B**) Dependence of the flow behaviour index *n* (Eq. 5.2) on pea protein concentration; (**C**) Dependence of RTB, the relative thixotropic structural breakdown, on pea protein concentration. Doted lines have no particular physical meaning and are only a guide for the eyes.

A significant departure from the Newtonian behaviour for the unprocessed dispersions was verified for protein concentrations above 7%, whereas for HPP treated dispersions this departure was already observed for concentrations above 3%. A decline in the *n* value after

HPP was also observed in soymilk, being more noticeable when the protein concentration was higher (Lakshmanan et al .2006). The relative difference between the viscosities obtained from the up and down curves, at the same shear rate of 50 $s^{-1}$, was used to approximately quantify the relative thixotropic structural breakdown (RTB) (Figure 5.15(C)). Clearly, HPP originated more structured dispersions and consequently higher RTB values within the whole range of protein concentrations analysed.

The enzymatic treatments with MTG, alone, only caused significant changes in the flow profile and viscosity of the PPI dispersions for protein concentrations greater than 9%, increasing the apparent viscosity and the degree of the shear-thinning behaviour, as illustrated in Figure 5.16. Equally to HPP, there is very little information on the effects of MTG on the viscosity of protein dispersions, particularly plant-based proteins. Still, the increase the apparent viscosity observed was expected, as the high molecular weight polymers, promoted by the crosslinking catalysed by MTG, can reduce mobility of the water in the protein network, increasing the flow resistance (Gaspar & de Góes-Favoni, 2015).

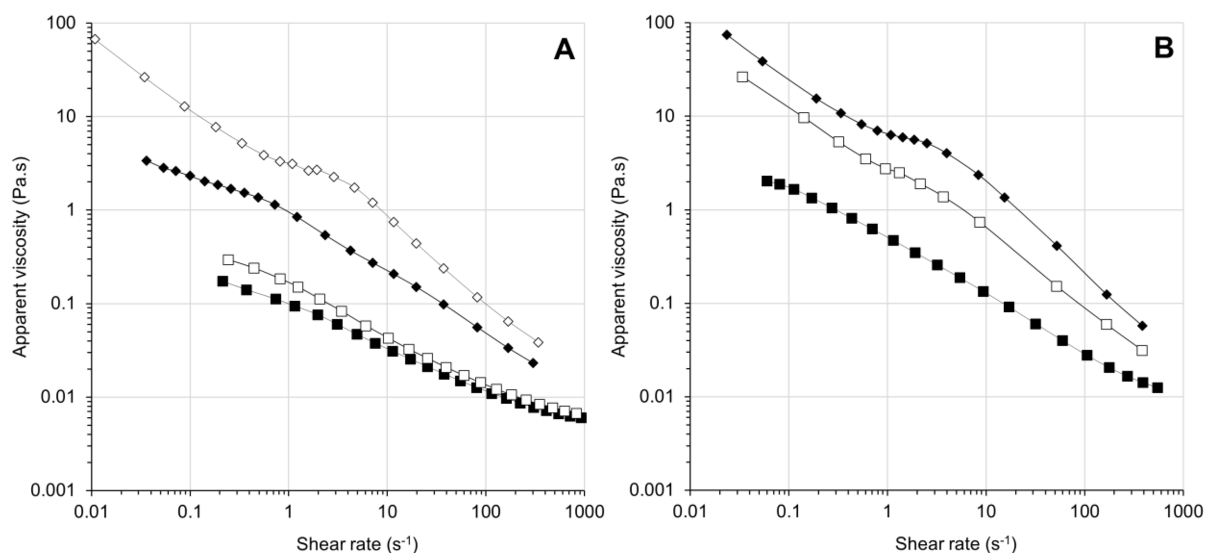



**Figure 5.16.** Flow curves (rising stress ramp) obtained for PPI dispersions at (A) 9 % and (B) 11 %, at 20 ºC, subjected to different treatments: (■) unprocessed samples; (□) MTG 30 U/(g protein); (◆) HPP 600 MPa, 15 min; (◇) MTG 30 U/(g protein) + HPP 600 MPa, 15 min. In (B), the 11% protein dispersion subjected to combined treatment by MTG and HPP gave rise to a gel.

Worth to note the flow profile observed for the 9% PPI dispersion subjected to the MTG+HPP combined treatment (Figure 5.16(A)) (similar to what is observed at 11% protein for

each treatment alone, Figure 5.16(B)). Besides the pronounced shear-thinning behaviour observed, these systems showed a tendency for a viscosity plateau around 1 $s^{-1}$ but a further (asymptotic-like) increase in viscosity as the shear rate decreased, suggesting the existence of significant yield stress, probably associated to the disperse nature of these systems. The combined MTG and HPP treatments seem to originate a synergistic effect, at least for pea protein concentrations above 7%, leading to a more pronounced increase in viscosity and on the shear-thinning flow profile. For 11% protein, the combined treatment even led to the formation of a gel.

Similar to the analysis previously performed for the other parameters under study (solubility, free SH, $H_0$), the relative variation (Eq. 5.3) of the measured apparent viscosity at a shear rate of 50 $s^{-1}$ caused by HPP, MTG or the combined MTG+HPP treatments, by comparison to that measured for the control solutions (PPI dispersions without any treatment), was also investigated, as a function of the protein concentration (Figure 5.17). Even for those protein concentrations where MTG alone did not cause any significant changes on viscosity and flow profile of the pea protein dispersions, the simultaneous MTG and HPP treatments lead to a much more significant increase in viscosity than HPP alone, probably related to the fact that the effect of the conformational alteration of proteins caused by HPP promotes the crosslinking action of the enzyme, leading to the formation of a larger number of aggregates and/or of a larger size, leading to a more noticeable increase in viscosity and in the shear-thinning character when compared to unprocessed dispersions or dispersions treated separately by each process.

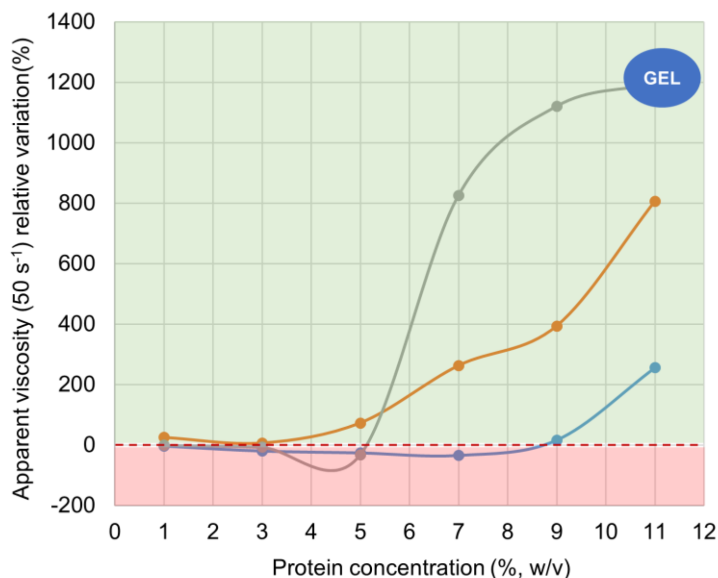



Figure 5.17. Relative variation (Eq. 5.3) of apparent viscosity measured at a shear rate of 50 $s^{-1}$, as a function of protein concentration, for pea protein dispersions (pH 7) under different processing conditions: (●) in the presence of 30 U/g protein MTG; (●) treated by HPP at 600 MPa, 15 min; (●) processed by a combination of both treatments (MTG+HPP).

## 5.4. CONCLUSION

This work has shown the combination of HPP and MTG may be an interesting tool to modify food proteins' function. Overall, the effects of MTG and HPP on the studied properties are dependent on the selected processing conditions and on the protein type and concentration. A series of synergistic and antagonistic effects between HPP and MTG were observed throughout this work, particularly at higher protein concentrations. For instance, the addition of MTG seems to counter the increase of solubility promoted by HPP. On the other hand, for higher pea protein concentrations, the combined application of HPP and MTG resulted in a higher apparent viscosity than when these treatments were applied individually. The possible dissociation of aggregates and protein conformational expansion promoted by HPP made the proteins more accessible to MTG, which further modified these proteins' properties. These findings would be useful for understanding how combined HPP and MTG treatments may help modify proteins' structure and consequently tailor their techno-functional properties, both by the synergistic and antagonistic effects of HPP and MTG.

## 5.5. REFERENCES


Achouri, A., & Boye, J. I. (2013). Thermal processing, salt and high pressure treatment effects on molecular structure and antigenicity of sesame protein isolate. *Food Research International*, *53*(1), 240–251.

Adebiyi, A. P., & Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (*Pisum sativum L.*) seed protein isolate. *Food Chemistry*, *128*(4), 902–908.

Agyare, K. K., Xiong, Y. L., & Addo, K. (2008). Influence of salt and pH on the solubility and structural characteristics of transglutaminase-treated wheat gluten hydrolysate. *Food Chemistry*, *107*(3), 1131–1137.

Babiker, E. E. (2000). Effect of transglutaminase treatment on the functional properties of native and chymotrypsin-digested soy protein. *Food Chemistry*, *70*(2), 139–145.

Baier, A. K., & Knorr, D. (2015). Influence of high isostatic pressure on structural and functional characteristics of potato protein. *Food Research International*, *77*, 753–761.

Beveridge, T., Toma, S. J., & Nakai, S. (1974). Determination of SH- and SS-groups in some food proteins using ellman's reagent. *Journal of Food Science*, *39*(1), 49–51.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Cardoso, C. L., Mendes, R. O., Saraiva, J. A., Vaz-Pires, P. R., & Nunes, M. L. (2010). Quality Characteristics of High Pressure-Induced Hake ( Merluccius capensis ) Protein Gels with and without MTGase. *Journal of Aquatic Food Product Technology*, *19*(3–4), 193–213.

Chao, D., Jung, S., & Aluko, R. E. (2018). Physicochemical and functional properties of high pressure-treated isolated pea protein. *Innovative Food Science & Emerging Technologies*, *45*(February 2017), 179–185.

Dube, M., Schäfer, C., Neidhart, S., & Carle, R. (2007). Texturisation and modification of vegetable proteins for food applications using microbial transglutaminase. *European Food Research and Technology*, *225*(2), 287–299.

Gaspar, A. L. C., & de Góes-Favoni, S. P. (2015). Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review. *Food Chemistry*, *171C*, 315–322.

Gharibzahedi, S. M. T., Roohinejad, S., George, S., Barba, F. J., Greiner, R., Barbosa-Cánovas, G. V., & Mallikarjunan, K. (2018). Innovative food processing technologies on the transglutaminase functionality in protein-based food products: Trends, opportunities and drawbacks. *Trends in Food Science and Technology*, *75*(December 2017), 194–205.

He, R., He, H. Y., Chao, D., Ju, X., & Aluko, R. (2013). Effects of High Pressure and Heat Treatments on Physicochemical and Gelation Properties of Rapeseed Protein Isolate. *Food and Bioprocess Technology*, *7*(5), 1344–1353.

Hu, X., Zhao, M., Sun, W., Zhao, G., & Ren, J. (2011). Effects of microfluidization treatment and transglutaminase cross-linking on physicochemical, functional, and conformational properties of peanut protein isolate. *Journal of Agricultural and Food Chemistry*, *59*(16), 8886–8894.

Jiang, S. J., & Zhao, X. H. (2010). Transglutaminase-induced cross-linking and glucosamine conjugation in soybean protein isolates and its impacts on some functional properties of the products. *European Food Research and Technology*, *231*(5), 679–689.

Jiang, Y., Tang, C. H., Wen, Q. B., Li, L., & Yang, X. Q. (2007). Effect of processing parameters on the properties of transglutaminase-treated soy protein isolate films. *Innovative Food Science & Emerging Technologies*, *8*(2), 218–225.

Jong, G. H. A. de, & Koppelman, S. J. (2002). Transglutaminase Catalyzed Reactions: Impact on Food Applications. *Journal of Food Science*, *67*(8), 2798–2806.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA) - Protein Structure*, *624*(1), 13–20.

Kato, A., Wada, T., Kobayashi, K., Seguro, K., & Motoki, M. (1991). Agricultural and Biological Chemistry Ovomucin-Food Protein Conjugates Prepared through the Transglutaminase Reaction Ovomucin-Food Protein Conjugates Prepared through the Transglutaminase Reaction. *Agricultural and Biological Chemistry Agric. Bio!. Chem*, *554*(554), 1027–1031.

Lakshmanan, R., de Lamballerie, M., & Jung, S. (2006). Effect of Soybean-to-Water Ratio and

pH on Pressurized Soymilk Properties. *Journal of Food Science*, 71(9), E384–E391.

Lauber, S., Krause, I., Klostermeyer, H., & Henle, T. (2003). Microbial transglutaminase crosslinks β-casein and β-lactoglobulin to heterologous oligomers under high pressure. *Eur Food Res Technol*, *216*, 15–17.

Lauber, S., Noack, I., Klostermeyer, H., & Henle, T. (2001). Oligomerization of β-lactoglobulin by microbial transglutaminase during high pressure treatment. *European Food Research and Technology*, *213*(3), 246–247.

Lee, K. H., Ryu, H. S., & Rhee, K. C. (2003). Protein solubility characteristics of commercial soy protein products. *JAOCS, Journal of the American Oil Chemists' Society*, *80*(1), 85–90.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2011). Effects of High Hydrostatic Pressure on Some Functional and Nutritional Properties of Soy Protein Isolate for Infant Formula. *Journal of Agricultural and Food Chemistry*, *59*, 12028–12036.

Li, H., Zhu, K., Zhou, H., & Peng, W. (2012). Effects of high hydrostatic pressure treatment on allergenicity and structural properties of soybean protein isolate for infant formula. *Food Chemistry*, *132*(2), 808–814.

Lorenzen, P. C., Schlimme, E., & Roos, N. (1998). Crosslinking of sodium caseinate by a microbial transglutaminase. *Nahrung*, *42*(3–4), 151–154.

Manassero, C. A., Vaudagna, S. R., Sancho, A. M., Añón, M. C., Speroni, F., Mar, A., & Cristina, A. (2016). Combined high hydrostatic pressure and thermal treatments fully inactivate trypsin inhibitors and lipoxygenase and improve protein solubility and physical stability of calcium-added soymilk. *Innovative Food Science and Emerging Technologies*, *35*, 86–95.

Menéndez, O., Rawel, H., Schwarzenbolz, U., & Henle, T. (2006). Structural changes of microbial transglutaminase during thermal and high-pressure treatment. *Journal of Agricultural and Food Chemistry*. 54, 1716−1721.

Partschefeld, C., Richter, S., Schwarzenbolz, U., & Henle, T. (2007). Modification of β-lactoglobulin by microbial transglutaminase under high hydrostatic pressure: Localization of reactive glutamine residues. *Biotechnology Journal*, *2*(4), 462–468.

Puppo, C., Chapleau, N., Speroni, F., De Lamballerie-Anton, M., Michel, F., Añón, C., & Anton, M. (2004). Physicochemical modifications of high-pressure-treated soybean protein isolates. *Journal of Agricultural and Food Chemistry*, *52*(6), 1564–1571.

Qin, Z., Guo, X., Lin, Y., Chen, J., Liao, X., Hu, X., & Wu, J. (2013). Effects of high hydrostatic pressure on physicochemical and functional properties of walnut (juglans regia l.) protein isolate. *Journal of the Science of Food and Agriculture*, *93*(5), 1105–1111.

Queirós, R. P., Saraiva, J. A., & da Silva, J. A. L. (2018). Tailoring structure and technological properties of plant proteins using high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition*, *58*(9), 1538–1556.

Ribotta, P. D., Colombo, A., & Rosell, C. M. (2012). Enzymatic modifications of pea protein and its application in protein-cassava and corn starch gels. *Food Hydrocolloids*, *27*(1), 185–190.

Schäfer, C., Zacherl, C., Engel, K. H., Neidhart, S., & Carle, R. (2007). Comparative study of gelation and cross-link formation during enzymatic texturisation of leguminous proteins. *Innovative Food Science and Emerging Technologies*, *8*(2), 269–278.

Sharma, R., Lorenzen, P. C., & Qvist, K. B. (2001). Influence of transglutaminase treatment of skim milk on the formation of ε-(γ-glutamyl)lysine and the susceptibility of individual proteins towards crosslinking. *International Dairy Journal*, *11*(10), 785–793.

Song, C. L., & Zhao, X. H. (2014). Structure and property modification of an oligochitosan-glycosylated and crosslinked soybean protein generated by microbial transglutaminase. *Food Chemistry*, *163*, 114–119.

Speroni, F., Beaumal, V., de Lamballerie, M., Anton, M., Añón, M. C., & Puppo, M. C. (2009). Gelation of soybean proteins induced by sequential high-pressure and thermal treatments. *Food Hydrocolloids*, *23*(5), 1433–1442.

Tang, C. H., Li, L., & Yang, X. Q. (2006). Influence of transglutaminase-induced cross-linking on in vitro digestibility of soy protein isolate. *Journal of Food Biochemistry*, *30*, 718–731.

Tang, C. H., Sun, X., Yin, S. W., & Ma, C. Y. (2008). Transglutaminase-induced cross-linking of vicilin-rich kidney protein isolate: Influence on the functional properties and in vitro digestibility. *Food Research International*, *41*(10), 941–947.

Trespalacios, P., & Pla, R. (2007). Synergistic action of transglutaminase and high pressure on chicken meat and egg gels in absence of phosphates. *Food Chemistry*, *104*(4), 1718–1727.

Tsevdou, M. S., Eleftheriou, E. G., & Taoukis, P. S. (2013). Transglutaminase treatment of thermally and high pressure processed milk: Effects on the properties and storage stability of set yoghurt. *Innovative Food Science & Emerging Technologies*, *17*, 144–152.

Tsevdou, M., Soukoulis, C., Cappellin, L., Gasperi, F., Taoukis, P. S., & Biasioli, F. (2013). Monitoring the effect of high pressure and transglutaminase treatment of milk on the evolution of flavour compounds during lactic acid fermentation using PTR-ToF-MS. *Food Chemistry*, *138*(4), 2159–2167.

Wang, J.-M., Yang, X.-Q., Yin, S.-W., Zhang, Y., Tang, C.-H., Li, B.-S., … Guo, J. (2011). Structural rearrangement of ethanol-denatured soy proteins by high hydrostatic pressure treatment. *Journal of Agricultural and Food Chemistry*, *59*(13), 7324–7332.

Yang, H., Yang, A., Gao, J., & Chen, H. (2014). Characterization of Physicochemical Properties and IgE-Binding of Soybean Proteins Derived from the HHP-Treated Seeds. *Journal of Food Science*, *79*(11), C2157–C2163.

Yasir, S. B. M., Sutton, K. H., Newberry, M. P., Andrews, N. R., & Gerrard, J. A. (2007). The impact of transglutaminase on soy proteins and tofu texture. *Food Chemistry*, *104*, 1491–1501.

Yin, S. W., Tang, C. H., Wen, Q. B., Yang, X. Q., & Li, L. (2008). Functional properties and in vitro trypsin digestibility of red kidney bean (Phaseolus vulgaris L.) protein isolate: Effect of high-pressure treatment. *Food Chemistry*, *110*(4), 938–945.

Zhang, H., Li, L., Tatsumi, E., & Kotwal, S. (2003). Influence of high pressure on conformational changes of soybean glycinin. *Innovative Food Science and Emerging Technologies*, *4*(3), 269–275.

Zhang, P., Hu, T., Feng, S., Xu, Q., Zheng, T., Zhou, M., Chu, X., Huang, X., Lu, X., Pan, S., Li-Chan, C. Y. & Hu, H. (2016). Effect of high intensity ultrasound on transglutaminase-catalyzed soy protein isolate cold set gel. *Ultrasonics Sonochemistry*, *29*, 380–387.

Zhao, Z. K., Mu, T. H., Zhang, M., & Richel, A. (2019). Effects of high hydrostatic pressure and

microbial transglutaminase treatment on structure and gelation properties of sweet potato protein. *LWT*, *115*(February), 108436.

# CHAPTER 6

## Concluding Remarks and Future Work

## 6.1. CONCLUDING REMARKS

The application of simultaneous HPP and MTG can be an attractive way to modify food proteins and create new products. However, it is essential to collect information not only on how these methods affect the properties of proteins *per se*, but also on how MTG is affected by HPP.

With this work it was possible to observe that the pressure inactivation is enhanced at pH 4. However, MTG proved to be relatively resistant to pressure when near its optimal pH, being required high pressure values (> 400 MPa) to achieve some inactivation. The inactivation rate constant increased with both pressure and temperature at pH 5, 6 and 7, although the opposite was observed at pH 4. Also, both pressure and temperature did not significantly affect the energy and volume of activation when at pH 4 and 5. In contrast, at pH 6 and 7, it seems that pressure and temperature had an antagonistic effect in the inactivation of MTG, related to the reduction of the energy and volume of activation with increasing pressure and temperature, respectively. Overall, taking into account all of the kinetic parameters obtained in this work, together with the data available in the literature, it can be concluded that the constructed models adequately describe the pressure inactivation of MTG. The results here obtained allowed to determine the HPP and environmental conditions in which MTG could be used simultaneously with pressure without being substantially inactivated.

Considering the individual effects of HPP on low concentration protein dispersions (1 % w/v), the results obtained in this work indicate that pressure may change the structure of pea and soy proteins enhancing some of their techno-functional properties. For instance, HPP increased the solubility and surface hydrophobicity of both types of proteins. Nevertheless, the pressure effects on the proteins' interfacial and emulsifying properties proved to be very determined by the processing conditions, being sometimes compromised by protein aggregation. On the other hand, at this concentration, MTG was unable to modify these same proteins' properties, most likely due to the low accessibility of the enzyme to glutamine residues buried within the protein tertiary structure. Nevertheless, when HPP and MTG were applied simultaneously, different results were obtained. Pressure possibly induced the dissociation of aggregates and promoted the unfolding of the proteins' structure, making them more accessible to MTG. The results obtained demonstrate both synergistic and antagonistic effects between

HPP and MTG, indicating the potential of combined HPP and MTG treatments to change the structure of proteins and therefore modify their technological properties.

The effect of the concentration of the proteins, from 1 to 9 % (w/v), was also considered. When applied individually, pressure improved the solubility of both proteins when these were in low concentrations. However, in high protein concentrations, the pressure seems to promote the formation of insoluble aggregates, reducing the amount of soluble protein. Similarly, the surface hydrophobicity increased when pressure was applied in dispersions with low concentrations of protein and the opposite was observed in high protein concentrations. Nevertheless, the effects of pressure on the content of free sulfhydryl groups appear to be little dependent on protein concentration. MTG, when individually applied, had more effects on dispersions with a higher concentration of proteins. Overall, at high protein concentrations, MTG activity reduced the solubility and surface hydrophobicity of both proteins, having the opposite effect on the content of free sulfhydryl groups. Like in 1 % protein dispersions, the changes promoted by HPP appear to make the proteins more accessible to MTG, resulting in further modifications in the proteins' properties.

Overall, the presented results reveal that the combination of HHP and MTG can be effectively used to improve and tailor pea or soy proteins to be used as technological ingredients in the formulation of food products.

## 6.2. Future Work

Further research is needed to improve our understanding of the effects of pressure on the inactivation of MTG under alkaline pH. Furthermore, it is necessary to explore how the inactivation of MTG is influenced by other food components, having in mind the real food matrices where the combined effects or HPP and MTG may have key applications. Future research should also focus on gathering fundamental knowledge of the effects of combined pressure and MTG on the structure of this type of proteins, its relationship with conformational changes and consequent changes in proteins' functionality. It is also fundamental to understand how other compounds interact and influence these alterations in order to adapt the use of these proteins to practical applications. In this way, it will also be necessary to investigate the behaviour of plant proteins when incorporated in food products treated with HPP and MTG, as well as in the development of new food products, which could make use of the potential

functionalities provided by this type of treatment. Protein systems with enough concentration to originate gels should also be studied to obtain a better understanding about the topological and internal structure of the protein aggregates and gels obtained by the combination of the HPP and MTG. The incorporation of said gels in food matrices for the development of new food products should also be the focus of future research. Crosslinking of proteins may also affect the physiological properties of the end-product when consumed by humans, for example, digestibility and allergenicity, but these issues concerning the physiological functionality of cross-linked proteins are far from thoroughly studied and more research is needed.

# ANNEXES

# Annex A

FIGURE A1 - Residual plots of the regression model obtained for pH 4.

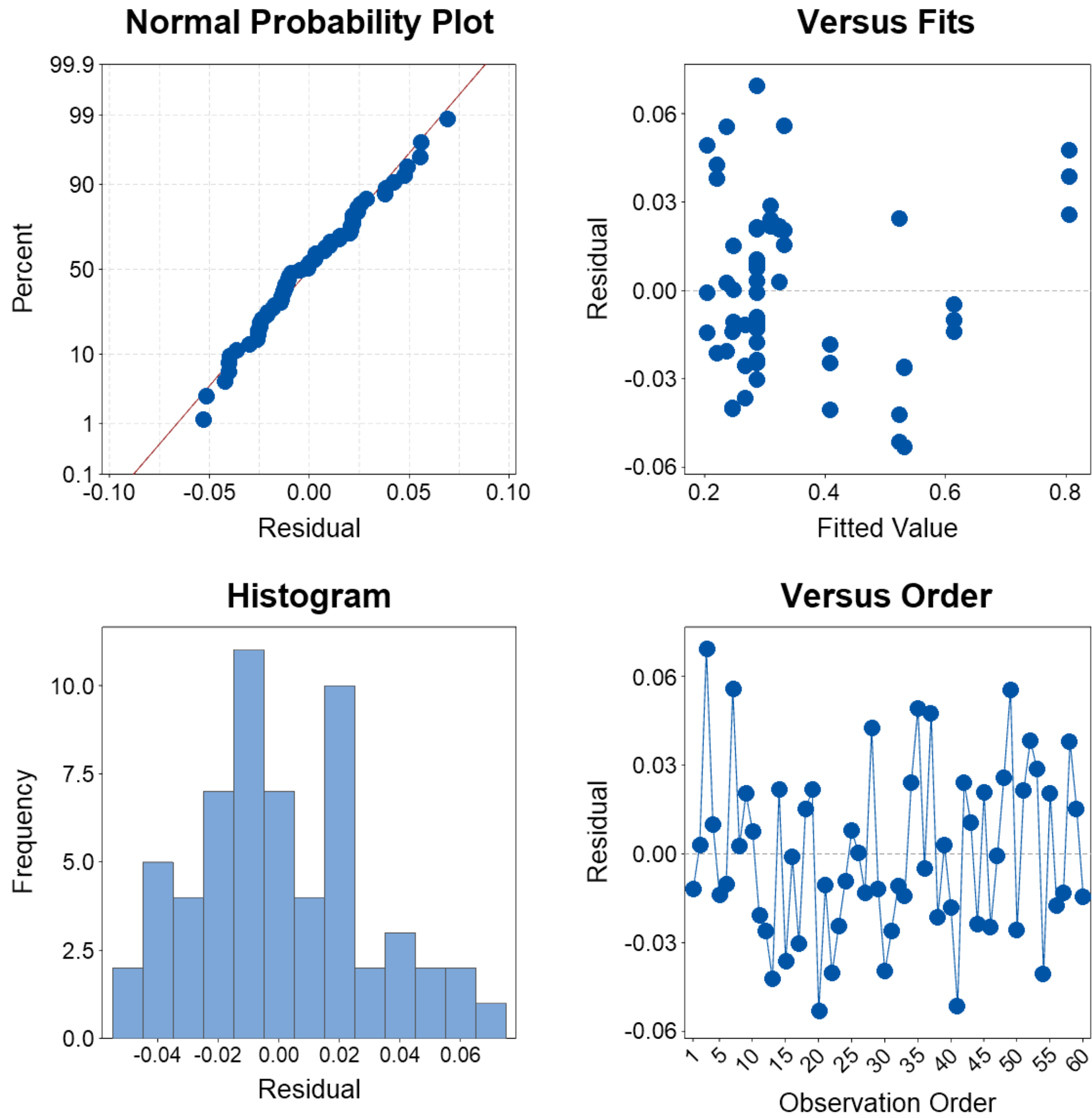


FIGURE A2 - Residual plots of the regression model obtained for pH 5.

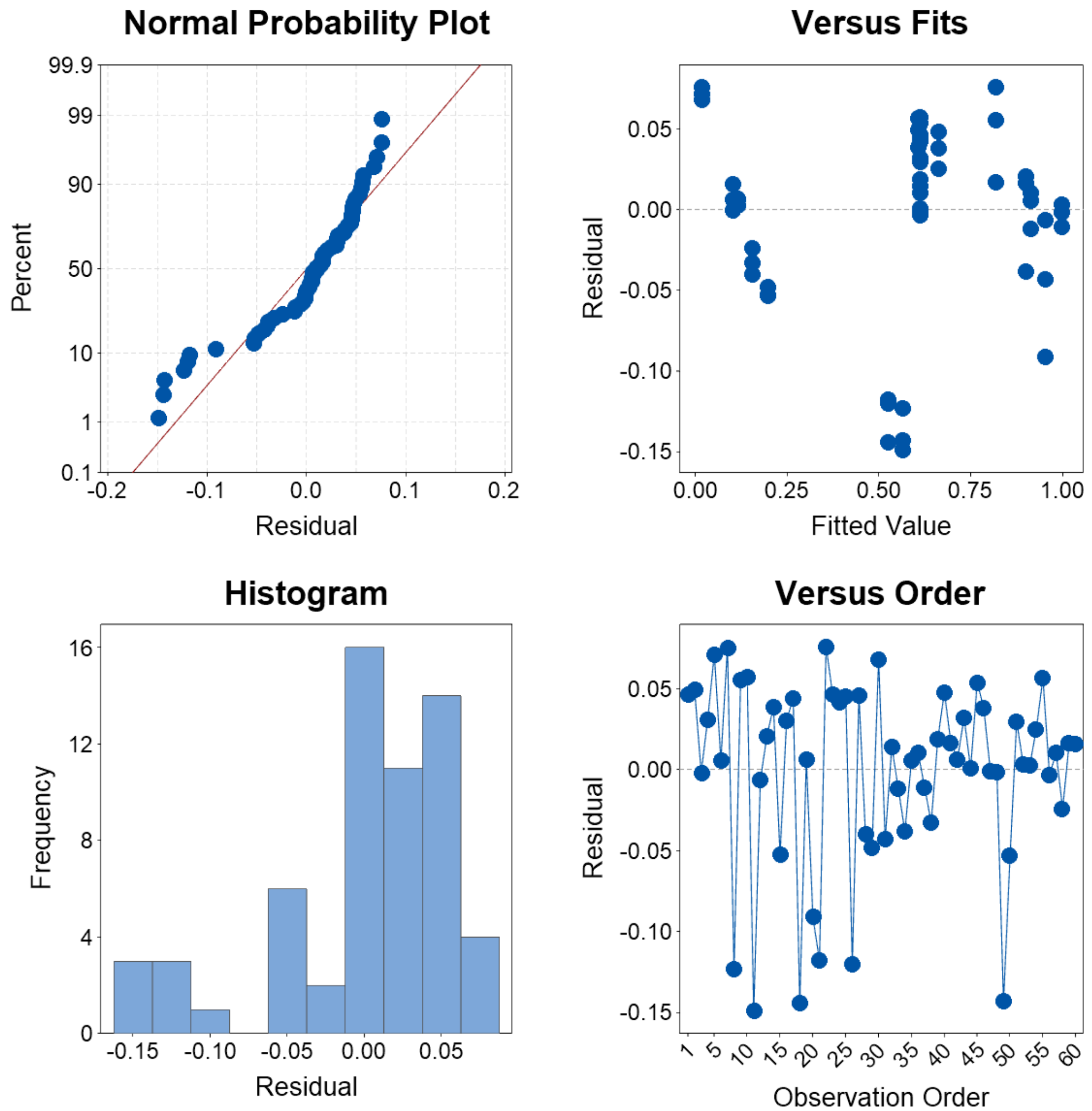


FIGURE A3 - Residual plots of the regression model obtained for pH 6.

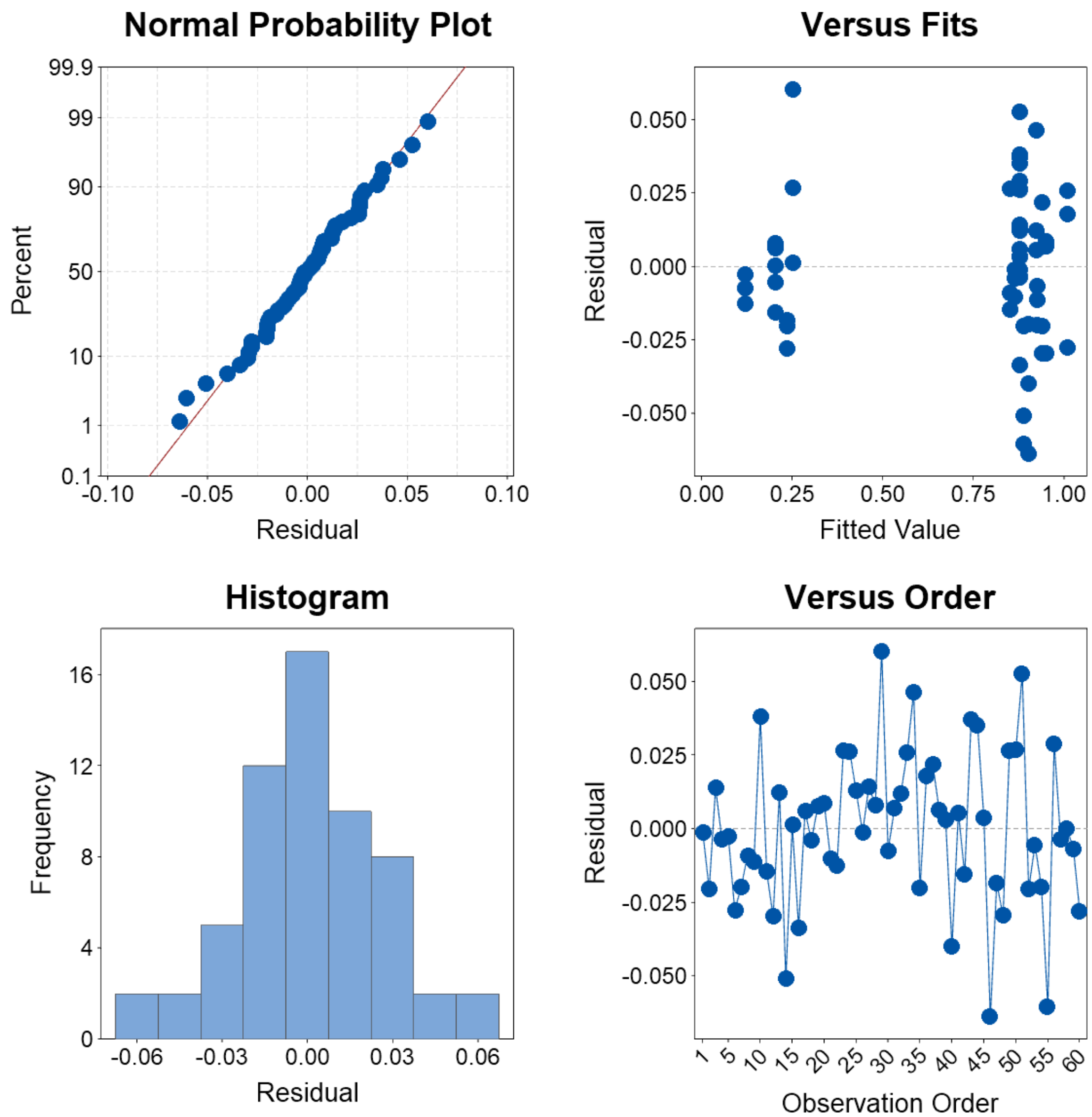
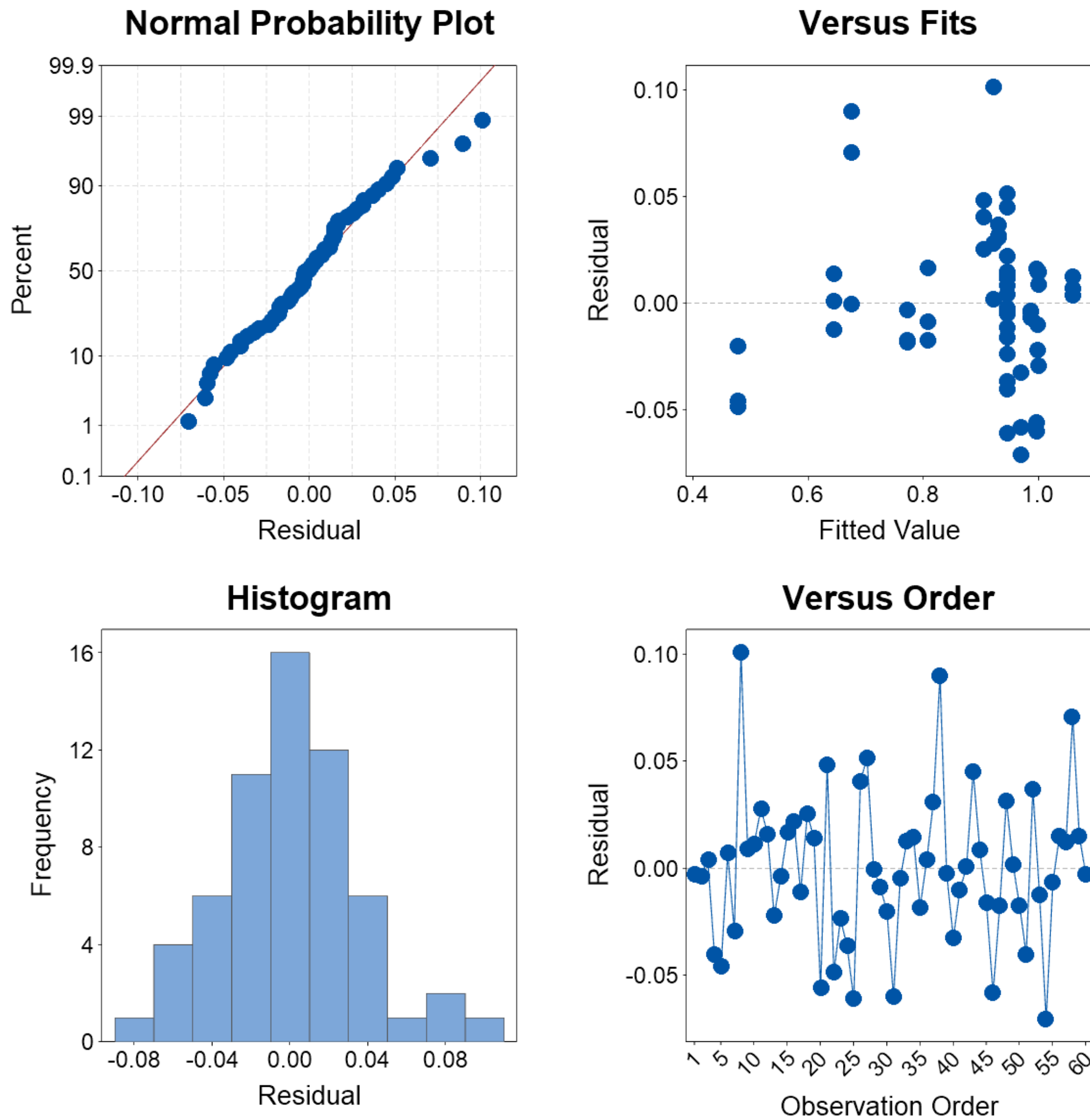


FIGURE A4 - Residual plots of the regression model obtained for pH 7.



# Annex B

FIGURE B1 - Residual plots of the regression model obtained for the solubility of pea protein isolate.

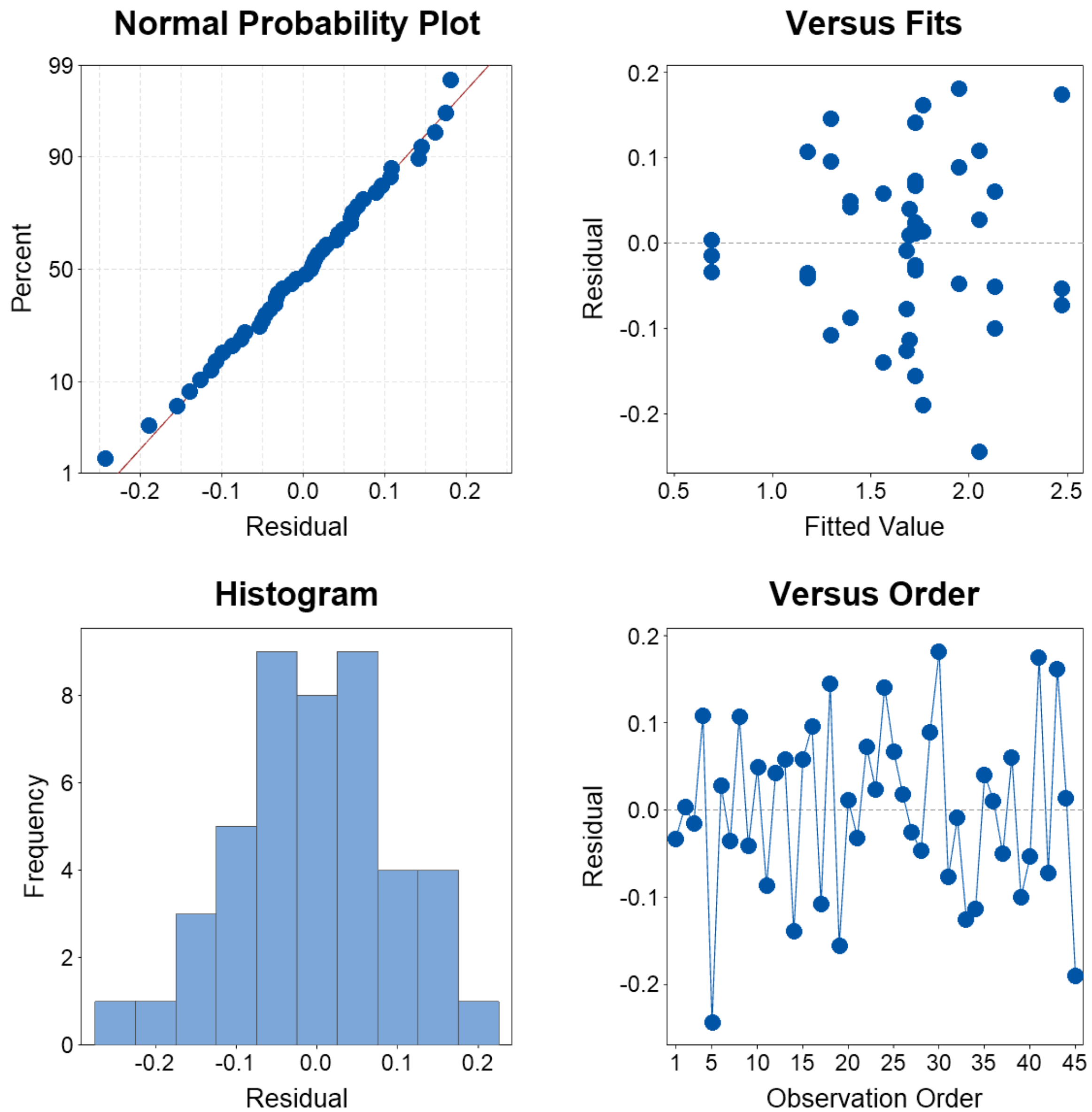


FIGURE B2 – Residual plots of the regression model obtained for the solubility of soy protein isolate.

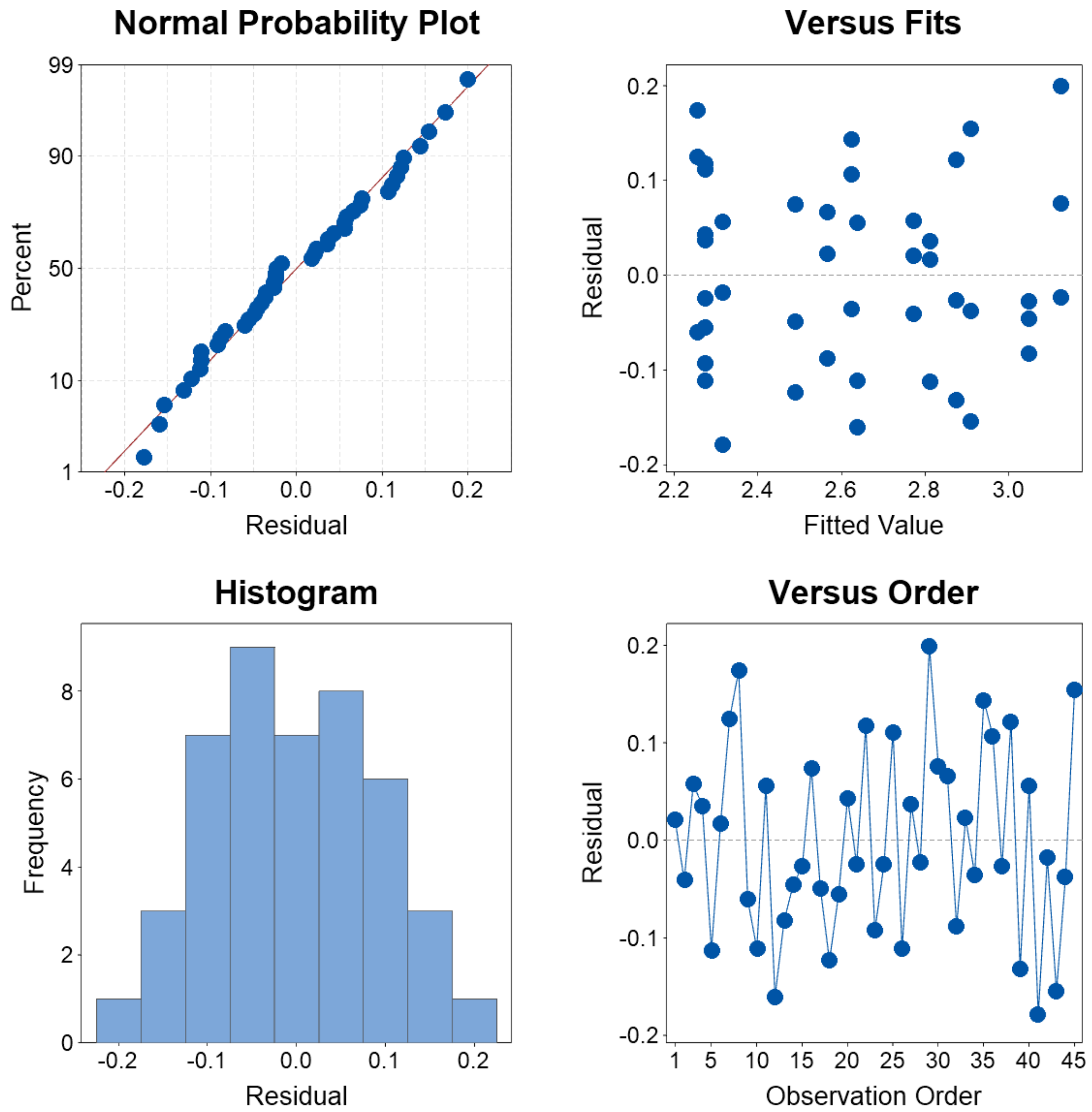


FIGURE B3 – Residual plots of the regression model obtained for the content of free sulfhydryl groups of pea protein isolate.

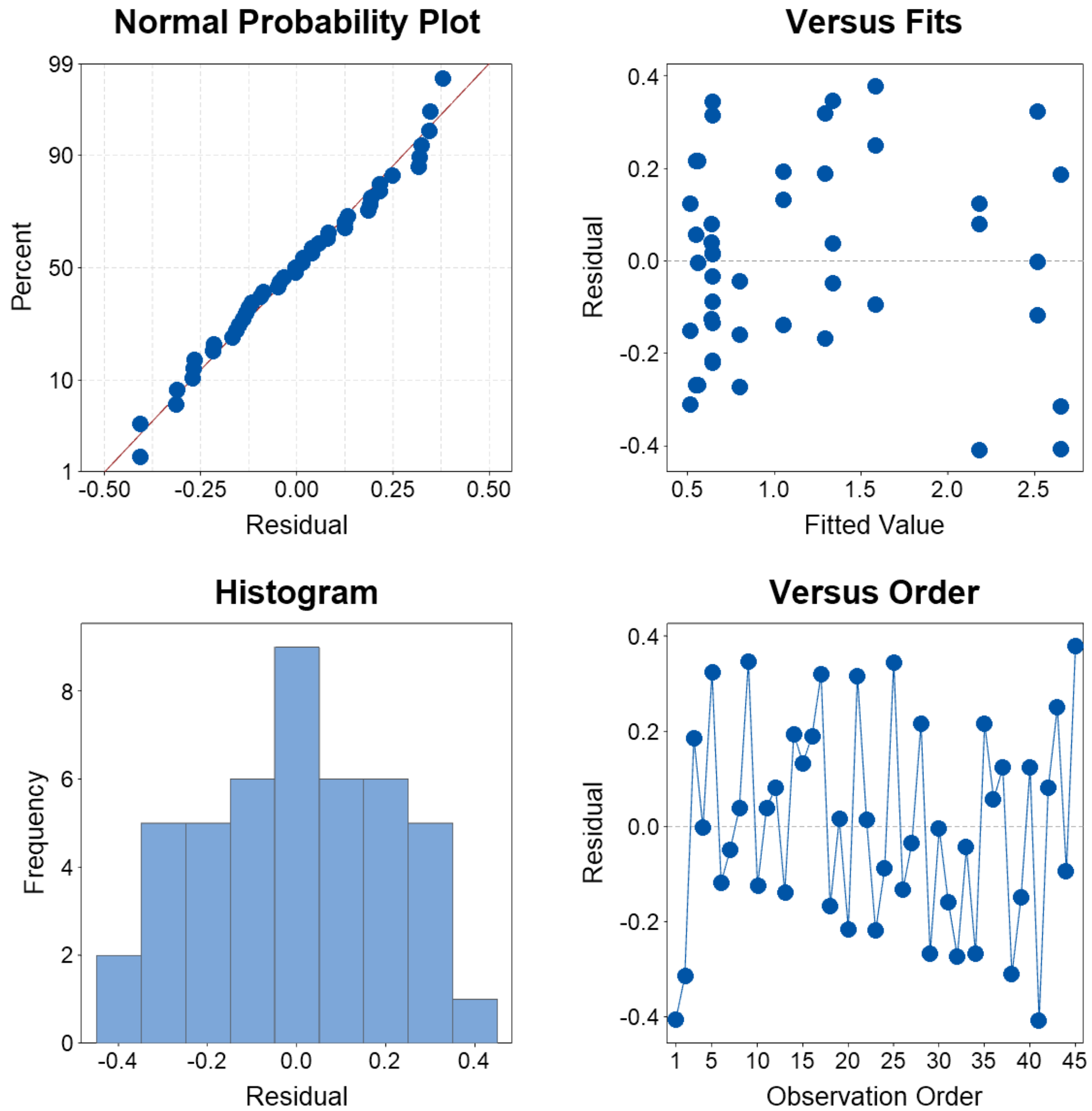


FIGURE B4 – Residual plots of the regression model obtained for the content of free sulfhydryl groups of soy protein isolate.

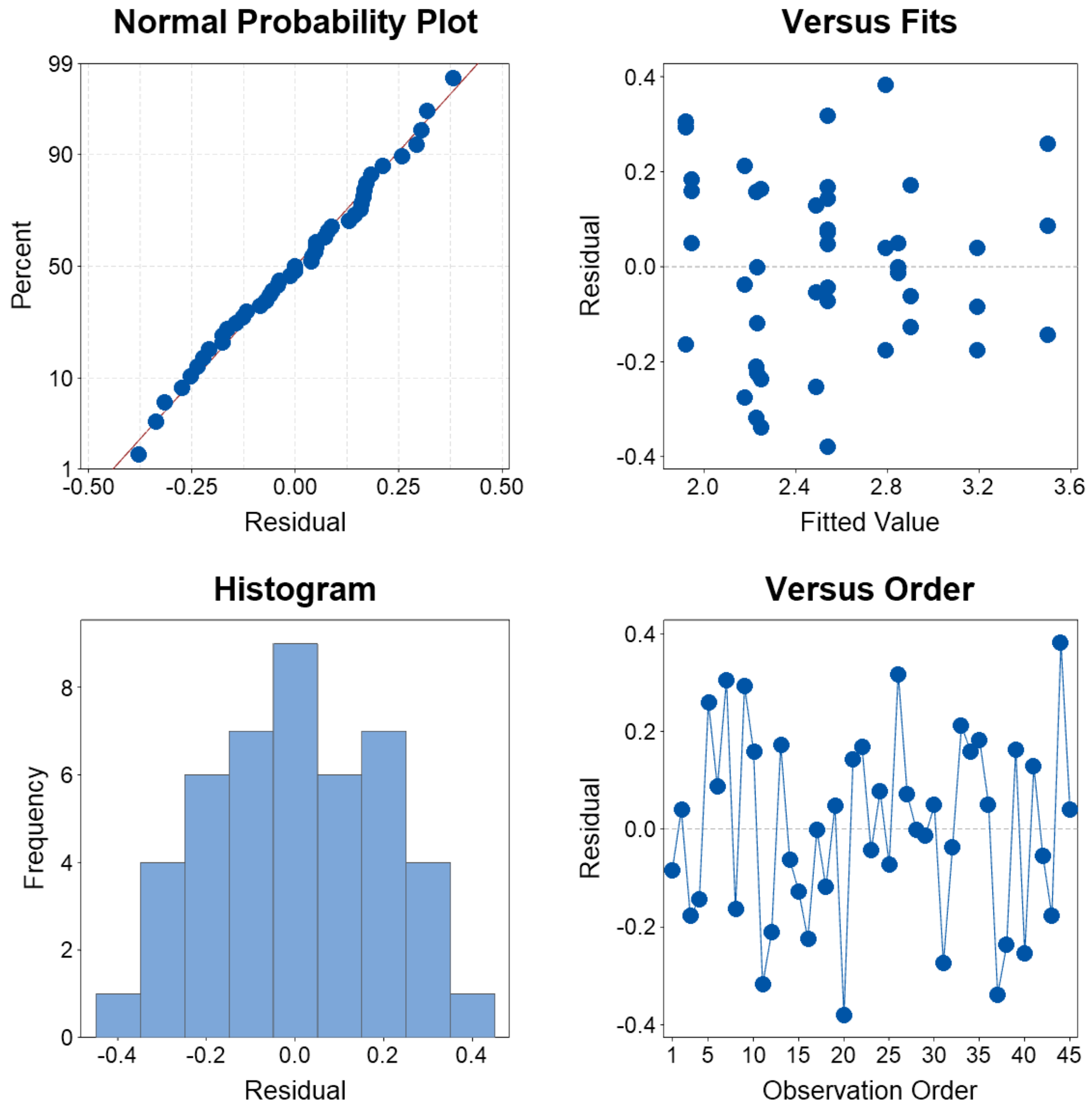


FIGURE B5 – Residual plots of the regression model obtained for the surface hydrophobicity of pea protein isolate.

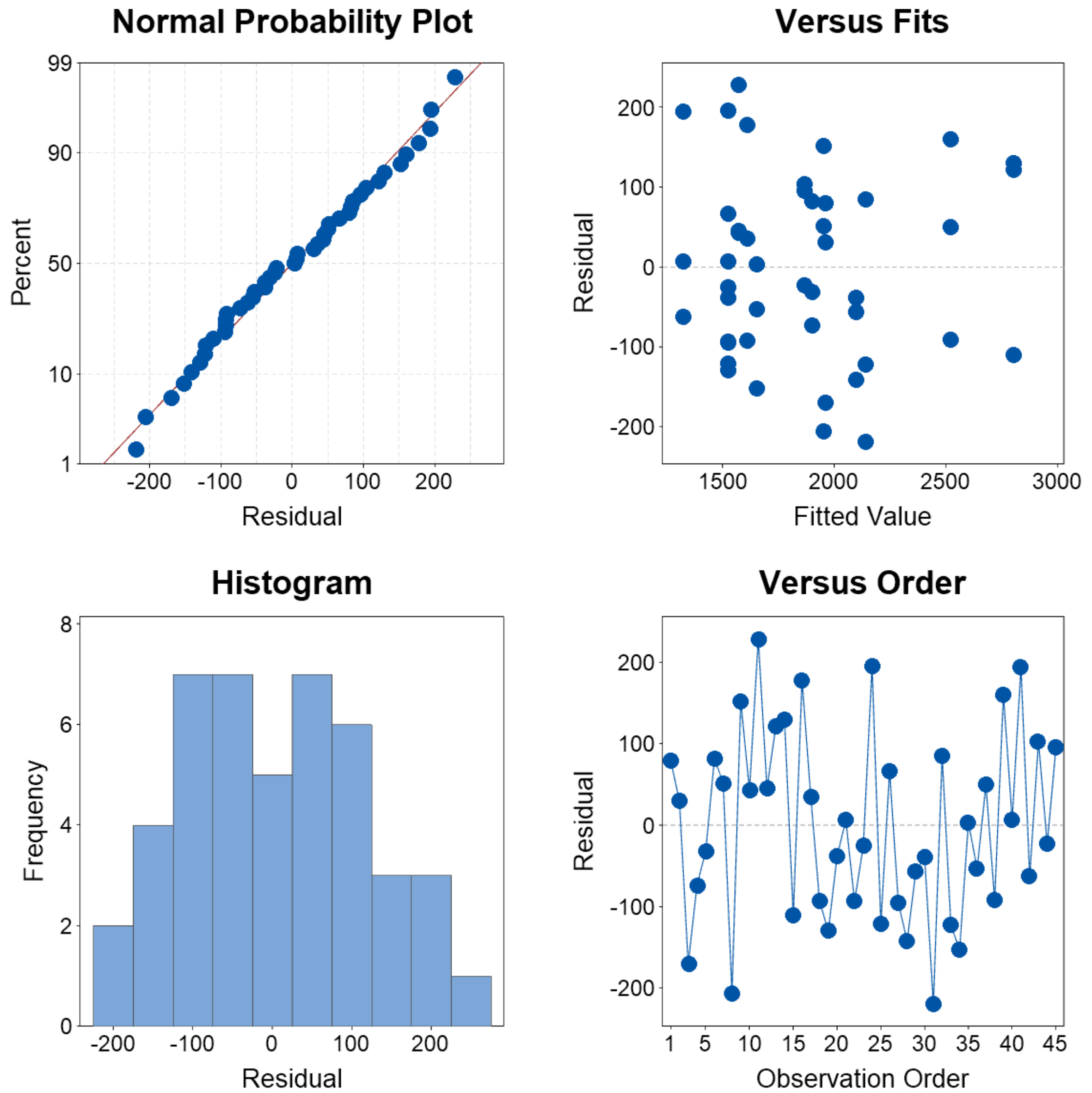
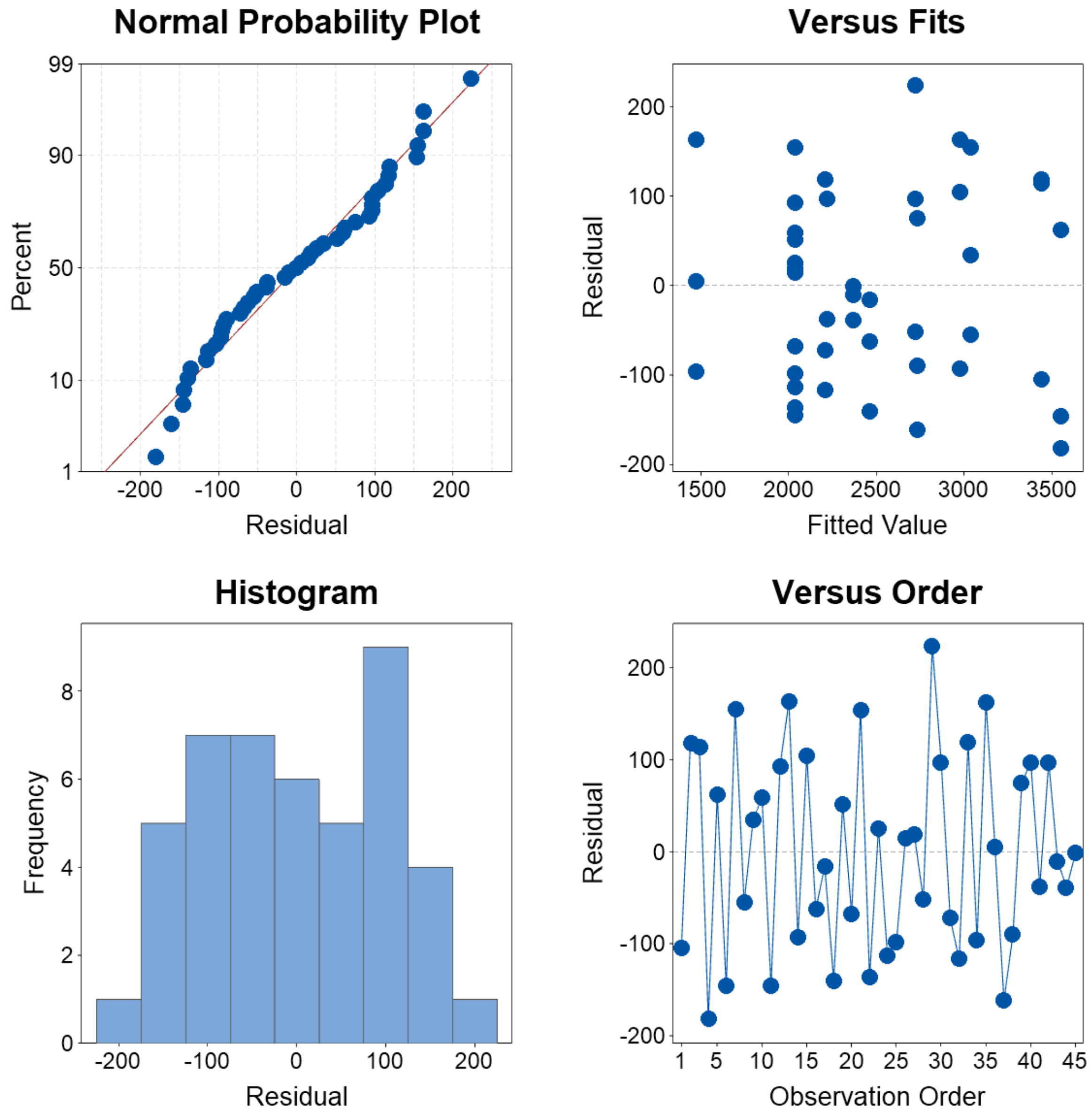


FIGURE B6 – Residual plots of the regression model obtained for the surface hydrophobicity of soy protein isolate.



# Annex C

## Microbial transglutaminase effects on selected properties of pea and soy protein isolates

The effects of microbial transglutaminase (MTG), 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 min at 37 °C, on the solubility, content of free sulfhydryl groups, surface hydrophobicity and emulsifying properties of pea and soy protein isolates (1 % w/v), at pH 6 and 7, were assessed – Figures B1 – B7. Generally, adding MTG to protein isolates resulted in little to no significant differences ($p > 0.05$) in all the parameters studied. As discussed throughout the document, the low concentration of soluble protein due to the formation of insoluble aggregates is a common characteristic of commercial protein isolates (Adebiyi & Aluko, 2011; Lee, Ryu, & Rhee, 2003). This low solubility and aggregates of the protein isolates, specifically in the case of globular proteins with a compact structure, may restrict the accessibility of MTG to glutamine and lysine residues, limiting the enzyme effects (Jong & Koppelman, 2002). Although it was verified a slight increase in the solubility of SPI, at pH 7, when 30 U·(g protein)$^{-1}$ was used. It was also observed a slight improvement in the emulsifying activity index of PPI when MTG was applied at pH 6. As discussed in the main document, MTG may catalyze the deamination of glutamine and asparagine, converting them into glutamic acid and aspartic acid, respectively. The resulting deamidated proteins have increased negative charges that may increase the amphiphilic nature of the protein. This change in the protein's hydrophobicity/hydrophilicity may lead to enhanced solubility or improved emulsifying properties (Agyare, Addo, & Xiong, 2009; Babiker, 2000).

## References

Adebiyi, A. P., & Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (*Pisum sativum* L.) seed protein isolate. *Food Chemistry*, 128(4), 902–908.

Agyare, K. K., Addo, K., & Xiong, Y. L. (2009). Emulsifying and foaming properties of transglutaminase-treated wheat gluten hydrolysate as influenced by pH, temperature and salt. *Food Hydrocolloids*, 23(1), 72–81.

Babiker, E. E. (2000). Effect of transglutaminase treatment on the functional properties of native and chymotrypsin-digested soy protein. *Food Chemistry*, 70(2), 139–145.

Jong, G. H. A. de, & Koppelman, S. J. (2002). Transglutaminase Catalyzed Reactions: Impact on Food Applications. *Journal of Food Science*, 67(8), 2798–2806.

Lee, K. H., Ryu, H. S., & Rhee, K. C. (2003). Protein solubility characteristics of commercial soy protein products. JAOCS, *Journal of the American Oil Chemists' Society*, 80(1), 85–90.

FIGURE C1 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the solubility of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.

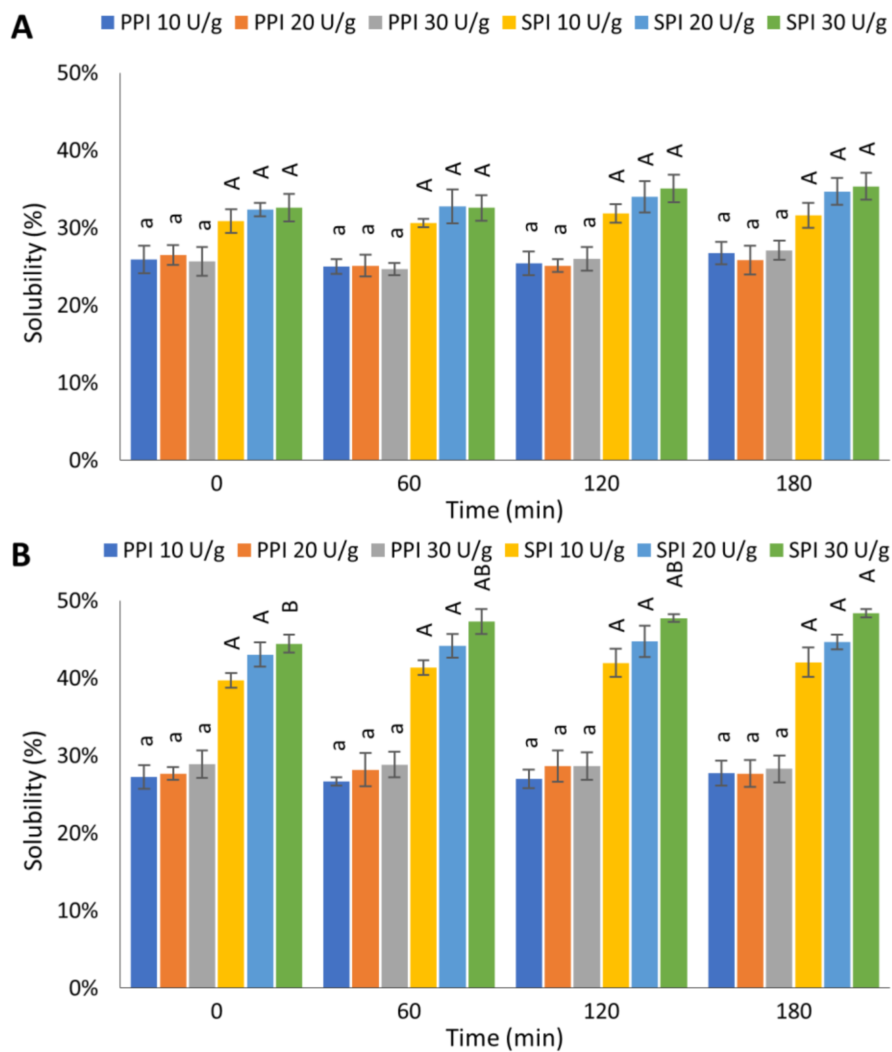


FIGURE C2 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the content of free sulfhydryl groups of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.

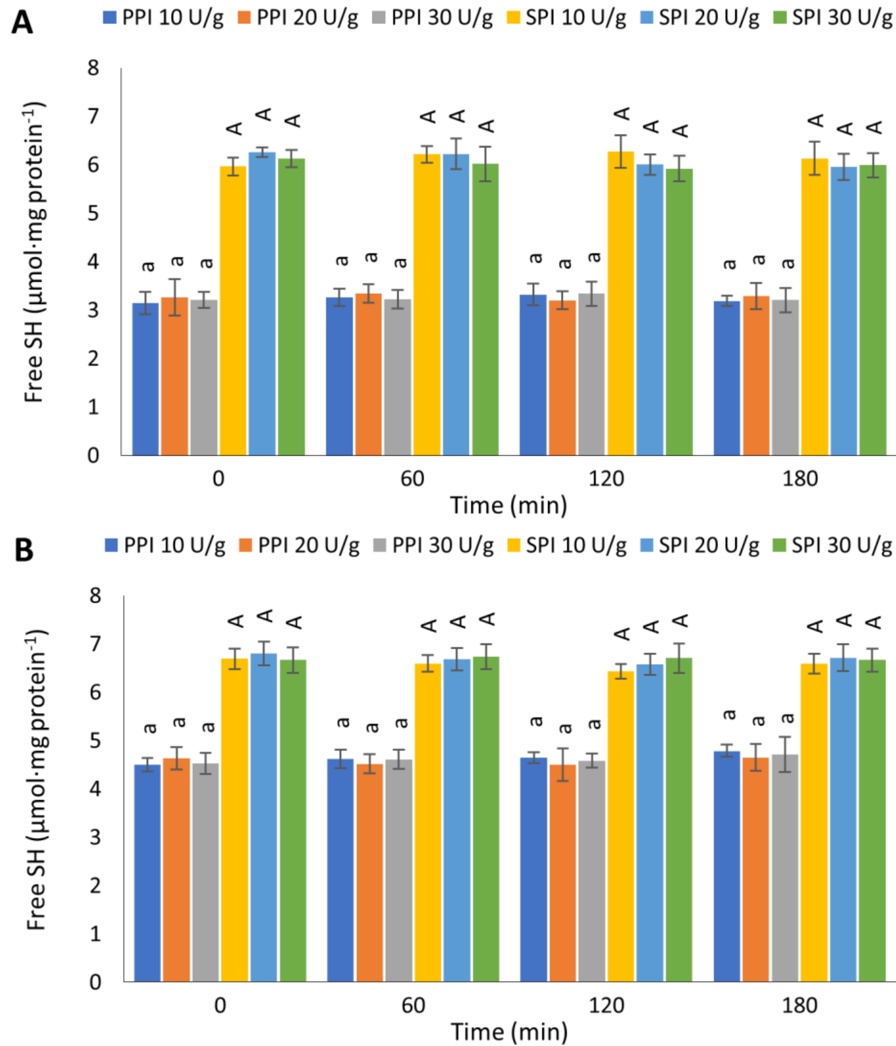


FIGURE C3 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the surface hydrophobicity of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.

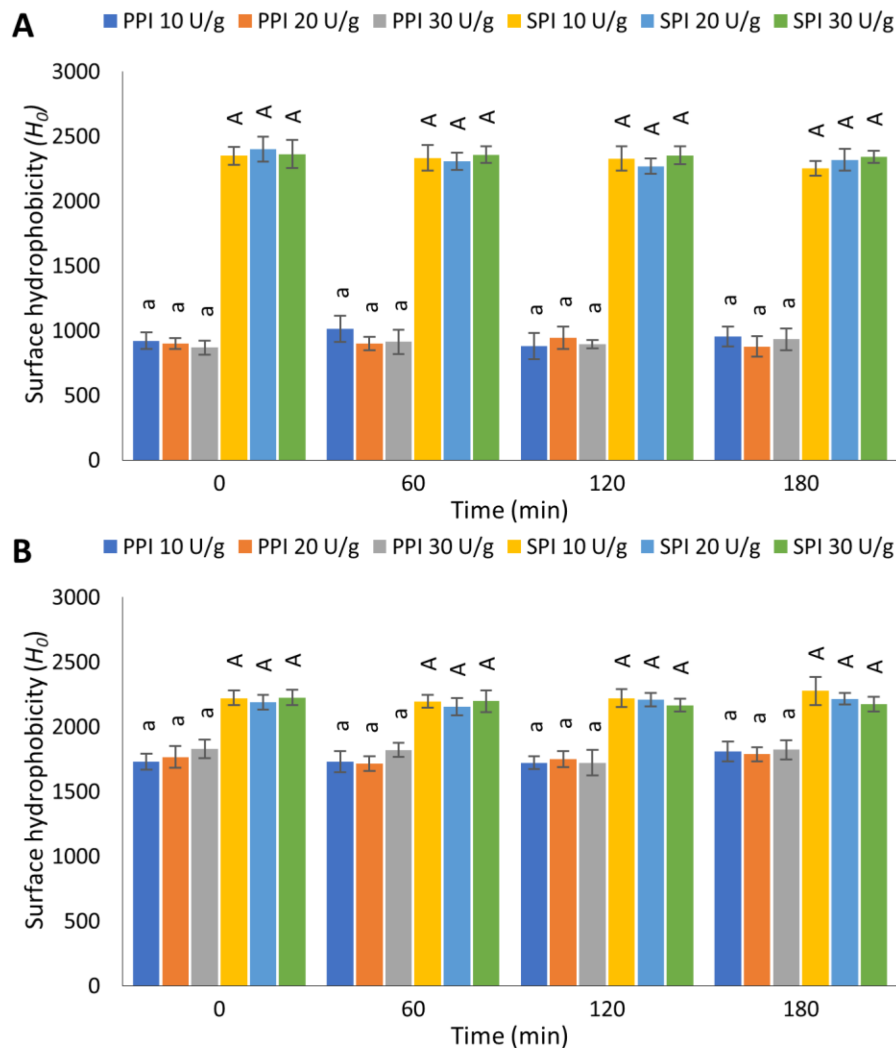


FIGURE C4 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the emulsifying activity index on the soluble fraction of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.

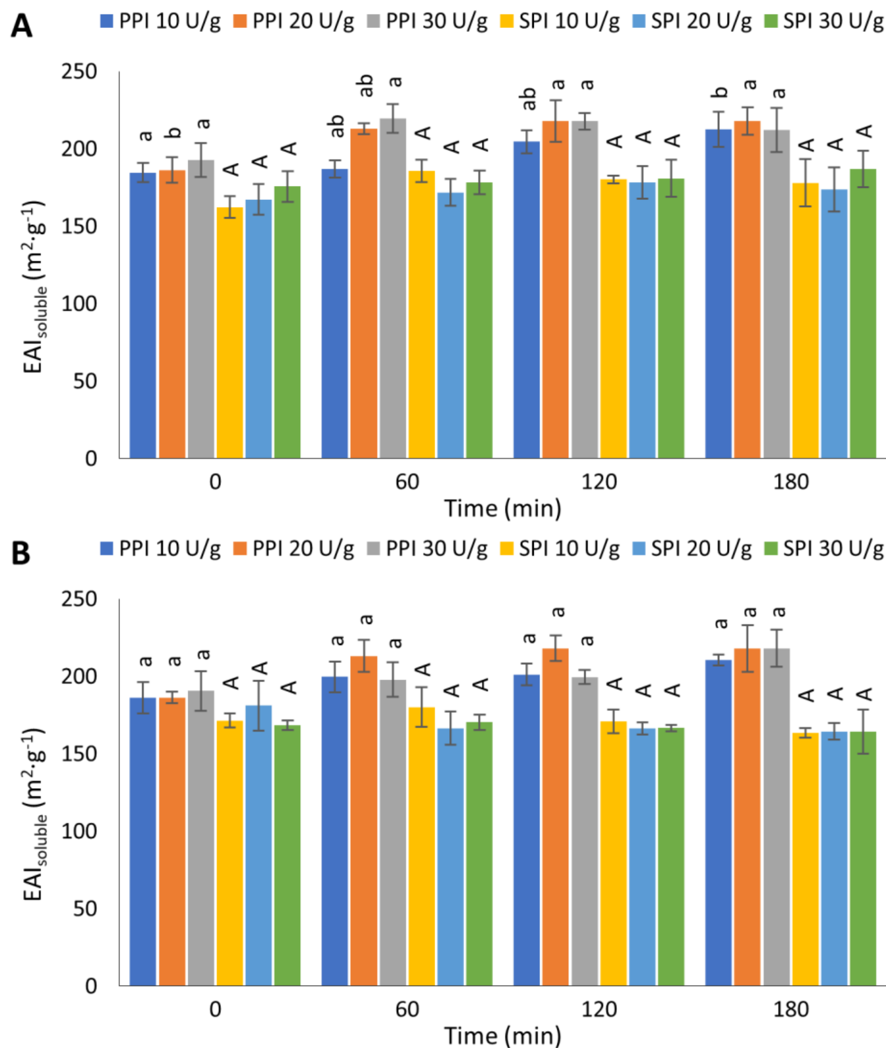


FIGURE C5 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the emulsifying stability index on the soluble fraction of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.

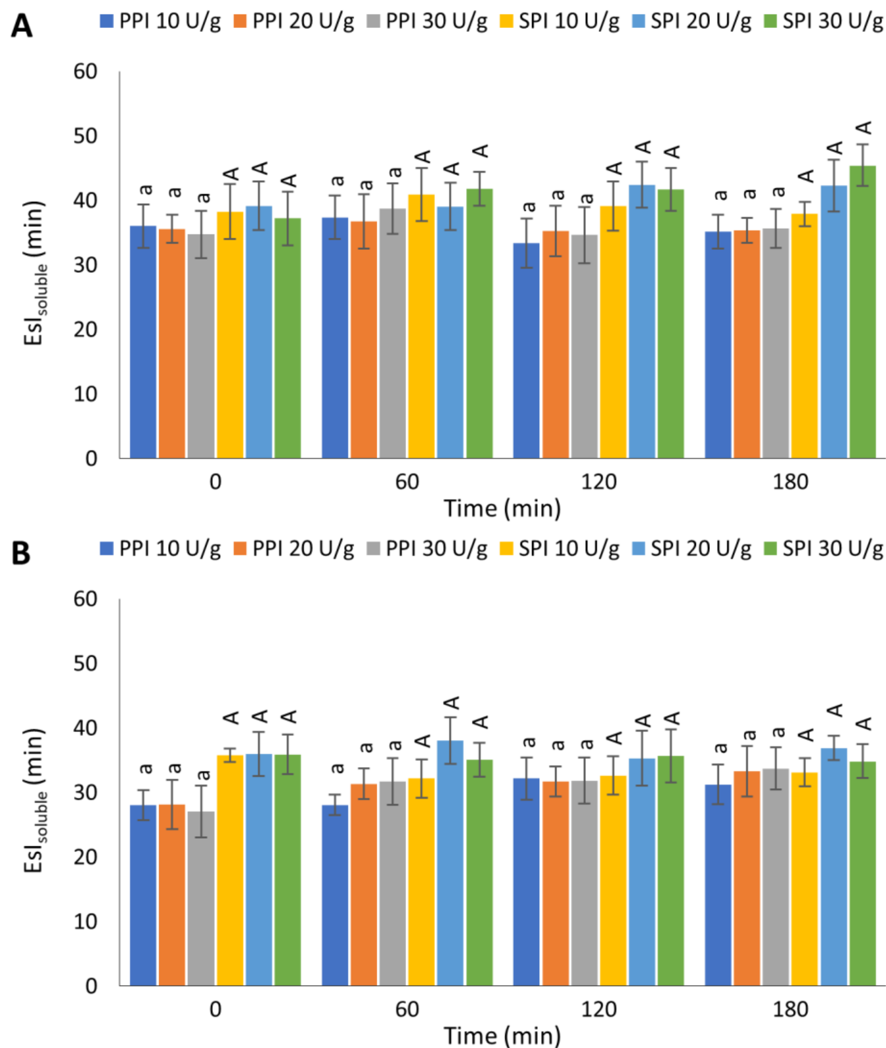


FIGURE C6 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the emulsifying activity index on the total fraction of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.

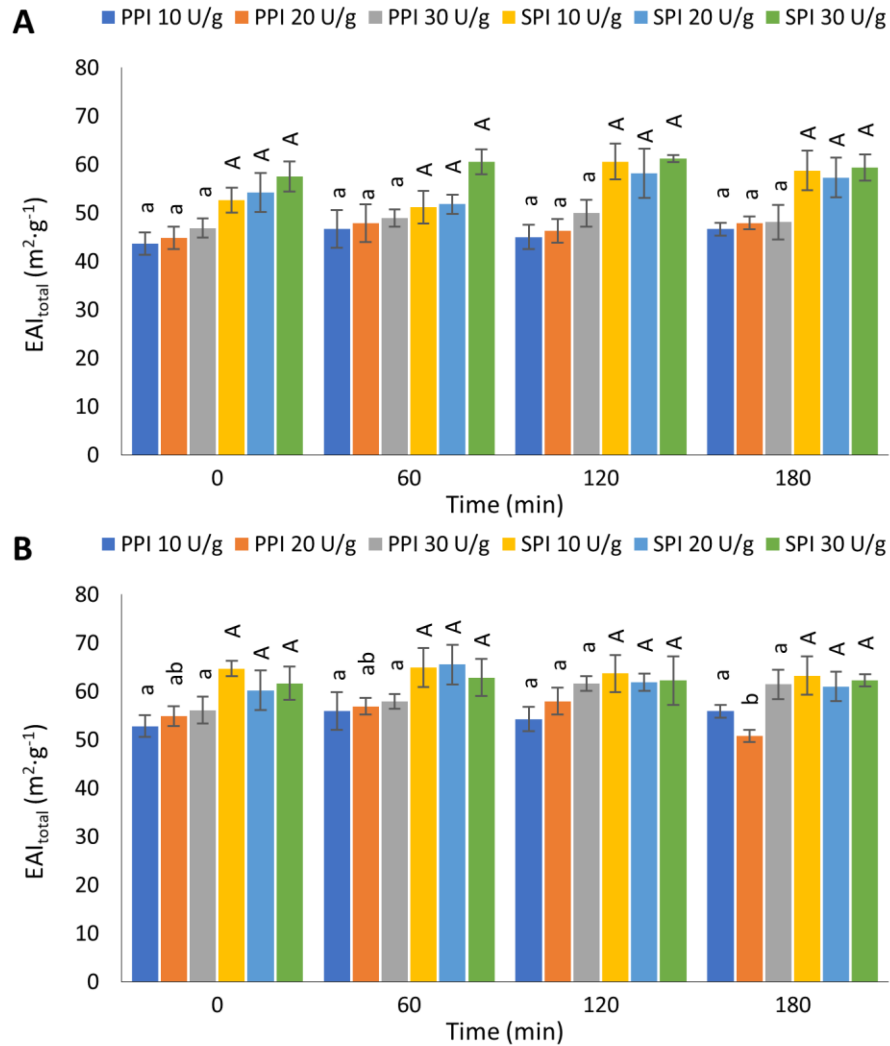


FIGURE C7 - Effects of microbial transglutaminase, 10, 20 and 30 U·(g protein)$^{-1}$ for 0, 60, 120 and 180 minutes at 37 °C on the emulsifying stability index on the total fraction of pea and soy protein isolates at pH 6 (A) and pH 7 (B). Different lowercase letters indicate significant differences ($p < 0.05$) between pea protein isolates. Different capital letters indicate significant differences ($p < 0.05$) between soy protein isolates.