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Soy protein gel systems with added phenolic acids: Effect of gel matrix on protein digestibility and phenolics bioaccessibility

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

This thesis studied how the physical properties of soy protein gels can affect proteolysis and the bioaccessibility of added phenolics. The types of gels developed were model systems for the commercial products firm and silken tofu, which differ in structure and composition.

To begin with, a variety of gels (known as pressed and unpressed) with different physical characteristics were developed. Next, the properties of these pressed and unpressed gels were assessed when they were formed using two different mechanisms - acidification with glucono δ-lactone (GDL) or coagulation with magnesium sulphate ($MgSO_4$).

Results showed that the gels formed with GDL (both pressed and unpressed) were firmer in texture and denser in microstructure than $MgSO_4$ gels, which resulted in a reduction in the extent of proteolysis. It was found that although the coagulation mechanism did not affect the total release of the phenolic acids on a percentage basis, GDL-induced pressed gels delivered larger masses of bioactives in the intestinal phase because they could retain more of the phenolics after pressing. It was hypothesised that at acidic pH the formation of complexes between phenolics and soy proteins is favoured which promotes their retention after pressing.

Overall, it was found that the release of the bioactives was rapid in the unpressed gels and more gradual with a higher final concentration in the pressed gels, which can be attributed to the dense protein network of the pressed gels. In terms of the soy protein itself, this work showed that the extent of protein hydrolysis and the amino acid bioaccessibility were higher in the unpressed/soft gels than in pressed / firm gels. These results pave the way for the formulation of soy products that can modulate not only the bioaccessibility of phenolics (pressed gels), which was the main objective of this work but also gives insights into the best type of format or product to promote amino acid release during digestion (unpressed gels).

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List of abbreviations

SP	Soy protein
SPI	Soy protein isolate
SPH	Soy protein hydrolysate
RDA	Recommended daily intake
LAL	Lysinoalanine
BCAA	Brached chain amino acid
MPS	Myofibrillar protein synthesis
PP	Polyphenols
PA	Phenolic acids
HCA	Hydroxycinnamic acids
HBA	Hydroxybenzoic acids
BLG	β-Lactoglobulin
GDL	Glucono δ-lactone
$MgSO_4$	Magnesium sulphate
$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
MTGase	Microbial transglutaminase
CLSM	Confocal laser scanning microscope
SDS-PAG	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
DTT	Dithiothreitol
PCA	Protocatechuic acid
GLA	Gallic acid
VNA	Vanillic acid
CFA	Caffeic acid
FRA	Ferulic acid

CMA	Coumaric acid (ortho, para, meta-)
OPA	o-phthalaldehyde
WHC	Water-holding capacity
DH	Degree of protein hydrolysis
FAA	Free amino acids
AA	Amino acid
SSF	Simulated salivary fluid
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
E:S	Enzyme-to-Substrate ratio

List of publications and conference presentation

I. Publications

Based on Chapter 5: Soy protein pressed gels: Gelation mechanism affects the *in vitro* proteolysis and bioaccessibility of added phenolic acids. **Marinea M.**, Ellis A., Golding M., Loveday S.M. ***Foods 10 (1), 154.***

Based on Chapter 6: Bioaccessibility of added phenolic acids from unpressed soy protein gels. **Marinea M.**, Ellis A., Golding M., Loveday S.M. ***In preparation***.

II. Conference presentations

Digestibility of silken and firm tofu». **Marinea M.**, Loveday S.M., Ellis A., Golding M. Poster presented at the 5th International Conference on "Food Structures, Digestion and Health", Rotorua, New Zealand, October 2019.

In vitro bioaccessibility of phenolic acids in soft and hard soy protein gels». **Marinea M.**, Loveday S.M., Ellis A., Golding M. Poster presented at the 8th International Symposium on "Delivery of Functionality in Complex Food Systems", Portugal, July 2019.

Effect of phenolic acids on rheological and structural characteristics of soy protein gels. **Marinea M.**, Loveday S.M., Ellis A., Golding M. Poster presented at the 7th International Symposium on "Delivery of Functionality in Complex Food Systems", New Zealand, November 2017.

Chapter 1

Introduction

1.1 General Introduction

Phenolic compounds are secondary metabolites of plants with various health benefits, such as antioxidant Pereira et al. (2009), anti-inflammatory (Ambriz-Pérez et al., 2016), antimicrobial (Raccach, 1984), anti-allergenic (Bellik et al., 2013), anticancerogenic (Wahle et al., 2010) and neuroprotective properties (Szwajgier et al., 2017). However, the bioavailability of many phenolics is low and it is not certain that they reach their target in the human body, after consumption (Bohn, 2014a). In the last few years, research has shown that food structure and matrix can affect nutrient uptake and bioavailability (Boland et al., 2014, McClements et al., 2009). Therefore, the study of the food structures can be useful for the development of carrier food matrices with targeted delivery properties that can improve aspects of phenolics bioavailability. Moreover, the release of endogenous phytochemicals might be affected by the food matrix, thus the knowledge of the effect of food structure on bioactives' bioavailability might inform food industry on the formulations and processing that ensure the highest bioavailabilities.

In this thesis, phenolics were embedded into two different types of soy protein gels which differ in texture and microstructure, and it was studied how the interaction of the phenolic bioactives with the soy proteins affect the physical properties of the gel systems, the protein digestibility and bioaccessibility of the phenolics. It was hypothesised that gel physical properties modulate the release of the added phenolics. These types of gels are an existing food format, and they are commercially known as silken and firm tofu. Also, we selected homologous phenolic acids as model bioactives, and we attempted to identify some structural features, that might affect the phenolics' bioaccessibility.

Soy protein gels were selected as a potential food matrix in our studies for a number of reasons. Firstly, soy proteins possess a balanced composition of polar, hydrophobic, and charged amino acids that allow the incorporation of a range of different bioactives. Soy proteins have a broad range of techno-functional properties and therefore a variety of microstructures could potentially be formed. There is also a significant industrial interest in soy as they form the basis of tofu and other soy-based foods such as tempeh and textured soy protein, but to date are still under-researched in respect to their digestibility and their potency as a delivery system for bioactives.

1.2 Objective of the thesis

- To produce a variety of soy protein gel structures, assess their physical characteristics and the effect of phenolic acid addition on the physical characteristics of the gels.
- To compare the protein digestibility of unpressed and pressed gel structures with different microstructural characteristics.
- To compare the bioaccessibility profile of phenolic acids from different types of soy protein gels.
- To use a homologous series of structurally related phenolic acids to investigate the structure-bioaccessibility profile relationship of phenolic acids embedded in soy protein gels.

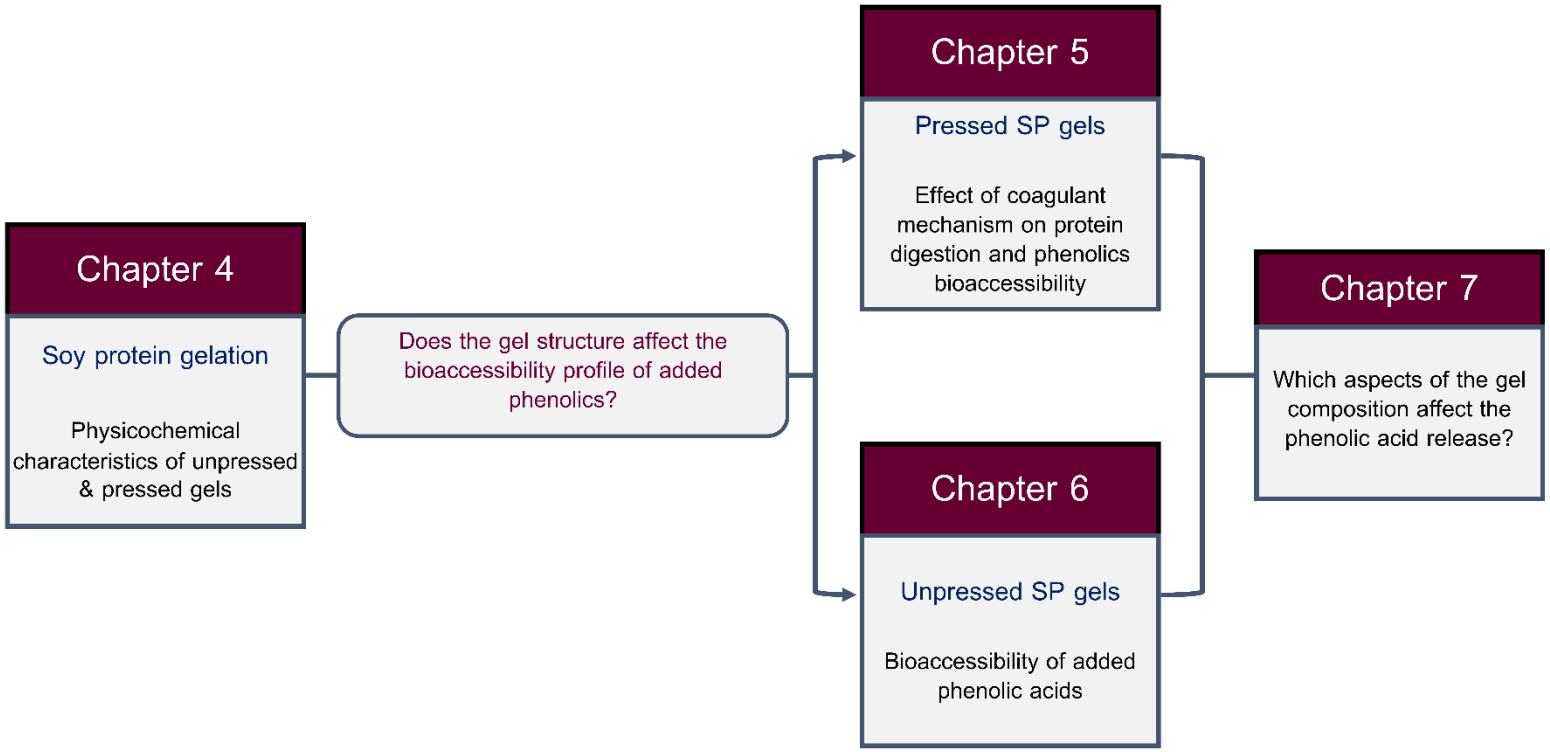


Figure 1.1 Outline of the PhD thesis.

Chapter 2

Literature Review

In the last few years, there has been an increasing interest concerning the consumption of plant proteins for both environmental and nutritional reasons. The production of plant proteins is more efficient and less resource-intensive than meat protein (Thrane et al., 2017). Soybeans produced by the plant *Glycine max* have been used in many food products for thousands of years in East Asia and are promising sources of protein. Some of the most well-known soybean food products are soymilk, tofu, tempeh, nattō, miso and textured soy protein. Soy proteins are of high nutritional quality and provide adequate amounts of essential amino acids (Rizzo and Baroni, 2018b).

2.1 Soybean composition and health benefits

The soybean seed composition on a dry basis is 40 % protein, 21 % fat, 34 % carbohydrates and 5 % of ash (Medic et al., 2014). Both protein and fat bodies are located on the cytoplasm of the cotyledon cells (Medic et al., 2014). There are also some secondary metabolites of special interest including a class of flavonoids with estrogenic and antioxidant properties, called isoflavones (Preedy, 2012), saponins and phytic acid (Liener, 1994).

In general, soybean products are considered very healthy and have been associated with various health benefits. However, some of the health claims are under debate in the literature. In this summary, only the recently evaluated claims will be mentioned. According to a meta-analysis from 2015 (Tokede et al., 2015), soy products positively affect the lipid profile and concentration in the serum, with whole soy products having a stronger effect (by 3 folds) than those supplemented with soy (Tokede et al., 2015). Thus, soy might have a protective effect against cardiovascular diseases (Li et al., 2020b, Tokede et al., 2015). Moreover, high intake of soy products has been associated with reduced risk of different types of cancer (Li et al., 2020b) and it has been stated that soy consumption could benefit women for both preventing and fighting breast cancer (Kucuk, 2017). Finally, it has been suggested that some polyphenol-rich foods, such as soy, might prevent osteoporosis disease and its progression (Chisari et al., 2019).

2.2 Soy proteins

The main fraction of soybean seed proteins are the storage proteins which account for 65 to 80 % of total protein composition (Medic et al., 2014). Generally, storage proteins are essential for the growing seedlings and their main function is to act as a storage reserve of nitrogen, carbon and sulphur (Krishnan and Coe, 2001).

In order to have a homogeneous and reproducible system, the raw material used in this study was a commercial soy protein isolate (SPI) powder, rather than soybeans. During the manufacturing process of the SPI, the defatted soybean meal is subjected to acidification (pH 4.5-4.8), where the more important and abundant fraction of the storage proteins precipitates (Nishinari et al., 2014). The supernatant is composed of the minor proteins γ-conglycinin and some contaminating proteins, including the following soy whey proteins: lipoxygenase, β-amylase, lectin and Kunitz and Bowman−Birk trypsin and/or chymotrypsin inhibitors (Iwabuchi and Yamauchi, 1987). A low concentration of the whey proteins can still be detected in the acid precipitate and thus in the isolate powders (Iwabuchi and Yamauchi, 1987). In the following summary, we will only focus on the major soy proteins that are found in the SPI.

2.2.1 Structure of major globulins

The main proteins found in the SPI can be classified into four groups based on their sedimentation coefficients measured in Svedberg units (S) 2S, 7S, 11S and 15S. Practically, the higher the S value the heavier the sedimented proteins. The 7S and 11S consist of more than 80% of the SPI proteins and their ratio is about 0.5 to 1.7 and varies among cultivars (Nishinari et al., 2014).

Glycinin (11S) is a hexamer which is formed by a face-to-face stacking of two trimers (Adachi et al., 2003) connected by non-covalent interactions (Adachi et al., 2003, Badley et al., 1975). Each trimer consists of three monomeric subunits which are composed of an acidic subunit (acidic pI) and basic subunit (basic pI) covalently attached by a disulphide bond (**Figure 2.1**) (Medic et al., 2014, Adachi et al., 2003). The molecular weight of glycine ranges from between 320-375 kDa (Badley et al., 1975). β-Conglycinin (7S) is a glycoprotein in a trimeric form which is constituted by

three subunits (α, α' and β) associated through non-covalent interactions. The molecular weight of β-conglycinin ranges between 150-200 kDa (Badley et al., 1975).

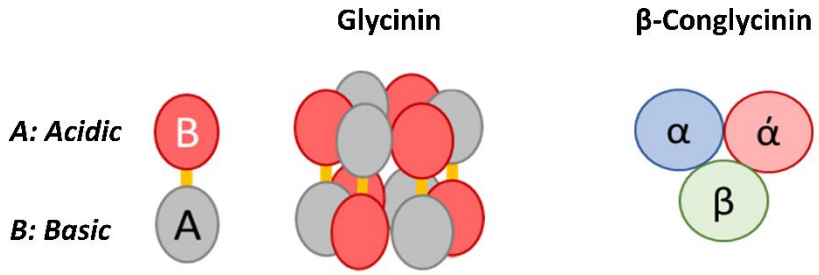


Figure 2.1 Primary storage proteins of soybeans, glycinin and β-conglycinin. Adapted with permission from (Badley et al., 1975).

2.2.2 Techno-functional properties of soy proteins

The functionality of the proteins depends on their structure. Depending on the primary structure, different intra- and inter-molecular forces can be developed which will affect the other levels of the protein structure. The main amino acid groups of soy proteins can be found in **Figure 2.2**. The hydrophobic and hydrophilic amino acids residues dominate in soy proteins, which can greatly affect functionalities such as solubility, emulsification, and gelation properties.

Both emulsification and foaming behaviours depend on the surface-active properties of the proteins. In particular, the proteins need to be able to diffuse and/or adsorb and stabilise an interface. The emulsification properties of soy proteins, with an emphasis on the role of conformational flexibility, have recently reviewed by Tang (2017). Soy proteins have an amphipathic nature and can act on the interface, although their use as emulsifiers is very limited due to solubility limitations (Tang, 2017). According to Tang the insoluble nature of soy proteins give a good potential to be used as Pickering stabilizers in an emulsion or emulsion gels (Liu and Tang, 2016).

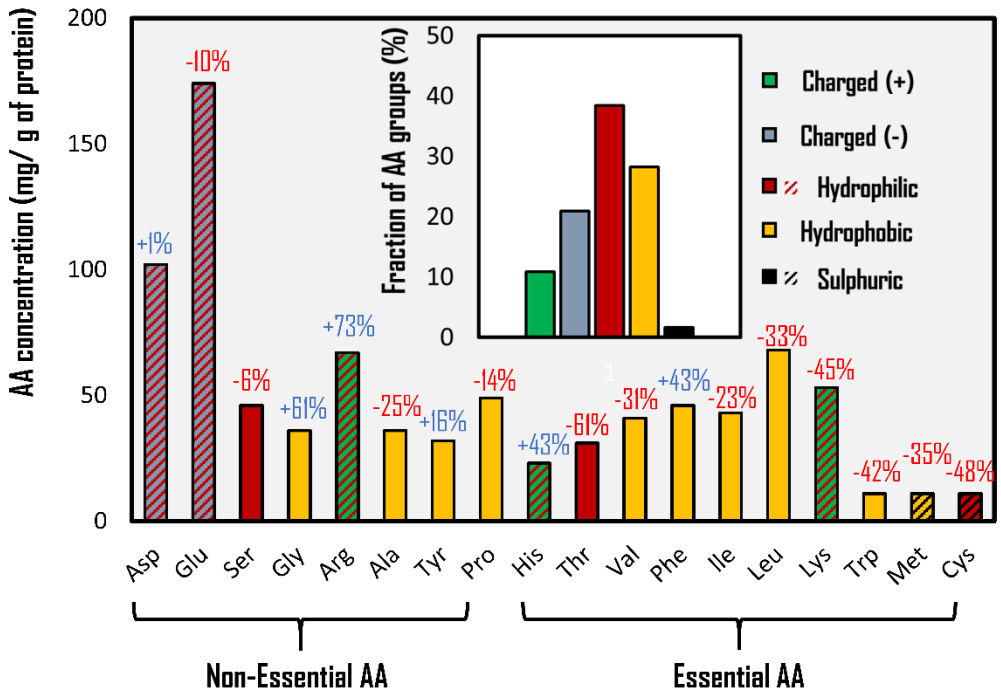


Figure 2.2 Amino acid (AA) composition of soybean proteins (Kalman, 2014). The colour coding indicates the AA grouping. The percentages above the bars represent the difference in the AA concentration (%) of soy proteins compared to whey proteins.

2.2.2.1 Soy protein solubility

Native SPIs exhibit good solubility at alkaline pH but this can be reduced as the pH decreases and, with precipitation occurring close to the isoelectric point (pI ~4.5) (Malhotra and Coupland, 2004) (**Figure 2.3**). Therefore, the solubility of soy proteins largely depends on the pH and ionic strength with their influence on the charge. However, the shape of the globular proteins reveals that a large fraction of the protein's hydrophobic region is buried into the core. Consequently, heat denaturation can expose the hidden hydrophobicity and negatively affect the solubility and form aggregates. Interestingly, Guo et al. (2012) found that the aggregation behaviour of soy protein (glycinin and β-conglycinin) is significantly different. Thermal processing caused aggregation in both soy protein fractions. However, the growth of these aggregates was limited and remained soluble for β-conglycinin because of the limited active sites in its structure. In contrast, glycinin contains more active aggregation sites and no polysaccharide attachment, thus the aggregates could grow large and

precipitate. As a result, the addition of β-conglycinin into glycinin dispersion limited the growth of the aggregates (**Figure 2.4**).

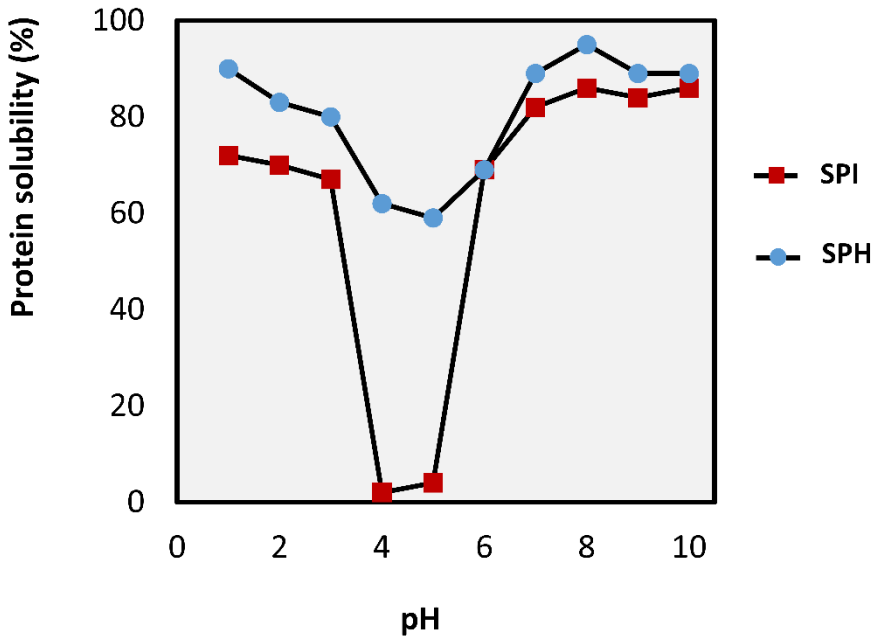


Figure 2.3 Protein solubility profiles of SPI (■) and soy protein hydrolysate (SPH) (•). Adapted with permission from (Achouri et al., 1998).

Wang et al. (2012) studied the effect of thermal treatment (90 and 120 °C) on the emulsification properties of soy proteins. It was found that heat treatment increased the surface hydrophobicity due to protein unfolding. However, only the 90 °C treatment showed a reduction in surface pressure during long-term adsorption. Mild protein hydrolysis is a promising technique that can improve both proteins solubility and emulsification properties. Molina Ortiz and Wagner (2002) showed that soy protein hydrolysates obtained by bromelain digestion had improved solubility and surface properties at acidic pH. Similarly, an increase in hydrolysate solubility has been found in other proteins, such as whey (Severin and Xia, 2006).

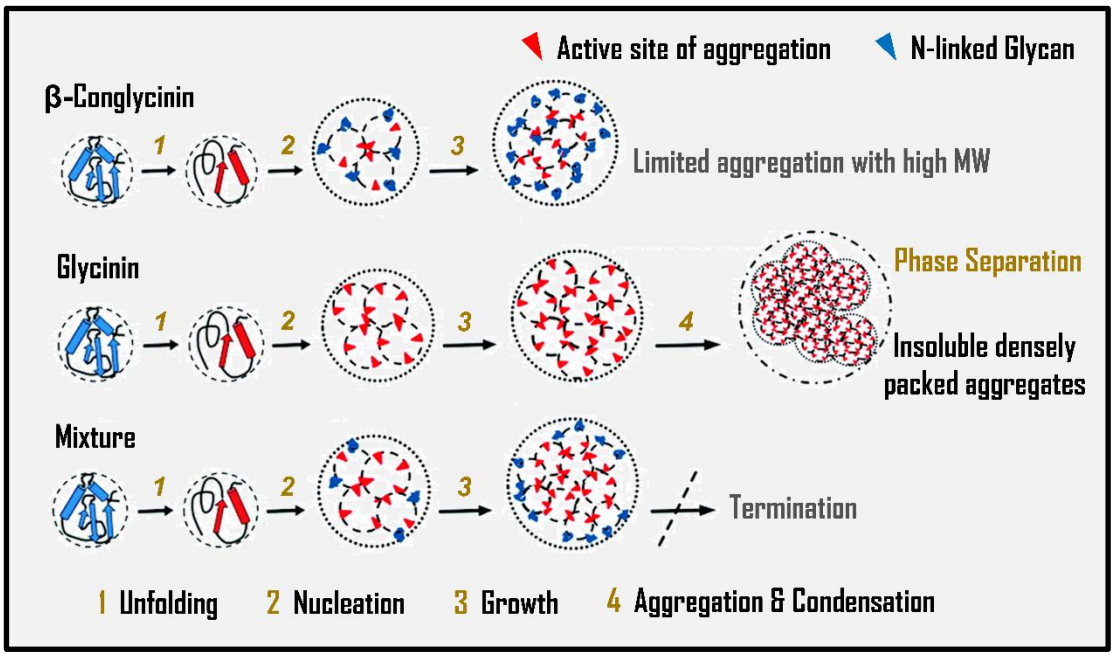


Figure 2.4 Thermal aggregation behaviours of soy proteins glycinin and β-conglycinin at neutral pH. Adapted with permission from Guo et al. (2012) American Chemical Society.

2.2.2.2 Soy protein gelation

Protein gelation was defined by Schmidt (1981) as "the protein aggregation phenomenon in which protein-protein and protein-water interactions occur and attractive and repulsive forces are so balanced that a well ordered tertiary network or matrix is formed". Although random aggregation is macroscopically the same with gelation, protein-protein interactions dominate over protein-water interactions (Schmidt, 1981).

Generally, the types of bonds involved in protein gels are covalent, such as disulphide bridges, and non-covalent, such as hydrogen, ionic and hydrophobic bonding. However, soy proteins contain only a small amount of sulphur-containing amino acids (**Figure 2.2**) (Saio and Watanabe, 1978).

A prerequisite for globular proteins to gel is heat-induced denaturation, where the buried reactive groups, such as hydrophobic residues become exposed, interact and form a gel network (hydrophobic effect) (Gosal and Ross-Murphy, 2000). Then the gelation can be induced in various ways.

2.2.2.2.1 Physical gelation: Thermally-induced

When the protein concentration is sufficient, further heating results in aggregation and subsequent gelation. According to Bikbow et al. (1979), the minimum SPI concentration (c_o) for heat-induced gelation was ~ 6.6 %. Catsimpoolas and Meyer (1970) proposed two gel states for soy proteins. First, the "progel state" which is formed due to the protein denaturation (*irreversible process*) and it can be detected as an increase in the mixture viscosity. Secondly, the formation of an actual "gel state" which occurs upon cooling (*reversible process*) (**Figure 2.5**). Gels reversibility indicates a low dependency on covalent interactions (Schmidt, 1981).

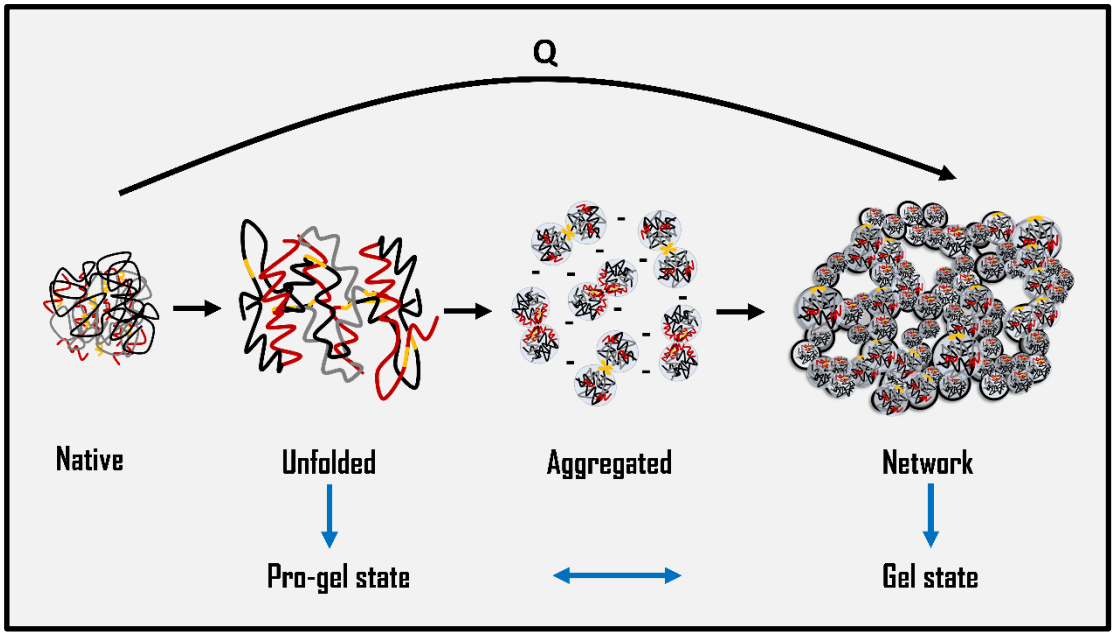


Figure 2.5 Heat-induced gelation of globular proteins and gel states of soy proteins. Adapted with permission from Altinǵ et al. (2003).

2.2.2.2.2 Chemical gelation: Ion and acid-induced

Gelation is mostly favoured when the net charge is close to zero and attractive protein-protein interactions dominate. Depending on the charge density, proteins rearrange into different networks which affect the texture and microstructure. At high ionic strengths and/ or low pH the electrostatic repulsion in a protein dispersion is partially or completely screened. Consequently, the proteins become unstable causing precipitation and the van der Waals attraction forces dominate due to particle

proximity (Verwey and Overbeek, 1947, Derjaguin and Landau, 1941). This sequence of events leads to rapid aggregation and the formation of a particulate microstructure. Under conditions where proteins have a small net charge, a more ordered microstructure is formed (Hermansson, 1986).

2.2.2.2.3 Soymilk gel formation: Tofu

Tofu is the most popular and versatile product made from soymilk and its production is based on the phenomena described above. The texture of tofu is important for consumer acceptance and ranges from very soft (ex. yoghurt) to very firm (ex. feta cheese) (Nishinari et al., 2018). To obtain firm tofu, the gel needs to be pressed; and in this way, both the whey serum that softens the gel and the water are removed (Shurtleff and Aoyagi, 1975). The pressing forces used in the traditional tofu making range between 20 –100 g/cm^2 (Shurtleff and Aoyagi, 2000). Among the factors affecting the texture of tofu are the soybean variety (Nishinari et al., 2018), protein profile, such as 7S to 11S ratio (Guo et al., 2012, Nishinari et al., 2014), protein and solid content and the processing conditions (Nishinari et al., 2014, Chang, 2006).

Another of the most crucial aspects is the type of coagulation agent used. According to the literature, there are four basic categories of coagulants used in tofu making and they are the following (Chang, 2006, Shurtleff and Aoyagi, 1975, Shurtleff and Aoyagi, 2000): firstly, the nigari salts, which refer mainly to chloride divalent salts such as magnesium and calcium chloride. Secondly, sulphate salts such as calcium sulphate (gypsum) and magnesium sulphate (Epsom salts). Both nigari and sulphate coagulants are based on the salt aggregation mechanism of gelation mentioned previously. Thirdly, glucono-δ-lactone (GDL), which is a δ-lactone of D-gluconic acid, can be used. GDL gradually hydrolyses to gluconic acid, and the produced protons reduce the pH of the mixture (Pocker and Green, 1973). Finally, the last category consists of natural acidifiers such as citrus juice, vinegar and lactic acid, but these have many disadvantages that limit their use (Chang, 2006).

2.2.3 Nutritional quality of soy proteins

The nutritional quality of proteins depends on the content, digestibility, absorption and utilization of the amino acids (Friedman and Brandon, 2001). Proteins deficient in one or more essential amino acids are considered of poor quality. Plant proteins tend to be inferior to meat proteins; for instance, lysine is lacking in grains such as wheat, and methionine is lacking in legumes (Friedman and Brandon, 2001). Also, the presence of digestive enzyme inhibitors or antinutritional factors such as Kunitz and Bowman−Birk inhibitors and phytic acid can significantly affect the digestibility of legume protein sources. Nevertheless, these inhibitors are inactivated by heat treatment due to the denaturation process and/or removed in purification (e.g. soy whey) (Yuan et al., 2008).

According to human nitrogen balance studies, soy protein is the only high-quality protein among the known plant proteins (Rizzo and Baroni, 2018b). Several methods are available for estimating protein quality in foods. World Health Organisation (WHO) on 1991 stated that the "Protein Digestibility Corrected for Amino Acid Score" (PDCAAS) method provides the "best estimation of digestibility and amino acid score" with 1.0 being the maximum score (FAO/WHO, 1991). However, PDCAAS was recently replaced by "Digestible Indispensable Amino Acid Score" (DIAAS) to overcome inaccuracies (FAO/WHO, 2013), although there are limited data available at the moment (Loveday, 2019).

Sarwar (1997) compared the protein digestibility profile of fourteen different protein sources using different methods, including the PDCAAS using both rats and humans. The proteins were either commercial isolates or defatted meals made from raw legumes. In **Table 2.1**, the combined results of the PDCAAS and DIAAS scores from different protein sources can be found (Rizzo and Baroni, 2018b, Sarwar, 1997, Loveday, 2019). These studies showed that SPI has a protein quality score close to meat proteins. Moreover, in a meta-analysis from 2003 when data from nitrogen-balanced studies were analysed, it was demonstrated that well-processed soy proteins are equivalent to animal proteins (Rand et al., 2003). It is worth mentioning that the term "well-processed" implied that the used soy material was in the form of protein isolate or concentrate (Istfan et al., 1983, Scrimshaw et al., 1983, Young et al., 1984).

Table 2.1 Comparison of the PDCAA and DIAAS score from different protein sources (Loveday, 2019, Rizzo and Baroni, 2018b, Sarwar, 1997).

Protein Source	PDCAA Score	DIAAS
Casein	1.00	n/a
Bovine milk	1.00	1.32
Lactalbumin	1.00	1.13
SPI	0.92-1.00	0.90
Beef	0.92	1.12
Egg	1.00	n/a
Wheat	0.25	0.41

2.2.4 Amino acid composition of soy proteins

Although most of the plant proteins provide sub-optimal proportions of essential amino acids (AA), soy and canola contain a well-balanced AA composition with high concentrations of essential AA (Day, 2013). The AA composition of soy proteins and comparison with whey proteins can be found in **Figure 2.2**. Generally, plant proteins have lower levels of lysine than animal sources (Day, 2013), yet, soybean provides adequate amounts of lysine (Day, 2013, Friedman and Brandon, 2001). The estimated recommended dietary allowance (RDA) for lysine according to FAO/WHO reports is 12 mg/Kg per day for adults (Council, 1989), thus around 16 g of SPI would be enough to reach the recommendation.

Methionine is another essential amino acid that is present in lower amounts in plant proteins, including soybean (Day, 2013, Friedman and Brandon, 2001). Moreover, methionine can be further chemically modified during food processing (Friedman and Brandon, 2001). Biotechnological strategies that increase the sulphur amino acid content in legume have been proposed in the literature (de Lumen et al., 1999). Food processing methods that involve treatment with pH >9 can irreversibly affect the nutritional quality of soy proteins. Friedman and co-authors investigated the formation of lysinoalanine (LAL), an unnatural amino acid, in soy proteins which causes lower digestibility of soy proteins (Friedman, 1978, Friedman et al., 1984, Friedman and Liardon, 1985).

High temperature and alkaline pH destroyed cysteine and lowered the amounts of arginine, lysine, serine and threonine residues, while the formation of LAL observed (Friedman et al., 1984). Furthermore, protein isolates from both plant and dairy sources that were treated under alkaline conditions, showed significantly negative results in terms of the digestibility of the proteins (Sarwar, 1997). The production of fibrillar proteins for the use as meat analogues involves alkaline treatment (Friedman and Brandon, 2001).

One of the most important groups of amino acids is the branched-chain amino acid (BCAA) which includes leucine, isoleucine, and valine. BCAA have been associated with multiple benefits related to muscle synthesis and growth (Kamei et al., 2020). Studies in the field of exercise research have shown that soy proteins do not perform equally well with dairy proteins regarding myofibrillar protein synthesis (MPS). Professor Philips and his group compared the effect of dairy and SPIs and drinks on MPS of both young and old individuals (Phillips et al., 2009, Tang et al., 2009, Yang et al., 2012).

Whey protein hydrolysate resulted in greater MPS at both resting and post resistance exercise in young healthy men. Soy protein performed better than caseinate but worse than whey (Tang et al., 2009). Similarly, whey protein isolate response on MPS was significantly higher than SPI in the elderly (Yang et al., 2012). The findings were explained by the lower postprandial leucinemia observed and the greater rates of amino acid oxidation following ingestion of soy (Yang et al., 2012). Leucine is primarily responsible for stimulating protein synthesis in skeletal muscle (Kimball and Jefferson, 2006) and whey protein has almost 34 % more leucine than soy (**Figure 2.2**).

2.2.5 Effect of food matrix on soy protein digestion

The majority of nutritional studies related to soy protein digestibility use highly simplified protein solutions, without considering the impact of the food structure. Nonetheless, protein digestibility can be affected by various aspects of the food structure. The molecular conformation of the protein, the intermolecular interactions between proteins, and the food microstructure are some of the factors that have been investigated (Golding, 2019).

Both of the major soy protein fractions (glycinin and β-conglycinin) share a high content of β-sheet conformation and a relatively low amount in α-helix (Damodaran et al., 2007). These conformation characteristics are common to almost all legume proteins. Carbonaro and co-workers showed that the β-sheets structures, which are hydrophobic, are linked to lower solubility and consequently lower protein digestibility (Carbonaro et al., 1997, Carbonaro et al., 2015).

The effect of soy protein food structures on protein digestibility is largely unexplored, there is only a small number of studies available addressing this topic. Rui et al. (2016) compared the protein digestibility of unpressed SPI gels induced by different coagulation mechanisms; acidification, salt and enzymatic gelation. They found that the proteolysis was significantly lower in the gels induced by enzymes and this was explained by the covalent bonding induced by the used enzyme, transglutaminase. It was also found that the harder the soy protein gels the higher the protein digestibility (Rui et al., 2016). In another study, it was demonstrated that the gel particles of soft tofu were disintegrated more easily and quickly than the firm tofu. These two types of tofu varied significantly on their textural properties (Kozu et al., 2014).

In a recent study, Reynaud et al. (2020) found that soy proteins from a soya juice matrix were more prone to *in vitro* proteolysis than fresh tofu. The tested soy protein matrices differed in both protein concentration (∼ 4 higher in tofu than juice) and structure (solid versus liquid). In general, the authors suggested that both the food matrix and the protein concentration could influence the *in vitro* proteolysis results (Reynaud et al., 2020). According to Luo et al. (2017) solid or dense matrices inhibit the diffusivity of pepsin and as a consequence reduces their proteolysis rates compared to liquid matrices. Moreover, the protein content affects the substrate to proteases ratio during *in vitro* digestion, which might affect the proteolysis results. Another study revealed β-conglycinin in tofu remained intact after 2 hours of gastric processing (in the presence of pepsin), while it was completely digested in soymilk (Adachi et al., 2009). The same authors found that β-conglycinin was the causative allergen of food-dependent exercise induced anaphylaxis and that the food matrix could affect an allergens response to patients (Adachi et al., 2009). Overall, the composition and physical characteristics of the soy protein matrices might affect their protein

digestibility. The latter is of great importance for people consuming soy products as a main source of proteins.

2.3 Phenolic acids

Phenolic acids are a subclass of a larger class of compounds referred to as phenolics, there are at least 8000 naturally occurring compounds that belong to this category (Stalikas, 2010). Phenolics are aromatic secondary metabolites of plants and essential for their interaction with the environment, reproductive strategy and their defence mechanisms (Cheynier et al., 2013).

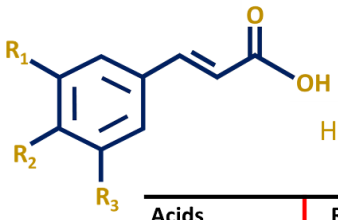
The common structural feature of the phenolics is the hydroxyl substituted, aromatic benzene ring. Other subclasses of phenolics are the following *(a)* flavonoids, *(b)* isoflavones, neoflavonoids and chalcones, *(c)* flavones, flavonols, flavanones and flavanonols, *(d)* flavanols and proanthocyanidins and lastly *(e)* anthocyanidins (Tsao, 2010). Flavonoids alone account for roughly two-thirds of the total dietary phenolics in plant-based foods while phenolic acids account for the remaining one-third (Kumar and Goel, 2019). Generally, phenolic compounds have been associated with various health benefits, such as antioxidant, anti-inflammatory, anti-viral, anti-bacterial and anti-allergenic properties. Additionally, they are known to reduce risk of cancer, heart disease and diabetes. In this thesis, however, we will only focus on the phenolic acid subclass.

2.3.1 Chemical structure and occurrence in nature

Phenolic acids are phenols possessing a carboxyl group with one or more hydroxyl (-OH) and/or methoxy ($-OCH_3$) groups attached to a benzene ring. They can be divided into two groups depending on the basic carbon framework, the hydroxycinnamic (C_6-C_3) and hydroxybenzoic (C_6-C_1) acids. There are a variety of phenolic acid structures found in plants and they differ only in the number and the position of the -OH and $-OCH_3$ on the benzene ring (**Figure 2.6**). They are also found in dimer, trimer or multimer forms.

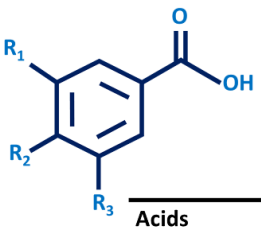
Hydroxycinnamic acids (HCA) are more abundant than hydroxybenzoic acids (HBA) and they are usually found esterified with quinic acid, tartaric acid or

carbohydrate derivatives (Lafay and Gil-Izquierdo, 2007). Chlorogenic acid, a well-known phenolic acid present in coffee, is an ester of caffeic and quinic acid. The most common phenolic acids in foods are caffeic, ferulic and coumaric acids (Clifford, 2000). Organic acid and glucose esters of phenolic acids naturally occur in fruits and vegetables (Herrmann and Nagel, 1989, Winter and Herrmann, 1986), mushrooms (Heleno et al., 2015), tea and coffee (Hodgson et al., 2004), cocoa (El-Seedi et al., 2012), as well as alcoholic beverages such as wine and beer (Stalikas, 2010). Herrmann and Nagel (1989) reviewed the occurrence and the concentration levels of both HCAs and HBAs derivatives in fruits, vegetables and herbal dry spices. Moreover, ferulic acid derivatives are the dominant phenolic acids in wheat bran (Laddomada et al., 2015) and therefore in whole grains (Călinoiu and Vodnar, 2018). Depending on the individuals' diet the consumption of cinnamates is estimated between 25 to 800 mg daily (Clifford, 1999).



Hydroxycinnamic acids (HCAs)

Acids	R_1	R_2	R_3
p-Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH_3	OH	H
Sinapic	OCH_3	OH	OCH_3



Hydroxybenzoic acids (HBAs)

Acids	R_1	R_2	R_3
Gallic	OH	OH	OH
Protocatechuic	OH	OH	H
Vanillic	OCH_3	OH	H
Syringic	OCH_3	OH	OCH_3

Figure 2.6 Basic phenolic acid structures found in plants. Adapted from Shahidi and Ambigaipalan (2015).

2.3.2 Bio-functional properties

Phenolic acids can act as antioxidants, this is due to the reactivity of the benzene ring and the hydroxyl groups attached to it (Kumar and Goel, 2019). An antioxidant can protect macromolecules (nucleic acids, protein, lipids) from oxidative damage by donating an electron to free radicals and thereby stabilising them (Leopoldini et al., 2004). The oxidation of a lipid molecule to hydroperoxide can be seen in **Table 2.2**. The oxidation process might be responsible for various diseases including cancer, diabetes, atherosclerosis, heart and neurodegenerative diseases (Pizzino et al., 2017).

There are two possible mechanisms of antioxidant action: *(A)* H-atom transfer in which the antioxidant provides a proton and to the radical, then the antioxidant is being transformed to a stable radical. *(B)* One-electron transfer, the antioxidant provides an electron to the free radical becoming itself a radical cation (Wright et al., 2001). Both modes of action result in the following structures.

Table 2.2 Oxidation pathway and free radical R• generation, lipid molecules (R−H) are converted into lipid hydroperoxide (ROOH) (Wright et al., 2001).

$RH \rightarrow R^{\bullet}$	Initiation
$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet}$	Addition of oxygen
$RO_2^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$	H-atom exchange

$RO_2^{\bullet} + \text{Antioxidant-OH} \rightarrow ROOH + \text{Antioxidant-O}^{\bullet}$

The antioxidant activity of phenolic acids depends on their structural features, which are the number of hydroxyl and methoxy groups, and the position of the hydroxyl groups on the aromatic ring; *ortho, para, meta* (Rice-Evans et al., 1996). The carboxylate group on HBAs has a negative effect on their antioxidant properties which makes the HCAs more effective.

Recently, Vinayagam et al. (2016) reviewed the antidiabetic effect of simple phenolic acids. The phenolic acids that might affect glucose metabolism are gallic, protocatechuic, ellagic, syringic, salicylic, caffeic, p-coumaric, sinapic, cinnamic, chlorogenic, and rosmarinic acid. Some of the antidiabetic mechanisms that were proposed are the inhibition of enzymes involved in carbohydrate digestion, stimulation

of insulin secretion from the pancreatic β-cells, modulation of glucose release and output from the liver, and activation of insulin receptor and glucose uptake in insulin-sensitive tissues (Vinayagam et al., 2016).

Specific phenolic acids have been found to exhibit a selective anti-microbial activity towards bacterial pathogens found in the intestinal tract, such as E. coli and coliform bacteria (Si et al., 2006). Phenolic acids are present in higher concentrations in the colon and faecal water (Jenner et al., 2005), possibly due to metabolic degradation of larger polymers such as flavonoids and proanthocyanidins (Déprez et al., 2000, Jenner et al., 2005). Moreover, a study showed that there is a structure-activity relationship between phenolic acids and their antimicrobial effect. Some phenolic acids were able to inhibit the growth of both pathogenic and beneficial bacteria, methyl and butyl esters seem to be more potent (Cueva et al., 2010).

Some recent studies have shown that phenolic acids have a positive role in numerous neurodegenerative diseases such as neuroinflammation, apoptosis, epilepsy, hyperinsulinemia-induced memory impairment, hearing and vision disturbances and Parkinson's disease. Interestingly, low concentration (<50 μmol/L) of phenolic acids were adequate for their bioactivity. Two recent reviews are available related to the benefits of phenolic acids on Alzheimer's disease (Szwajgier et al., 2018) and their neuroprotective role in general (Szwajgier et al., 2017).

Lastly, HCAs showed promising results regarding their cytoprotective effects in animal studies. The studies mainly focused on colon, gastric, liver, breast and lung cancer cell trials and the results suggested that HCAs can have an inhibitory effect on cancer invasion and metastasis (Weng and Yen, 2012). A review of the anti-cancer properties of HCAs was published by Rocha et al. (2012)

2.4 The fate of dietary phenolic acids

The route that any xenobiotic substance follows after entering the human body is known by the acronym LADME which stands for liberation, absorption, distribution, metabolism and excretion (Caldwell et al., 1995). The gastrointestinal system is the site of absorption for any orally administrated substance. In general, most bioactive compounds can be released either in the mouth after mastication or in the stomach during gastric digestion. Then, the compound will be transferred to the small intestine, which is the main site of absorption of most substances (Blake et al., 2019). A molecule is absorbed when it can cross the epithelial cell membranes and eventually enter the systemic circulation. Nevertheless, the crossing of the cell membranes can be complicated because it depends on the structural characteristics of the molecule.

There are two major mechanisms of cell crossing: ***(a)*** passive transport or diffusion due to concentration gradient and ***(b)*** active transport through a proteinic carrier (Blake et al., 2019). The occurring mechanism depends on the size, lipophilicity and charge of the molecule (Caldwell et al., 1995). Eric Lipinski attempted to correlate the structural and physical properties of a molecule so that poor absorbance could be predicted. He came up with some simple rules that became known as the "rules of five" because each rule involves a multiple of 5 (**Table 2.3**) (Lipinski et al., 2001).

Table 2.3 The molecular properties of molecules that are more likely to be absorbed by cell membranes, "rule of five" (Lipinski et al., 2001)

Molecular property	Maximum value
Molecular weight (MW)	500
Lipophilicity (LogP)	5
Hydrogen Bond Acceptors (HBA)	10
Hydrogen Bond Donors (HBD)	5

After a molecule is absorbed i.e. reaching systemic circulation, it will be distributed to the body tissues. Then, any molecule including phenolics will be extensively metabolised by phase II enzymes in the liver. The main purpose of the metabolism is the increase of hydrophilicity and thus the easier elimination. This is occurring through conjugation reactions and more specifically: methylation,

sulphation and glucuronidation (Heleno et al., 2015). Lastly, the metabolites are excreted into the urine via the kidneys. Some of the phenolics, however, can be re-excreted to the small intestine through the bile, then transferred to the colon, catabolised from gut microbiota and finally excreted to faeces (enterohepatic circulation) (Ferreira et al., 2017) **(Figure 2.7)**.

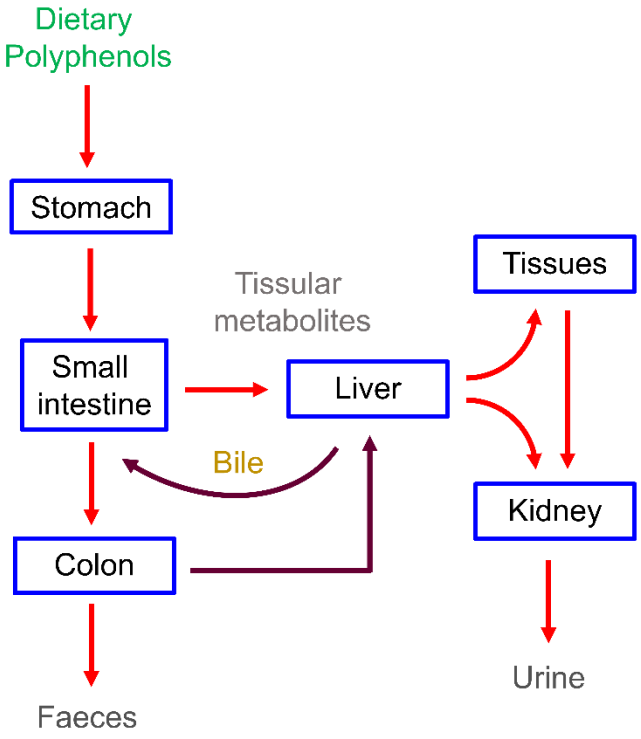


Figure 2.7 Fate of dietary phenolics in the human body after consumption. Adapted with permission from Scalbert et al. (2002).

Bioavailability is a term that is often used to describe the fraction of a nutrient in food that is used by the body for physiological functions (Bohn, 2014a, Fairweather-Tait and Southon, 2003). This term is very difficult to be quantified and various techniques have been proposed such as *in vitro* cell culture techniques, balance studies and bioassays that directly measure the molecule of interest or its metabolites in tissues, blood, or urine (Fairweather-Tait and Southon, 2003). The food structure and the physicochemical form of the phenolics are important factors for determining bioavailability (Bohn, 2014a, Fairweather-Tait and Southon, 2003).

For a molecule to be bioavailable, it needs to first become bioaccessible. Bioaccessibility describes the fraction of a molecule that is liberated from the food matrix during gastrointestinal conditions and it is available for absorption (Rein et al.,

2013). Bioaccessibility can be influenced by various factors such as the composition and physicochemical properties of the food matrix (Rein et al., 2013).

2.4.1 Liberation of phenolic acids from the food matrix

The food matrix seems to play a significant role in the release and thus the absorption of the phenolic acids. Studies have shown that caffeic and ferulic acid can be absorbed after consumption of tomatoes (Bourne and Rice-Evans, 1998), beer (Nardini et al., 2006), prunes, bark and herbal extract (Graefe and Veit, 1999, Virgili et al., 2000) but not from whole bran cereals (Kern et al., 2003). This is mostly because of the structure of phenolic acids present in cereals. They are mainly esterified to the cell wall of the plant which limits their release and bioavailability. Food bioprocessing can improve the bioaccessibility of phenolic acids by increasing their free form. Some successful examples mentioned in the literature are the use of enzymes such as esterases, xylanases and cellulases (Bento-Silva et al., 2019). Hole et al. (2012) increased the content of free phenolic acids significantly in whole grain barley and oat groat after fermentation with lactic acid bacteria. A similar approach was followed by de Queirós et al. (2021), where a protease treatment was used in order to increase the extractability of isoflavones from soy flour. Also, the use of enzymes can facilitate the biotransformation of glycoside isoflavones into aglycones, which are their biologically active forms in soymilk and can lead to enhanced anti-inflammatory properties (Hiramatsu et al., 2020, de Queirós et al., 2020). Other strategies that could increase the amount of endogenous unbound phenolic acids are germination (seed sprouting) or thermal and hydrothermal processing such as roasting, boiling and extrusion cooking. These strategies were discussed in a recent review by Shahidi and Yeo (2016).

Finally, another factor that could affect the bioaccessibility of phenolics in the GI tract is their interaction with salivary proteins during mastication (de Freitas and Mateus, 2012) which results in the perception of astringency. Evidence that small phenolics such as PA can interact with salivary proteins is scarce. A recent study, however, revealed that wine PA (gallic acid, protocatechuic acid, caffeic acid, p-coumaric acid) can interact with a proline-rich peptide fragment ($IB7_{12}$) (Ferrer-Gallego et al., 2017).

2.4.2 Absorption of phenolic acids

Phenolic acids in their natural form (esterified with glucose, organic acids or bound in fibre matrix) are poorly bioavailable (Bento-Silva et al., 2019). It has been suggested that the glucosides are converted to aglycon forms by brush border enzymes (Bohn, 2014a). The free phenolic acid forms, on the other hand, appear to be released and absorbed better and faster than the conjugated forms (Bohn, 2014a). However, there is still debate about the site of absorption of free phenolic acids. On the one hand, rat studies have indicated that free phenolic compounds are absorbed in the stomach (Konishi et al., 2006). This was mainly suggested due to the rapid postprandial appearance of the compounds in the plasma. In contrast, research that studied the availability of apple polyphenols using healthy ileostomy patients showed that some of the polyphenols, including phenolic acids, reach the small intestine unmetabolized (Kahle et al., 2005). One more controversy in the literature is related to the absorption mechanism. Konishi and co-workers used human intestinal caco-2 cells and rat trials to study the absorption mechanism of various phenolic acids (Konishi et al., 2005, Konishi et al., 2003, Konishi and Shimizu, 2003). According to the caco-2 cells, the absorption mechanism is structure-dependent. Coumaric and ferulic acids were absorbed via facilitated transport by monocarboxylic acid transporters (MCTs), while gallic, caffeic, and rosmarinic acid were absorbed by paracellular passive diffusion (Konishi et al., 2003, Konishi and Shimizu, 2003). A factor affecting phenolic acid absorption is the structure of the molecule. For instance, caffeic acid has a good absorbance in the human body, but its ester with quinic acid, widely known as chlorogenic acid has low absorption and is thought to end up in the colon (Bento-Silva et al., 2019).

2.4.3 Distribution, metabolism, and excretion of phenolic acids

Following transport in the bloodstream, polyphenols can be found distributed in most tissues and can cross the blood-brain barrier (Bohn, 2014a). Phenolic acids such as gallic, protocatechuic acid and benzoic acid derivatives have been detected in small amounts in rat brains after administration of plant extracts (Del Bo et al., 2010, Margalef et al., 2015, Zhang et al., 2011).

Phenolic acids are extensively metabolized in the human body (Heleno et al., 2015, Scalbert et al., 2002). Nardini and co-workers studied the metabolism of phenolic acids from beer and white wine. They found that HCA's present as tartaric acid esters in white wines are metabolized into glucuronide and sulphate conjugates, while the parent compounds were practically undetectable (Nardini et al., 2009). Similarly, after the consumption of beer, the predominant forms of ferulic, vanillic and caffeic acids found in plasma were the glucuronate and sulphate metabolites (Nardini et al., 2006). The authors also found a relationship between the extent of conjugation and the chemical structure of the phenolic acids. It seems that the conjugation is favoured to the dihydroxy over monohydroxy phenolic acid derivatives (Nardini et al., 2006). In another study in which cranberry juice was given to healthy men, many phenolic metabolites were detected in plasma and urine (Feliciano et al., 2016). Cranberry juice is rich in phenolic acid derivatives with benzoic, p-coumaric and chlorogenic acid being the most abundant of this phenolic subclass. The results showed at least 24 metabolites of cinnamic acid derivatives, including sulphates and glucuronides of ferulic, caffeic, isoferulic, and their dihydro derivatives. Also, a high concentration of caffeic acid was detected which was a result of intestinal esterase activity on chlorogenic acid (Feliciano et al., 2016). These studies indicate that the disposition of phenolics is a very complicated topic and the extent of metabolism should be considered. Besides, more studies should be done using less complicated phenolic mixtures, to clarify the parent compounds of the metabolites. For instance, polyphenols such as flavonoids and proanthocyanidins can be degraded to phenolic acids and further metabolized. Furthermore, there is evidence that the bioactivity of the metabolites is different from the one found in the parent compounds (Heleno et al., 2015).

Phenolic compounds, depending on the degree of polymerization and their lipophilicity, will either excreted in bile or urine through the kidneys. Simple phenolics such as phenolic acids are usually excreted in the urine. The recovery percentage in urine is often used to study bioavailability (Pérez-Jiménez et al., 2010). This is mainly due to the availability of renal excretion data which allows the comparison of different polyphenols levels present in diets (Scalbert et al., 2002). Caution is needed, however, since the metabolites have not been considered in many studies (Bohn, 2014a, Heleno et al., 2015). Free phenolic acids exhibit a higher urinary recovery than other phenolic substances. A review of 97 bioavailability studies showed that the recovery of gallic acid in urine was between 36 to 39 % from both tablets and tea formats (Manach et al., 2005). Soy isoflavones also resulted in high recoveries ranging from 5 to 62 % depending on the food matrix (Manach et al., 2005). Hydroxycinnamic acids such as caffeic and ferulic acids varied from 3 to 60 % again depending on the product formulation, while chlorogenic acid was much lower 0.3 to 6 % (Manach et al., 2005). Anthocyanins had the lower recoveries among the phenolics which counted for barely 0.1 % (Bohn, 2014a). The number of studies for phenolic acid bioavailability or absorption is very small and it is usually focusing on caffeic, ferulic and chlorogenic acid. The food matrix and structural form seem to be of great importance, although it is still under-researched.

2.5 Study of digestion using *in vitro* systems

After the food is consumed it is converted into a bolus and directed to the GI tract for further digestion. As can be seen from **Figure 2.9**, the pH conditions and geometry of the different parts of the GI tract vary. The onset of digestion for proteins and lipids is in the stomach where proteins are hydrolysed into peptides by the action of pepsin and hydrochloric acid (pH 1.0-2.5). Moreover, triglycerides are hydrolysed into diglycerol and free fatty acids (short and medium chain), by the action of gastric lipase. The digestion of starch starts earlier, during mastication due to the presence of amylases in the saliva (Boisen and Eggum, 1991).

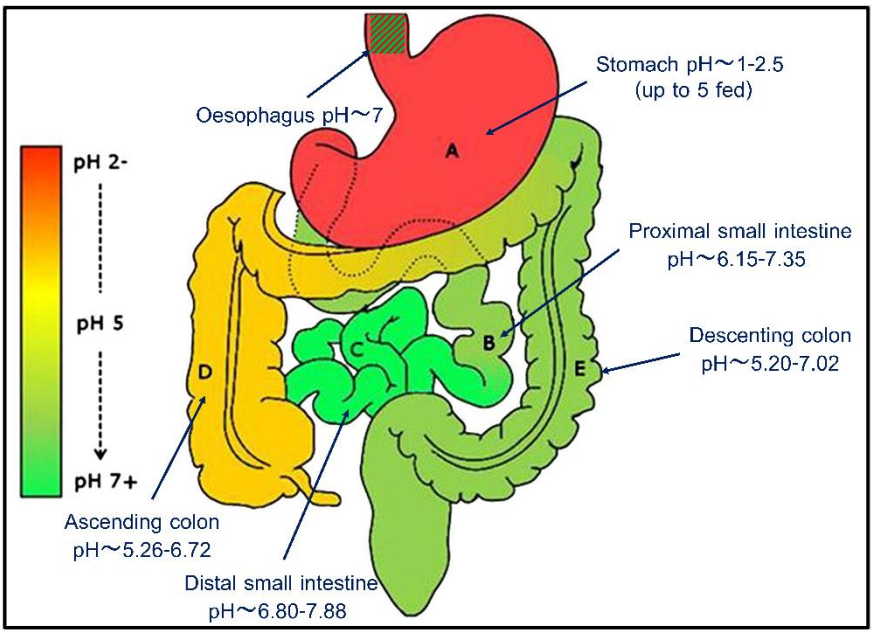


Figure 2.8 Parts of GI tract and pH variation. Adapted with permission from (Cook et al., 2012).

The main site of digestion for all the macronutrients is the small intestine (pH 6-8). The products released in the stomach (peptides, diglycerides and starch polymers) are subjected to further hydrolysis by the action of more specialised enzymes found in the pancreatic juices, lumen and enterocytes of the small intestine (Boisen and Eggum, 1991). The products of the intestinal processing are 1) free amino acids and di or tri-peptides originated by proteins, 2) free fatty acids and glycerol originated by triglycerides and finally, 3) di- and mono-saccharides originated by starch.

Several *in vitro* digestion protocols have been developed to study the digestibility of macronutrients in foods (Dupont et al., 2019). While it is understood that human digestion can never be completely mimicked with *in vitro* models, they offer several advantages such as rapid screening of samples, cost-effectiveness, they are less laborious and do not need ethical approval (Boisen and Eggum, 1991, Minekus et al., 2014). In 2014, the international network INFOGEST proposed a general standardised static protocol (Minekus et al., 2014) which was recently improved (Brodkorb et al., 2019). The authors aimed to harmonise the *in vitro* digestion models by identifying key digestion factors from human in vivo data. Some of the harmonised parameters of the INFOGEST protocol are the salt concentration, pH, digestion time and activity of digestive enzymes, among other factors (Minekus et al., 2014). The INFOGEST protocol has been extensively used in recent years by numerous groups around the world, which has allowed the comparison of *in vitro* digestion results from a variety of food matrices.

In general, *in vitro* digestion models can be divided into static and dynamic. Static models are simple, and they simulate mainly the chemical conditions of the GI tract. Also, they are useful to study the digestion of a single substance or simple meal (Minekus et al., 2014). The *in vitro* dynamic models are more sophisticated and they allow the simulation of both chemical and physical procedures such as shear, digesta emptying, and regulation of enzyme concentration and pH over time (Dupont et al., 2019). Recent studies have shown that there is a good correlation in the endpoints of milk protein digestion between the in vitro INFOGEST protocol with in vivo human and animal systems (Egger et al., 2017, Sanchón et al., 2018).

Some limitations of the *in vitro* digestion systems are that anatomical and geometrical characteristics of the GI tract are not considered (Li et al., 2020a). Also, under physiological conditions, the digestive enzymes involved in macromolecules digestion will adapt to any changes in substrate, such as enzymatic activities (Corring, 1980, Corring et al., 1989).

2.6 Soy protein food matrices as a delivery vehicle of bioactives

The release (bioaccessibility) of native or added phenolics have been studied by various food matrices. Some of the existing food formats that have been fortified with phenolics are mainly dairy products, such as milk (Lamothe et al., 2014, Moser et al., 2014), yoghurt (Chouchouli et al., 2013, Georgakouli et al., 2016, Helal and Tagliazucchi, 2018, Karaaslan et al., 2011, Lamothe et al., 2014, Petrotos et al., 2012), cheese (Giroux et al., 2013, Han et al., 2011, Helal et al., 2015, Lamothe et al., 2016, Lamothe et al., 2014) and ice cream (Çam et al., 2014). The food structure can either facilitate the release of polyphenols or impede it

Due to the industrial significance of soy, the gelation of soy proteins has attracted much attention over the years. Although many studies are focusing on the complexation of fat-soluble bioactives (curcumin) into soy protein nanoparticles (Tang, 2019), there are only a few articles that study the potential use of SP gels as a delivery system. Maltais and co-workers encapsulated riboflavin (0.05 mM) into tableted cold set SPI hydrogels induced by different concentrations of $CaCl_2$ which resulted in a distinct microstructure, filamentous and particulate. They studied the effect of riboflavin on the mechanical properties of gels and its release profile during GI processing (Maltais et al., 2009, Maltais et al., 2010). Results showed that the addition of riboflavin deteriorated the mechanical properties of the gels, because of crystal formation. Interestingly, filamentous gels delayed the release of the bioactive during intestinal conditions. In contrast, particulate gels gave a more rapid release, which was explained by the higher porosity of the latter which favoured the diffusion of the bioactive (**Figure 2.9**) (Maltais et al., 2009).

In another study, Maltais et al. (2010) compared riboflavin's release profile from cold set SPI filamentous hydrogels and SPI tablets produced by lyophilized filamentous gels. Despite the gels' matrix, riboflavin released faster under intestinal conditions (pH 7.5) compared to gastric conditions (pH 1.2) (Maltais et al., 2010). The authors found that gels' swelling was the principal mechanism of riboflavin release from tablets at intestinal conditions, while bioactive-protein interactions reduced this release at gastric conditions. Both tablet and hydrogel were digested completely within 6 hours of intestinal conditions in the presence of pancreatin. Although the end riboflavin release was 100 % in both systems, the hydrogel showed a slower release than the tablet (Maltais et al., 2010).

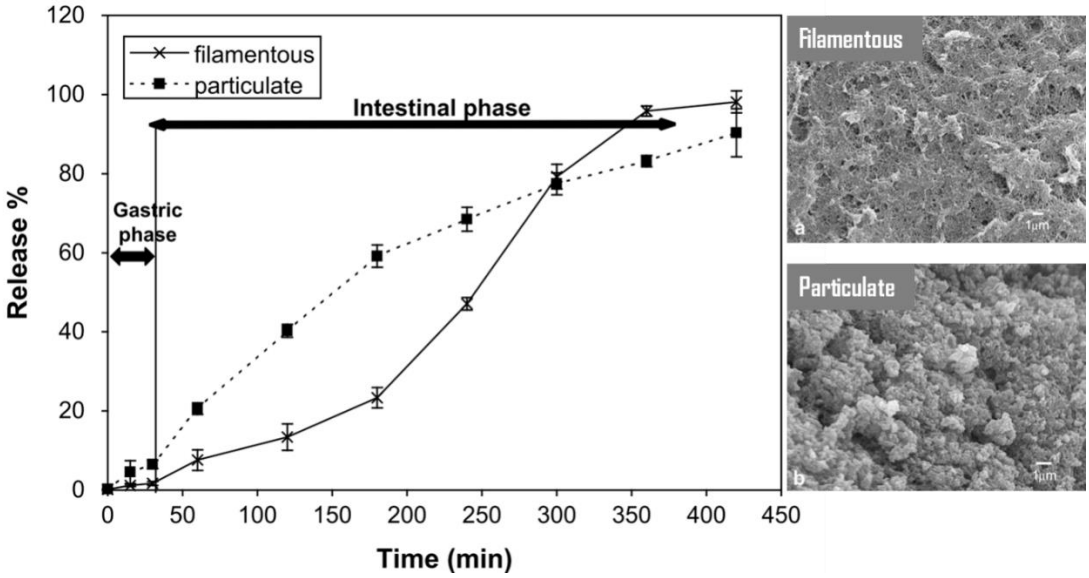


Figure 2.9 Impact of GI conditions on riboflavin release from filamentous and particulate gels. Adapted with permission from (Maltais et al., 2009).

These two studies provide important information about the behaviour of different types of SPI gels during digestion (Maltais et al., 2009, Maltais et al., 2010). Unfortunately, these studies aimed to use SPI gels as tablets for drug delivery, and, therefore, the mastication process was excluded, which is important in the case of food products. Moreover, only riboflavin, which is a hydrophobic compound was tested; thus, the behaviour of hydrophilic compounds within soy protein gels is largely unknown. Lastly, SPI gelation can be induced by other mechanisms, such as acidification, salt bridges and enzymatic cross-linking in combination with high temperature (if non-heat labile bioactives are tested). So, there is a range of different conditions that give different gel microstructures and could be tested as potential delivery vehicles of bioactive compounds.

2.7 Protein-phenolics interactions

Studies have shown that phenolics present in foods can interact with macromolecules; proteins, carbohydrates and lipids under certain conditions (Le Bourvellec and Renard, 2012). In this study, the gels formed were consisted almost exclusively of proteins and thus it is useful to review how the potential binding of the added phenolics can affect the protein matrix.

The protein-phenolic interactions can be either reversible or irreversible depending on the nature of the interactions that occur; non-covalent or covalent. Such interactions might affect many aspects of the foods such as sensory characteristics, reduction of vanilla flavour and astringency perception, for instance (Haslam et al., 1988). Moreover, the colour could be changed into darker hues because of oxidation and condensation reactions. Undesirable turbidity and colloidal haze have been observed in products such as beer and fruit juices (Siebert, 1999). Another consequence of the protein-phenolic interactions is the reduction of the nutritional value because the amino acid that participates in the interactions might be less bioavailable (Rawel et al., 2002b). On the other hand, the beneficial properties of the phenolics such as antioxidant activity might be reduced. Finally, the binding of phenolics with proteins might alter the physicochemical and techno-functional characteristics of the proteins such as solubility, isoelectric point, gelation, and emulsification properties (Zhang et al., 2020).

A prerequisite for the formation of covalent interactions between proteins and phenolics is the oxidation of phenols to quinones. The oxidation of phenolics is possible either enzymatically or chemically. Enzymatic oxidation is possible after the release of phenolics from vacuoles of the cells, due to disruption of the food matrix and their subsequent interaction with oxygen and oxidative enzymes such as polyphenol oxidases, which are physically present in foods. The chemical oxidation takes place under alkaline conditions (i.e., pH 9.0) in the presence of oxygen. The highly reactive quinones can interact covalently in two ways. Firstly, they undergo condensation, resulting to higher molecular weight (MW) pigments (tannins) (Ozdal et al., 2013) which can further react with proteins (Hagerman, 1992). Secondly, their phenolic ring can react covalently with sulfhydryl and amino groups of proteins to form C-N or C-S bonds (Arts et al., 2002). The presence of more sulphur-containing amino acid in a protein (cysteine) will enhance the binding of quinones due to covalent

bond (C-S) thus, the composition of the protein is important (Hassan, 2013). The topic of covalent interactions between phenolics and proteins has been reviewed by Kroll et al. (2003).

Non-covalent interactions are the most common interactions found in nature and they include hydrophobic-, van der Waals-, ionic, hydrogen and π- bonding (Zhang et al., 2020). The hydroxyl groups of the phenolics form hydrogen bonds with the carboxyl group of the amino acid residues (Ozdal et al., 2013). Hydrophobic interactions are more stable than the ionic and hydrogen interactions and they can be formed at high temperatures (Sastry and Rao, 1990).

There is only a limited number of articles studying the interactions between soy proteins and phenolic compounds. Most studies are performed using only tea, coffee, and chocolate as phenolic compound sources and milk as the protein source (Ozdal et al., 2013). Gan et al. (2016a) assessed the interactions between β-conglycinin (7S) and four phenolic acids, it was found that the binding is structure-dependent. According to fluorescence spectra results, caffeic and gallic acid bind more tightly to β-conglycinin than p-coumaric acid and chlorogenic acid. This can be explained by the larger number of –OH groups on ring A (Gan et al., 2016a). A recent study showed a structure-affinity relationship between PA and β-lactoglobulin (BLG) after studying 71 HBAs (Wu et al., 2018). The authors found that methylation of -OH groups (except C_3 position), enhanced the binding affinity towards BLG. In general, methylation increases the hydrophobicity and enable PA to penetrate into Trp-rich hydrophobic regions of BLG (Cao et al., 2013). In addition, Wu et al. (2018) found that the presence of-OH groups in -C_2 and -C_4, reduced, while in -C_3 increased the binding affinity towards BLG. The latter could be explained by either hydrophobic or hydrophilic interactions. More studies are needed to demonstrate the structure-affinity relationship between PA and SP since structural and compositional differences in proteins will certainly affect the binding.

Regarding the effect of PA-SP interactions on protein digestibility, Gan et al. (2016a) found that there was not any effect on 7S *in vitro* proteolysis. On the other hand, Rawel et al. (2002a) found that phenolic compounds react with soy glycinin (11S) by binding their lysine, tryptophan, and cysteine residues, which can have nutritional consequences. In this study, the binding affinity was estimated by the total amount of the protein groups that were blocked from phenolics and in this respect,

caffeic acid and quercetin were the most reactive compounds (Rawel et al., 2002a). It was also observed that the isoelectric point of the complexes was shifted to lower pH values (from pH 5 to 3.5). In the case of further cross-linking between the proteins and phenolics, the net charge can be changed which can directly influence the solubility of the derivatives. Finally, the protein surface becomes more hydrophilic due to alteration in secondary and tertiary structure (Rawel et al., 2002a). Thus, differences in emulsion and gelation properties could be observed.

Rodríguez-Roque et al. (2013) evaluated the changes of phenolic compounds during *in vitro* digestion in soy products. The levels of total phenolics, isoflavones and total antioxidant activity in soymilk were compared, during gastric and intestinal processing. They found that the release of all phenolic compounds from soymilk was improved by 35% during gastric digestion (Rodríguez-Roque et al., 2013). However, the concentration of those compounds was significantly dropped during intestinal processing. The authors suggested that phenolics form complexes with proteins, which are low in solubility and/or large in molecular weight and as a result they cannot cross the dialysis membrane used for the experiments.

2.8 Gaps in the literature

Although phenolic acids are the most abundant phenolic structures in nature, they are largely unexplored, especially when delivered in a protein food structure. Some of the gaps identified in the literature are related to their bioaccessibility, bioavailability and metabolism in the human body. Nevertheless, this study focused only on the following technological topics:

- Soybean and soy protein gels are food matrices that naturally deliver isoflavones in sufficient amounts in the human body. However, soy proteins have not been studied as potential delivery systems of other phenolic structures.
- It is unclear whether the different soy protein gels (silken and firm tofu) are equally digestible as the SPIs that are usually used in nutritional studies. This is important information for people consuming plant proteins.

Chapter 3

Material and Methods

3.1 Materials

SPI (Wilpro G300) was purchased from Wilmar International (Singapore). The coagulants; glucono-δ-lactone (GDL), magnesium sulphate ($MgSO_4$ x $7H_2O$), the *in vitro* digestion materials; pepsin (P7000), pancreatin (P7545) and bile extract (B8631) that were of porcine origin, the pepsin inhibitor pepstatin A (P5318) and the phenolic acids, protocatechuic acid (PCA) (37580, purity ≥ 97 %) and o-coumaric acid (o-CMA) (H22809, purity 97 %), p-coumaric acid (p-CMA) (H52406, purity 98 %), m-coumaric acid (m-CMA) (H23007, purity 99 %), vanillic acid (VNA) (94779, purity 97 %), gallic acid (GLA) (G7384, purity 98.5 %), caffeic acid (CFA) (C0625, purity 98 %), ferulic acid (FRA) (128708, purity 99 %) were purchased from Sigma-Aldrich Co. (New Zealand). The trypsin inhibitor AEBSF hydrochloride (ab141403) was obtained from abcam PLC (New Zealand). Ethanol, methanol and trifluoroacetic acid were purchased from Merck (New Zealand).

3.2 Methods

3.2.1 Raw material analysis

3.2.1.1 SPI composition

The protein content of the SPI powder was determined using the Kjeldahl method (Helrich, 1990) with a conversion factor of 6.25 and the moisture content determined by oven-drying at 105 °C for 24 hours.

3.2.1.2 Determination of ζ-potential

A series of soy protein dispersions (0.01 % w/w) was prepared within the pH region of 2 to 9. After the pH was adjusted with HCL 0.1 M the dispersions were centrifuged at 4000 g for 15 min and the supernatant was collected to determine the electric charge. The ζ-potential was measured using a Zetasizer (Nano ZS, Malvern Instruments Ltd, UK) at 25 °C using a DTS1060 cell.

3.2.1.3 Protein solubility

A series of soy protein dispersions (0.01 % w/w) was prepared within the pH region of 2 to 9. After the pH was adjusted with HCL 0.1 M the dispersions were centrifuged at 4000 g for 15 min and the supernatant was collected to determine the protein solubility using the Bradford reagent according to the Sigma-Aldrich technical bulletin, 96 well plate assay protocol (Sigma-Aldrich). Briefly, 5 μL of Bovine serum albumin (BSA) standards (0.1–1.4 mg/ml), unknown samples and blank samples were mixed with 250 μL of Bradford reagent (B6916). The mixtures were incubated for 5 to 30 min at room temperature and then the absorbance was measured at 595 nm. The soluble protein concentration of the unknown samples was expressed as BSA equivalents.

3.2.1.4 Gel electrophoresis

Tricine - sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed on SPI dispersions. The dilutions were mixed with denaturing tris-tricine load buffer (pH 6.8 with SDS/Dithiothreitol). The gel buffer had a pH of 8.45 and the staining was performed using 0.04 % (v/v) colloidal Coomassie Blue G-250. The final soluble protein mass was 10 μg. For the image analysis, the software Image-J (IJ 1.46r, NIH, USA) was used, where the coloured image (RGB) was converted to 8-bit and binarized, then the density of the bands was measured and divided by the total band density.

3.2.2 Preparation of soy protein gels

In general, all gels were heat-induced and the main steps involved were the following: 1) heating of the SPI dispersion to unfold the protein (pre-treatment step-T1), 2) addition of PA or the equivalent volumes of ethanol in the case of the control samples, 3) addition of a coagulant and 4) heating for the formation of the gels (T2). The SPI concentration that was selected in this thesis was 4.5 % w/w because is close to the protein content of the silken tofu (unpressed gel) (Nutririondata, 2021). Although the gelation principles were the same in all chapters, there was a need for optimizing the steps leading to SPI gelation, which was the objective of **Chapter 4**. Therefore, factors such as pre-treatment step temperature (T_1), gelation temperature (T_2), types of coagulants and their concentration as well as PA concentration were tested in **Chapter 4**.

3.2.2.1 Unpressed SPI gels: silken tofu model system

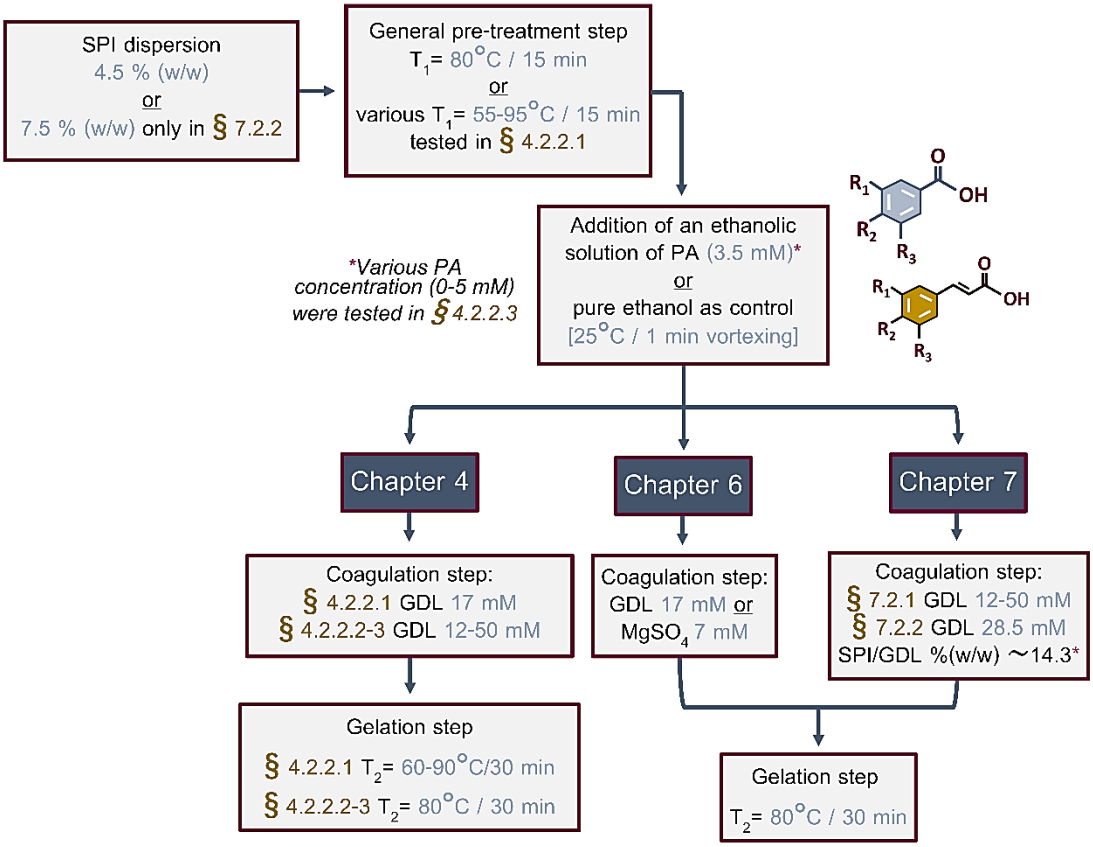


Figure 3.1 Overview of the gelation conditions for the production of unpressed gels.

A two-step heating gelation method was used for the gelation of the SPI to form silken type tofu. Initially, 250 g of 4.5 % (w/w) SPI dispersion was heated in a water bath at 80 °C for 15 min (**pre-treatment step-T1**) to unfold the soy protein (Bryant and McClements, 1998, De Wit, 1990, Peng et al., 2016) and subsequently cooled at room temperature until a final temperature of approximately 25 °C. Various pre-treatment temperatures, between 55 to 95 °C, were tested in **section 4.2.2.1**.

Then, 15 mL of SPI dispersion was distributed in plastic tubes of 20 mL. A volume of ~ 0.3 mL a concentrated PCA or CMA (-o, -m, -p) or CFA or FRA or GLA or VNA ethanolic solution (300 mmol/Kg) was added to the mixture and it was vortexed for 1 minute at ambient temperature. Fresh, concentrated GDL (950 mmol/Kg) or $MgSO_4$ (730 mmol/Kg) solution was added into the mix and vortexed

for 5-10 seconds (**coagulant step**) and subsequently heated at 80 °C for 30 min in a water bath (**gelation step-T_2**). Various gelation temperatures, between 60 to 90 °C, were tested in **section 4.2.2.1**. Generally, the final concentration of the coagulants was 17.12 ± 0.09 mmol/Kg for GDL, 7.09 ± 0.05 mmol/Kg for $MgSO_4$ and 3.44 ± 0.04 mmol/Kg for all PA tested. Various CFA and PCA final concentrations (0-5 mmol/Kg) were tested in **section 4.2.3.3**.

The coagulant concentrations corresponded to a SPI/GDL (% w/w) ratio of 14.3 and a SPI/$MgSO_4$ (% w/w) ratio of 24.9, respectively. In **section 4.2.2.2,** the water holding capacity (WHC) of SPI- gels with the following GDL concentrations were tested, 11.84 ± 0.01 mmol/Kg, 17.34 ± 0.00 mmol/Kg, 24.58 ± 0.00 mmol/Kg, 30.52 ± 0.00 mmol/Kg, 40.46 ± 0.00 mmol/Kg, and 50.74 ± 0.00 mmol/Kg. The gels were immediately stored at 5 °C before further analysis.

Finally, in **section 7.3.2**, 7.5 % (w/w) SPI- GDL gels were developed with the same method as it was described above. The only difference was that the GDL concentration used was 28.5 ± 0.05 mmol/Kg which corresponded to a SPI/GDL (% w/w) ratio of 14.3 as the gels induced by 4.5 % (w/w) of SPI.

3.2.2.2 Pressed SPI gels: firm tofu model system

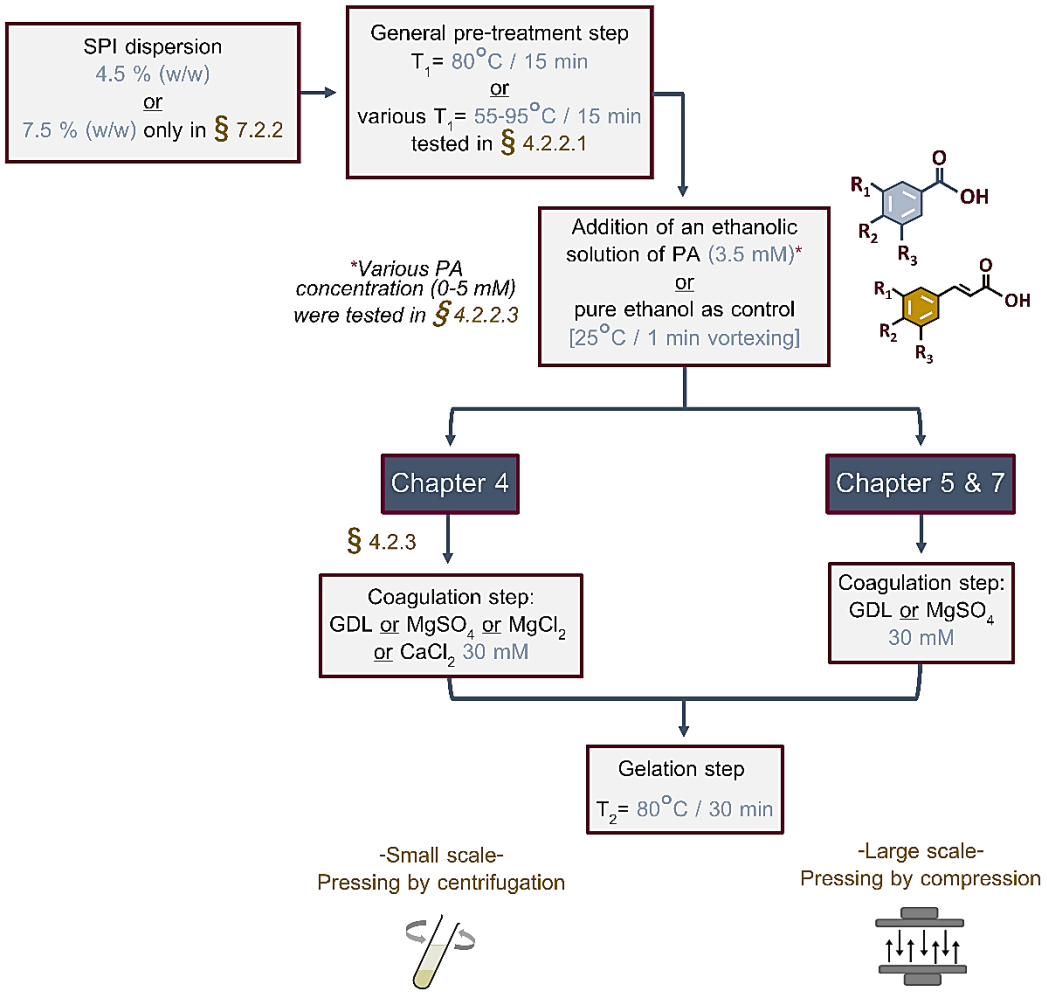


Figure 3.2 Overview of the gelation conditions for the production of pressed gels.

3.2.2.2.1 Small-scale gelation, pressing via centrifugation (Ch. 4)

A mass of 40 g of 4.5 % (w/w) SPI dispersion was heated in a water bath at 80 °C for 15 min **(pre-treatment step - T_1)** and subsequently cooled at room temperature until a final temperature of approximately 25 °C was reached. Then, approximately 1.00 mL of o-CMA or PCA or FRA or CFA ethanolic solution containing 185 mmol/Kg was added to the SPI dispersion, and the mixture was vortexed for 30 sec at room temperature. A volume of approximately 2.5 mL of a concentrated GDL or $CaCl_2$ or $MgCl_2$ or $MgSO_4$ (480 mmol/Kg) solution was poured into the mix and was vortexed for 5-10 sec ***(coagulant step)*** and subsequently heated at 80 °C for 30 min

in a water bath ***(gelation step – T_2)***. The final concentration of the coagulants was approximately 30.0 mmol/Kg and of the phenolic acids 3.5 mmol/Kg. The cooled mixture was centrifuged at 4000 g for 20 min. A volume of the supernatant (0.3 mL) was transferred to a pre-weighed into a tube containing two volumes of ethanol (0.6 mL), then it was centrifuged for 3 min at 11,000 rcf before immediately filtering through a polytetrafluoroethylene (PTFE) syringe filters, 0.20 μm (NTSF2513-4, ThermoScientific). The samples were stored at -20 °C prior to analysis and they were used to determine the retention of phenolic acids in the gels after pressing (**section 3.2.4.6**). The precipitant (protein curd/ gel) was stored at 5 °C and it was used for texture analysis (**section 3.2.4.7**) and surface characterization using SEM (**section 3.2.4.11**). This pressing method was only used in **Chapter 4** and it was selected to allow fast screening of different experimental conditions.

3.2.2.2.2 Large-scale gelation, pressing via compression (Ch.5)

Approximately 850 g of 4.5 % (w/w) SPI dispersion was heated in a water bath at 80 °C for 15 min (**pre-treatment step-T_1**) and subsequently cooled at room temperature until a final temperature of approximately 25 °C was reached. Then, approximately 10.3 g of o-CMA or PCA ethanolic solution (300 mmol/Kg) was added to the SPI dispersion, and the mixture was stirred for 5 minutes at room temperature. A mass of approximately 28 g of concentrated GDL or $MgSO_4$ (950 mmol/Kg) solution was poured into the mix and agitated gently (**coagulant step**) and subsequently heated at 80 °C for 30 min in a water bath (**gelation step-T_2**). The final concentration of the coagulants was approximately 30.32 ± 0.38 mmol/Kg and of the phenolic acids 3.54 ± 0.07 mmol/Kg. The gel was then cooled down at room temperature for 30 min and poured into a plastic tofu press mould with dimensions 14 cm x 10 cm x 9 cm (Mangocore, Amazon, USA).

For the pressing procedure used in **Chapter 5,** after the gel was transferred in the tofu mould, weights of 700 g, 1500 g, and 2300 g were placed on the top for 15, 30 and 60 min respectively, which were equivalent to pressures of 6.5, 13.8 and 21.3 g force/cm^2. After pressing, the whey was drained off, and parameters, such as final weight and whey pH were recorded. The gels were stored at 5 °C before further analysis. For the straining procedure used in **section 7.3.4**, the curd remained in the mould without placing any weight on the top for 12 hours.

In all cases, a volume (0.3 mL) of the drained whey was transferred to a pre-weighed tube containing two volumes of ethanol (0.6 mL), then it was centrifuged for 3 min at 11,000 rcf and it was immediately filtered with polytetrafluoroethylene (PTFE) syringe filters, 0.20 μm (NTSF2513-4, ThermoScientific). The samples were stored at -20 °C prior to analysis and were used for determining the retention of phenolic acids in the gels after pressing or draining (**section 3.2.4.6**). The gels were used for further physical characterisation (**section 3.2.4**) and *in vitro* digestion using a static protocol (**section 3.2.5**).

3.2.3 Preparation of phenolic acid ethanolic solutions

Most of the phenolic acids used in this thesis were easily dissolved to pure ethanol after 2-5 min of vortexing at room temperature. Caffeic acid ethanolic solution was an exception since it was heated first at 70 °C for 10-15 min to facilitate its dissolution and then was vortexed for 1-3 min.

3.2.4 SPI gels characterisation

3.2.4.1 Composition and pH

The protein content of the gels was measured by Kjeldahl analysis (Helrich, 1990) in triplicate, using a conversion factor of 6.25. The yield of pressed gel was calculated according to **Equation 3.1**.

$$\text{Yield } (\%) = \frac{\text{Weight of gel}}{\text{Weight of SPI dispersion}} \times 100 \qquad \textbf{3.1}$$

3.2.4.2 Moisture content

A known mass (g) of gels was placed to pre-weighed aluminium dishes and were drying with an air oven at 105 °C for 24 to 48 hours. Then the mass of the dry

residue was weighed again after they were cooled and in a desiccator. The experiment was performed in triplicate.

3.2.4.3 pH and ζ-potential measurement

The pH of the unpressed-soft gel and the whey serum of the pressed gels was measured by a benchtop Orion 3-star, pH-meter (Thermo electron corporation). The charge of the whey serum of the pressed gels was measured using a Zetasizer (Nano ZS, Malvern Instruments Ltd, UK) at 25 °C using a DTS1060 cell. The experiment was performed in triplicate.

3.2.4.4 Water-holding capacity

The water-holding capacity of the SPI-gels in **Chapter 4** was estimated by **Equation 3.2**. Where W_{H_2O} Total is the total mass of the water contained in the gel and W_{H_2O} RCF, the mass of water released after centrifugation at 4000 g for 30 min. Tissue paper was also used to absorb the residues of water on the surface of the gels. Control SPI-GDL gels contained the equivalent volume of pure ethanol that was used when gels contained added PA. The experiment was performed in duplicate.

$$\text{WHC (\%)} = \frac{W_{H2O}\,\text{Total} - W_{H2O}\,\text{RCF}}{W_{H2O}\,\text{Total}} \times 100 \qquad \textbf{3.2}$$

3.2.4.5 HPLC method for detecting phenolic acids

The recovery of the PA in whey was analysed using LC-20AD, Prominence UFLC, Shimadzu, Japan. Data analysis was performed with LabSolutions software (version 5.73, Shimadzu Corporation, Japan). Samples (5 µL) were injected onto a Grace™ Alltech™ Prevail™ column, 150 mm × 4.6 mm i.d.; particle size 5.0 µm (Thermo Fisher Scientific, USA). The column oven temperature was set at 25 °C. Elution of phenolic acids (0.5 mL/min) was performed using aqueous TFA solution (0.02 % v/v) (eluent A) and methanol containing 0.02 % (v/v) TFA (eluent B) (Wen et al., 2005). The elution gradient was as follows: 0–5 min, 25 % B; 5–10 min, 25–30

% B; 10–16 min, 30–45 % B; 16–18 min, 45 % B; 18–25 min, 45–80 % B; 25–30 min, 80 % B; 30–40 min, 80-25 % B; 40-50 min, 25% B. Quantification of phenolic acids in the gels was based on the calibration curves that were obtained by the standard compounds (purity ≥ 96 %) in a concentration range between 0.01 to 0.13 mg/g. For the calibration curve, phenolic acids were diluted in an aqueous solution of 63 % (w/w) ethanol. The equations and the coefficient of determination (R^2) and the wavelength (λ) at which the peak areas were recorded can be found in **Table 3.1**. A new calibration curve was prepared with fresh standard solutions using the PA of interest before each experiment.

Table 3.1 Chromatographic information of phenolic acid standards

Compound	Calibration equation	R^2	λ (nm)	Retention
Gallic acid	$y = 2.86 \times 10^7 - 1.44 \times 10^3$	0.9998	280	6.4
Protocatechuic acid	$y = 1.76 \times 10^7 + 2.36 \times 10^4$	0.9999	295	11.0
Vanillic acid	$y = 1.89 \times 10^7 + 3.47 \times 10^3$	0.9993	295	18.7
Caffeic acid	$y = 7.10 \times 10^7 + 2.28 \times 10^4$	0.9998	325	19.3
p-Coumaric acid	$y = 5.25 \times 10^7 + 3.07 \times 10^4$	0.9999	325	23.5
Ferulic acid	$y = 4.74 \times 10^7 + 5.93 \times 10^4$	0.9999	325	24.2
m-Coumaric acid	$y = 1.48 \times 10^7 - 1.97 \times 10^2$	0.9999	325	24.7
o-Coumaric acid	$y = 3.08 \times 10^7 + 1.01 \times 10^4$	0.9994	325	25.7

3.2.4.6 Retention of phenolic acids in the gels after pressing

The retention of the phenolic acids (PA) in the formed gel was calculated by subtracting the PA amount detected in whey (**Equation 3.3),** from the total concentration of PA initially added to SPI dispersion, using the **Equation 3.4**. The total amount of PA in the whey serum was calculated with the standard curves' linear equations obtained by HPLC **section 3.2.4.5** (**Equation 3.5**), where y was the peak area, a and b were the slop and the intercept respectively and F was the dilution factor of the samples.

$$\text{Retention (\%)} = \frac{\text{PA in gel (mg)}}{\text{Total amount of added PA (mg)}} \times 100 \qquad \textbf{3.3}$$

$$\text{PA in gel} = \text{Mass of added PA (mg) - Mass of PA found in whey serum (mg)} \qquad \textbf{3.4}$$

$$\text{Mass of PA in whey (mg)} = \left(\left(\frac{y-b}{a}\right) \times F\right) \times \text{Mass of whey} \qquad \textbf{3.5}$$

3.2.4.7 Texture profile analysis of pressed SPI gels

Pressed gels were removed from the refrigerator and cut with a stainless cylinder cutter with a diameter of 22 mm and a height of 1.5 cm. Fracture stress and strain were measured with a TA XT plus (Stable Micro Systems, Surrey, UK) mounted with a 51 mm flat cell loaded with 50 kg. Samples were compressed to 80 % of their initial height at a constant deformation speed of 4 mm/s. The experiment was performed in triplicate on each of the 6 gels from different batches. The gels produced with the small-scale method (**section 3.2.2.2.1**) were analysed with the same procedure described above. The true stress (σ) (**Equation 3.6**) and Hencky's strain (ε_h) (**Equation 3.7**) were calculated at each time point on the force-displacement curve after compression according to Steffe (1996):

$$\sigma = \frac{F}{Ao}\left(\frac{L}{Lo}\right) \text{ (Pa)} \qquad \textbf{3.6}$$

$$\varepsilon_h = \int_{Lo}^{L} \frac{dL}{L} = \ln \frac{L}{Lo} \ (-) \qquad \textbf{3.7}$$

$F\ (N)$ is the force recorded per unit of sample area $Ao\ (m^2)$. The stress is corrected by including the ratio of the cylinder (sample) lengths in the stress calculation. Lo is the initial length and L the deformed length of the sample. The true strain is negative for compression experiments but is expressed as an absolute value. The fracture stress was measured by the local maximum of the stress over the strain curve (Havea et al., 2009) (**Figure 3.3**). Fracture strain is the one that corresponds to the fracture stress (Havea et al., 2009) (**Figure 3.3**). Young's modulus, E (Pa) was calculated from the linear part of the stress over strain curve within the region of 0.05-0.01 of fracture strain and is defined according to **Equation 3.8**.

$$E = \left(\frac{d\sigma}{d\varepsilon_h}\right) \qquad \textbf{3.8}$$

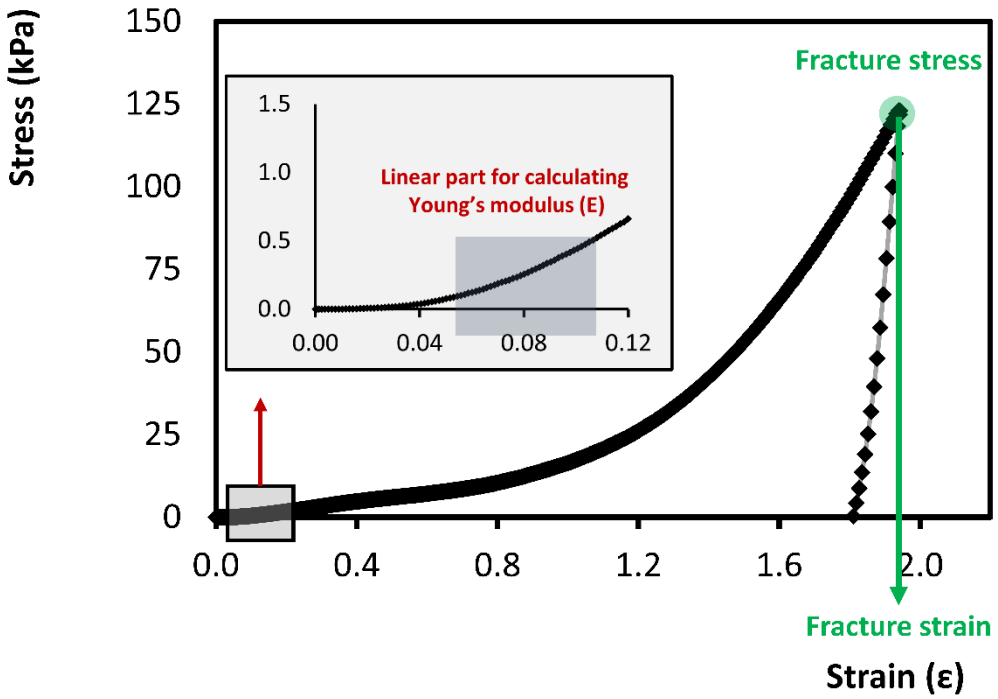


Figure 3.3 Example of a stress-strain curve and the textural parameters tested.

3.2.4.8 Viscoelastic properties of the unpressed SPI gels

A controlled stress dynamic rheometer (Physica MCR 301, Anton Paar, Inc., AU) coupled with a double gap cylinder geometry (C-DG26.7/T200, Anton Paar) with an internal diameter 23.822 mm and an external diameter of 27.594 mm was used to investigate the dynamic viscoelastic properties of gels during the gelation step. The pre-treatment step, the cooling and the addition of the phenolic acids and coagulants were conducted outside of the rheometer as described in **section 3.2.2.1**. The mixtures were immediately loaded into the rheometer after the addition of the fresh coagulants (GDL or $MgSO_4$) using a 5 mL pipette and they heated at 80 °C for 30 min. In **section 4.2.2.1**., where various gelation temperatures were tested (60 to 90 °C), the temperature in the rheometer was adjusted accordingly. Then, the gels were cooled to 5 °C at a cooling rate of 4 °C/min and maintained under these conditions for 30 min. The measurements were performed at a constant strain of 0.1, which was within the linear-viscoelastic region (0.05 to 5) (results not shown), and at a frequency of 1.0 Hz. To prevent solvent evaporation, a thin layer of soy oil was put on top of the mixtures. The coagulants and PA were freshly prepared and immediately stored at -20 °C before their use. The general temperature profile within the rheometer can be seen in **Figure 3.4**. The experiment presented in **chapter 4** was performed in duplicate with 2 gels from different batches, while the one in **chapter 6** was performed in triplicate or more using 3-5 gels from different batches.

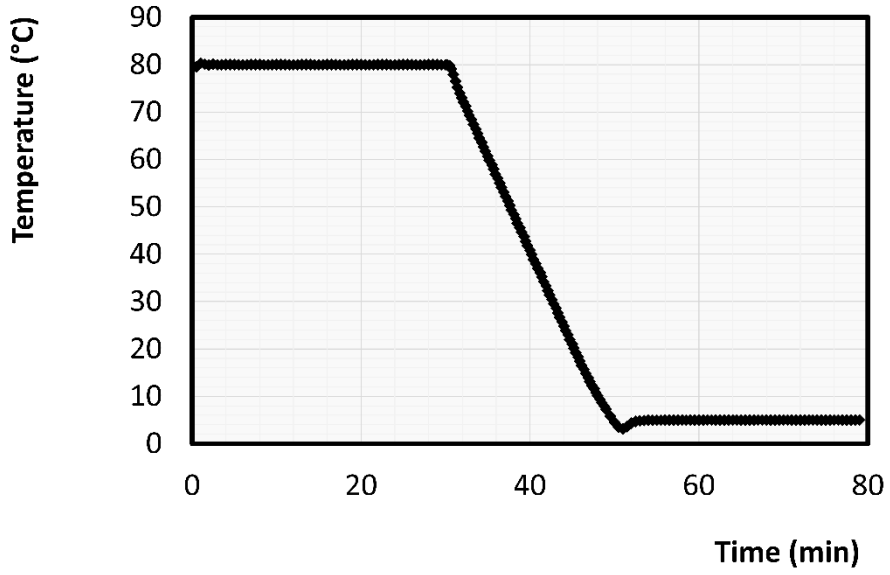


Figure 3.4 General temperature profile of the rheological conditions used in Ch. 6.

3.2.4.9 Surface hydrophobicity of unpressed SPI gels

The binding of bromophenol blue salt (BPB) method was used to measure the surface hydrophobicity of the soy protein gels. The method was developed by Chelh et al. (2006) and was slightly modified from Lorieau et al. (2018). Briefly, a mixture of soy protein gel with a volume of phosphate buffer (20 mM) at pH 6 was prepared to a final protein concentration of 2 mg/mL. The mixture was vortexed for approximately 10 min until dispersion was formed. Then, 1 mL of the mixture or phosphate buffer (control) was transferred to a vial and 80 µL of BPB (1 mg/mL) was added and vortexed for 1 min. Samples and control were agitated for 10 min at room temperature using an orbital shaker and then, they were centrifuged at 2000 g for 15 min. The supernatant was diluted approximately 10 times and the absorbance was measured at 595 nm. The amount that was bound on BPB was calculated using **Equation 3.9**, the higher the bound BPB, the higher the hydrophobicity. The experiment was performed in triplicate.

$$\text{Bound BPB } (\mu g) = 80\ \mu g \times \frac{\text{Abs}_{595\ nm}(\text{Control-Gel})}{\text{Abs}_{595\ nm}\ \text{Control}} \qquad \textbf{3.9}$$

3.2.4.10 Confocal microscopy (CLSM) on unpressed gels

A thin layer (~ 1 mm) of unpressed gels was cut and immediately stained with 0.1% fluorescent dye Rhodamine B (excitation wavelength 561 nm and emission spectral 570–725 nm) to visualize the protein network (Urbonaite et al., 2014). Imaging was performed at 25 °C using a Leica SP5 DM6000B scanning confocal microscope (Heidelberg, Germany). Multiple CLSM images were taken at different magnifications on different parts of each gel.

3.2.4.11 Scanning electron microscopy (SEM) on pressed gels

Pressed gels were cut into pieces (2-3 mm) and then were fixed in 0.1 M phosphate buffer containing 3% (w/v) glutaraldehyde and 2% (w/v) formaldehyde (pH 7.2) for 24 h at room temperature. The samples were washed three times for 10 min

each in 0.1 M phosphate buffer (pH 7.2) followed by ethanol dehydration using a series of solutions of increasing ethanol concentrations: once at 25 %, 50 %, 75 % and 95 % for 10 min each time and 100 % for 1 hour. All the samples were critical point dried in a Polaron E3000 series II apparatus (manufacturer, country), using liquid carbon dioxide as the critical point fluid and 100 % ethanol as the intermediary. Samples were torn to expose the inside of the cube and mounted on the aluminium stubs using double-sided tape. The dried samples were then sputter-coated with 100 nm of gold (Baltec SVD 050 sputter coater) and viewed in the FEI Quanta 200 environmental scanning electron microscope at an accelerating voltage of 20 kV. Multiple SEM images were taken at different magnification (x 6,000, x 12,000, x 40,000) on different parts of each gel.

3.2.4.12 Transmission electron microscopy (TEM) on pressed and unpressed gels

Tubes of pressed gels (2-3 mm) were fixed in 0.25 M glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2 for 16 h at 4 °C. Unpressed gels were too weak to be cut into tubes and therefore they were encapsulated into a 3% w/w aqueous solution of agarose before their fixation. After fixation, specimens were rinsed 3 times in 0.1 M sodium cacodylate buffer, before post-fixation with 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. After post-fixation, the samples were rinsed 3 times in 0.1 M sodium cacodylate buffer. The samples were dehydrated through a graded series of acetone for 45 min in each step (25 %, 50 %, 76 %, 95 % and 100 %) and were embedded in 50:50 resin: acetone and incubated overnight. The mixture was replaced with 100 % epoxy resin (Procure 812, ProSciTech Australia) and incubated for 8 h (repeated twice). The samples were then embedded in moulds with fresh resin and cured at 60 °C for 48 hours.

Light microscope sections of 70 nm thickness were cut from the resin blocks and were mounted on copper grids. Grids were stained in Saturated Uranyl Acetate in 50 % Ethanol for 4 min, washed with 50 % ethanol and MilliQ water and then stained in Lead Citrate (Venable and Coggeshall 1965) for a further four minutes and washed with MilliQ water. Samples were viewed using a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI Corp., Brno-Černovice, Czech Republic).

Twenty TEM images were taken at a magnification of x 20,500 on different parts of each gel. Image processing and analysis were conducted using Image-J software. The porosity of the protein network of the gels was characterised according to the procedure proposed by Silva et al. (2015). Briefly, the black top-hat filter (200 x 200) from MorphoLibJ plugin was used and then the Otsu automated threshold was applied, which resulted in a binary image, which allowed the measuring of the total area of the black and white area.

3.2.5 *In vitro* digestion static protocol

In vitro digestion experiments were conducted as stated in the INFOGEST consensus protocol for static *in vitro* digestion, with minor modification (Minekus et al., 2014).

3.2.5.1 Oral phase

The gel bolus was obtained by mixing 5 g of gel samples with 4 mL of simulated salivary fluid (SSF), 0.025 mL of 0.3 M $CaCl_2$ and 0.975 mL of distilled water in an amber glass bottle for 5 min. The pressed gels were coarsely grated using a box grater and the unpressed gels which were weaker were simply poured within the bottle (**Figure 3.5)**. In **section 7.3.3**, pressed gels were diluted with 1 (F=2) and 2 volumes (F=3) of water in order to test how the different gel-to-SF ratio or (enzyme to substrate ratio) affects the *in vitro* proteolysis and the PA bioaccessibility. The appearance of the gels before the start of the oral phase can be seen in **Figure 3.5.**

The salivary α-amylase was excluded from our experiments due to a lack of starch in the samples (Lorieau et al., 2018). The gel bolus was then kept at 37 °C under shaking at 40 rpm in a shaking water bath. Aliquots of 0.5 mL of digesta was withdrawn at the end of the oral phase for further analysis (**section 3.2.5.4**). In **section 7.3.1** the unpressed SPI gels induced by various GDL concentrations were grounded for 10 seconds using a coffee grinder (Sunbeam MultiGrinder™ II EM0405, New Zealand).

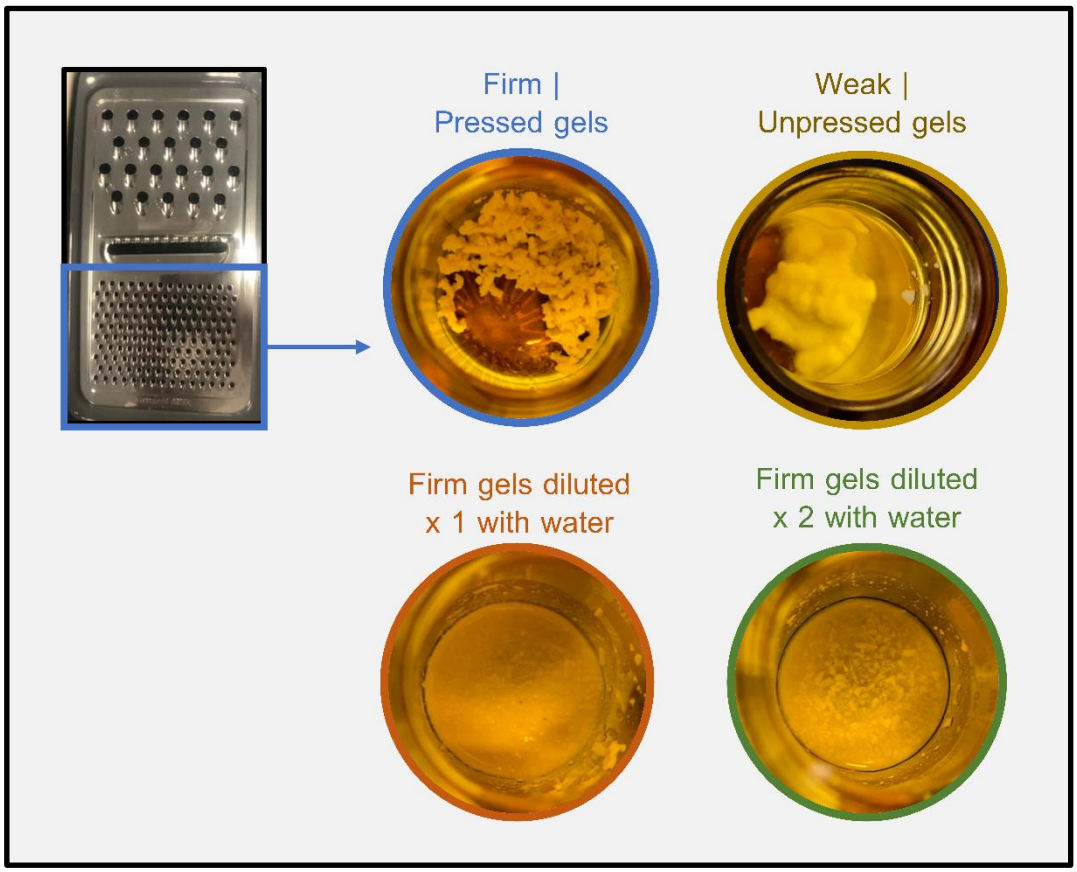


Figure 3.5 Appearance of the pressed, unpressed gels and diluted with water pressed gels before the addition of the SSF.

3.2.5.2 Gastric phase

The residual 9.50 g of the simulated oral bolus was mixed with 7.6 mL of simulated gastric fluid (SGF), 4.8 μL of 0.3 M $CaCl_2$, and the pH was adjusted to 3.0 by adding 0.25 to 0.55 mL of 1 M HCl. The rest of the volume was filled with 0.69 to 0.39 mL of distilled water and 0.95 ml of porcine pepsin stock solution (40,000 U/mL) to achieve an activity of 2000 U/mL in the final mixture. Then, the mixture was incubated for 2 h in a shaking water bath at 37 °C (40 rpm). Aliquots of 0.4 mL were withdrawn at times 30, 60, 120 min of the gastric processing for further analysis **(section 3.2.5.4)**.

3.2.5.3 Intestinal phase

The residual 17.80 g of the simulated gastric chyme was mixed with 9.76 mL of simulated intestinal fluid (SIF), 2.24 mL of bile (approximately 160 mM in SIF) and 36 μL of 0.3M $CaCl_2$, the pH was adjusted to 7.0 by adding 0.30 to 0.50 mL of 1 M NaOH. The rest of the volume was filled with 1.00 to 0.80 mL of distilled water and 4.47 mL of pancreatin stock solution (800 U/mL) to achieve a trypsin activity of 100 U/mL in the final mixture. Both pancreatin and bile salts are not readily dissolved in simulated fluids, thus they were vortexed and magnetically stirred for approximately 20 min under cool conditions, prior to intestinal processing. The simulated digesta was incubated for 2 h in a shaking water bath at 37 °C (40 rpm). Aliquots of 0.4 mL were withdrawn at times 5, 15, 30, 60, 120 min of the intestinal phase for further analysis (**section 3.2.5.4**).

3.2.5.4 Sample collection

In the case of the control trials (digesta of unfortified gels), the enzymatic reactions in the digesta were stopped using proteases inhibitors such as pepstatin A diluted in pure methanol at a final concentration of 0.5 mg/mL for the gastric aliquots and aqueous solution of 4-benzenesulfonyl fluoride hydrochloride (ABSF) of 0.1 M for the intestinal aliquots, respectively (Lorieau et al., 2018). After the addition of the inhibitors, the samples were immediately vortexed and centrifuged for 5 min at 11,000 rcf. All the samples were stored at -20 °C prior to further analysis (**sections 3.2.5.2 and 3.2.5.3**).

Aliquots from the digesta of the fortified gels (contained PA) were treated without inhibitors due to adverse interactions. It was observed that the addition of the ABSF was significantly reduced the levels of PCA upon addition (results not shown). Thus, the withdrawn digesta were immediately centrifuged for 5 min at 11,000 rcf, then the supernatant was transferred to a pre-weighed tube containing two volumes of ethanol, the tube was weighed again to determine the exact amount of sample and finally, it was centrifuged again for 3 min at 11,000 rcf. The dissolved samples were immediately filtered with polytetrafluoroethylene (PTFE) syringe filters, 0.20 μm (NTSF2513-4, Thermo Scientific) (**Figure 3.6**). All the samples were stored at -20 °C

prior to further analysis (**section 3.2.5.3**). The *in vitro* digestions were performed in triplicate with 3 different gels from different batches.

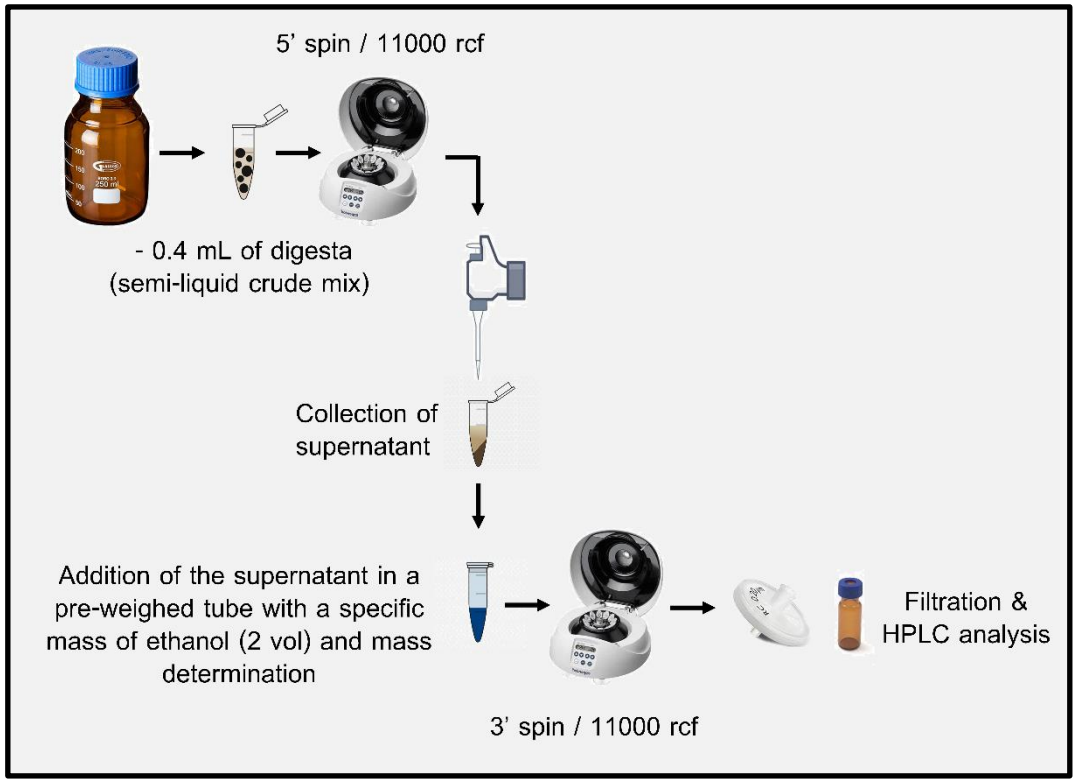


Figure 3.6 Preparation of aliquots collected during *in vitro* digestion destinated for PA bioaccessibility determination.

3.2.6 Digesta analysis

3.2.6.1 Total content of amino acids

Gel samples (undigested material) were hydrolysed using hydrochloric acid (6 mol/L) containing 0.1 % (w/v) phenol at 115 °C for 24 h under vacuum. Tryptophan could not be detected following acid hydrolysis due to oxidative degradation.

3.2.6.2 Degree of protein hydrolysis

The degree of protein hydrolysis was determined using the o-phthaldialdehyde (OPA) assay as reported by Nielsen et al. (2001). The OPA solution consisted of 80 mL of 125 mM sodium tetraborate (BORAX) and 4.3 mM of sodium dodecyl sulphate (SDS), 2 mL of 300 mM OPA in methanol, 2 mL of 285 mM dithiothreitol (DTT). The final volume was adjusted to 100 mL with distilled water. The assay was performed by mixing 0.1 mL of diluted digesta with 1 mL of OPA solution. The mixture was vortexed and held at room temperature for 2 min, followed by measurement of absorbance at 340 nm. The concentration range that was used for the calibration curve of L-Serine was 0.007-0.144 mM. The absorbance of the blank samples (0.1 mL of water with 1 mL of OPA solution) was always below 0.02.

The degree of protein hydrolysis was calculated using **Equation 3.10** (Schasteen et al., 2007), where NH_2 (final) is the concentration of free amino groups of the digested sample, NH_2 (initial) is the concentration of free amino groups in the undigested sample (oral phase), and NH_2 (acid) is the total amount of free amino groups in the gel samples (without digestive enzymes) after acid hydrolysis as it is described **section 3.2.6.1**.

$$\mathrm{DH}\ (\%) = \frac{\mathrm{NH_2(final)\text{-}\ NH_2(initial)}}{\mathrm{NH_2(acid)\text{-}\ NH_2(initial)}} \times 100 \qquad \textbf{3.10}$$

The total free amino acid concentration was found on average 7.05 ± 1.065 mmol Serine equivalents/ g of protein for GDL gels and 7.71 ± 0.713 mmol Serine equivalents/ g of protein for $MgSO_4$ gels. The experimental values were comparable with the theoretical value (7.67 mmol of total amino acids/ g of protein) as it was

calculated from the amino acid composition of soybeans reported in the literature (Day, 2013). The experiment was performed in triplicate.

3.2.6.3 Amino acid bioaccessibility

Free amino acid (FAA) determination is based on the protocol from Liu and co-authors (Liu, 2000). Briefly, the samples that were collected after *in vitro* digestion were deproteinized by ultrafiltration (Vivaspin 3000 MWCO) at 14,000 rcf, 20 °C for 1 hour. Norvaline was used as an internal standard. Amino acid concentrations were determined with the use of HPLC (Agilent 1200SL, Agilent Technologies, Santa Clara, CA, USA), Eclipse plus C18 Narrow Bore column, 150 mm × 2.1 mm i.d.; particle size 3.5 µm (Agilent Technologies, Santa Clara, CA, USA). The column oven temperature was set at 40 °C. Elution of amino acids (0.42 mL/min) was performed using an aqueous solution consisting of 10 mM sodium phosphate dibasic, 10 mM Borax and 0.5 mM of sodium azide in pH 8.2 (eluent A) and a mixture of methanol/ acetonitrile/ water in ratio 45/45/10 (eluent B). The elution gradient was as follows: 0-3 min, 100 % A; 3 min, 100 % A; 3 - 10.4 min, 100 - 81.5 % A; 10.4 - 23.0 min, 81.5 - 43.0 % A; 23.0 - 23.1 min, 43.0 - 0 % A, 23.1 - 28.0 min, 0 % A, 28.0 - 28.5 min, 0 - 100 % A, 28.5 - 30 min, 100 % A. The experiment was performed in triplicate. The AA bioaccessibility was calculated according to **Equations 3.11** and **3.12**.

$$[\text{AA}] \text{ in digesta} = \frac{\frac{\text{AA µmol}}{\cancel{\text{mL Digesta}}} \times \cancel{\text{Total volume of digesta mL}}}{\text{Available protein (g)}} = \frac{\text{AA µmol}}{\text{g of protein}} \qquad \textbf{3.11}$$

$$\text{AA bioaccessibility (\%)} = \frac{\text{AA concentration in digesta } \left(\frac{\text{µmol}}{\text{g of protein}}\right)}{\text{Total AA concentration } \left(\frac{\text{µmol}}{\text{g of protein}}\right)} \times 100 \qquad \textbf{3.12}$$

3.2.6.4 Phenolic acid bioaccessibility

The quantification of phenolic acids (PA) in digesta was obtained by HPLC analysis (**section 3.2.4.5**). The PA release was calculated according to **Equations 3.13 and 3.14**, where y was the peak area, a and b were the slop and the intercept of the linear equation found from the calibration curve of the PA of interest. F was the dilution factor of the samples.

$$\text{PA release pressed gels (\%)} = \frac{\frac{\text{y-b}}{\text{a}} \text{ x F x Mass of digesta (mg)}}{\text{Total mass of PA retained in gel after pressing (mg)}} \times 100 \quad \textbf{3.13}$$

$$\text{PA release unpressed gels (\%)} = \frac{\frac{\text{y-b}}{\text{a}} \text{ x F x Mass of digesta (mg)}}{\text{Total mass of PA added in the gel (mg)}} \times 100 \quad \textbf{3.14}$$

3.3 Statistical analysis

Most of the experiments were conducted in triplicate, the results were reported as means ± standard deviations of the measurements. Data were analysed using Minitab 17 statistical software (Minitab Inc., USA). The statistical analysis was conducted using the student t-test for two-sample comparison, one-way ANOVA for comparison of the means of two groups, and the linear regression model (ANOVA) for multi-factor comparison followed by Tukey's pairwise test. The correlations were considered significant when $p < 0.05$.

Chapter 4

Soy protein gel formation

Abstract

The main aim of this chapter was to understand the physical characteristics of soy protein gels by developing a range of different gel forms or structures. Two types of model gel systems were developed using a commercial SPI: 1) silken tofu (unpressed gel) and 2) firm tofu (pressed gel).

All protein gels were formed with a double heating step for denaturation of soy proteins and gelation enhancement. The rheological analysis suggested that the use of a temperature range between 70 to 85 °C for both heating steps formed the firmest gels. A coagulant concentration between 12 to 18 mM was enough to produce silken-like tofu (unpressed gels). At higher concentrations (>20 mM), gels had a reduced water-holding capacity and were more prone to phase separation, which was a useful property for the firm-like tofu (pressed gels) formation. The addition of phenolic acids in the unpressed gels reduced their hydration capacity by 10 to 30 % and increased their porosity. Pressed gels were formed with both salt and acid coagulants. GDL-induced pressed gels were significantly firmer than the salt-induced counterparts, and firmness correlated well with their water-holding capacity. Finally, GDL pressed gels were able to retain approximately 60 % of the phenolic acid PCA, which was almost double the performance of salt-induced gel

4.1 Introduction

This chapter aimed to develop and characterise reproducible tofu model systems for making gels with a range of microstructures, textures, and chemical properties. Another objective was to understand the physical characteristics of soy protein gels and determine which gels show the potential to be good candidates as delivery systems for phenolic acids. Two types of gels were developed using a commercial SPI: 1) silken tofu or unpressed gels and 2) firm tofu/ pressed gels model systems.

4.2 Results and discussion

4.2.1 Characterisation of SPI

An overview of the experimental design used for the SPI characterization can be seen in **Figure 4.1**. The SPI used contained 87.7 % (w/w) of protein on a dry basis and 5.2 % (w/w) moisture content. According to the manufacturer the fat content was below 1.0 % (w/w) and the ash was below 6.0 % (w/w).

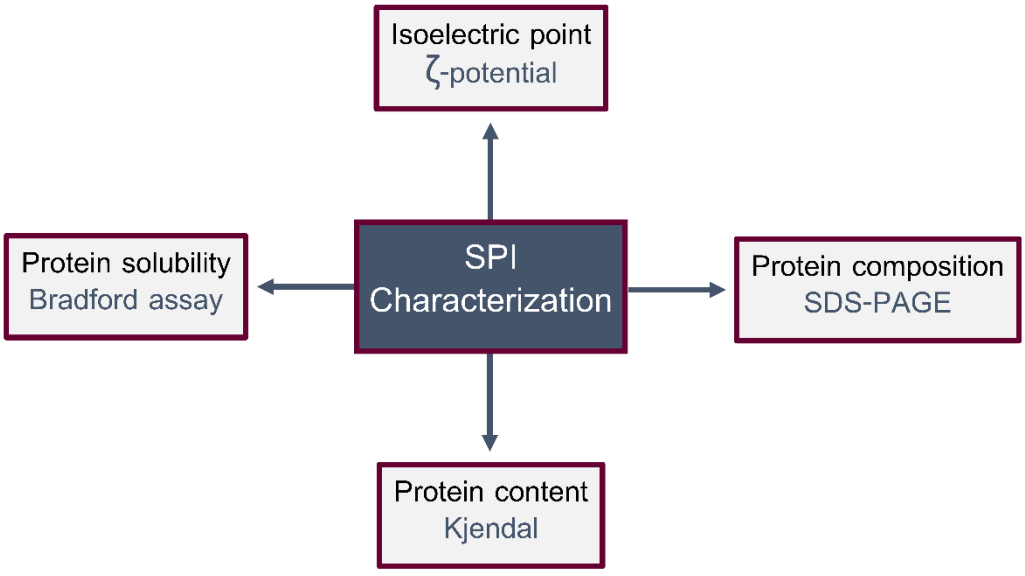


Figure 4.1 Overview of the experimental design of section 4.2.1.

The two major soy proteins are glycinin and β-conglycinin (Nishinari et al., 2014). The hexameric glycinin contains both acidic and basic proteins that are linked with disulphide bonds. The β-conglycinin are trimeric glycoproteins (Nishinari et al., 2014). Both glycinin (11S) and β-conglycinin (7S) were identified in the SDS-PAGE analysis (**Figure 4.2 A**). Density analysis after image binarization revealed a combined 11S and 7S purity of approximately 88 % with 11S/7S protein ratio of 1.30, which was slightly lower compared to other studies (Cai and Chang, 1999). The bands between 50 to 80 kDa corresponded to the three proteins of β-conglycinin (α, α', β) (Hsia et al., 2016). Also, the band at around 35 kDa corresponded to the acidic proteins, while the intense band close to 20 kDa correlated with the basic proteins of glycinin. One of the acidic glycinin proteins appeared as a faint band between 35 and 50 kDa (Hsia et al., 2016) (**Figure 4.2 A**).

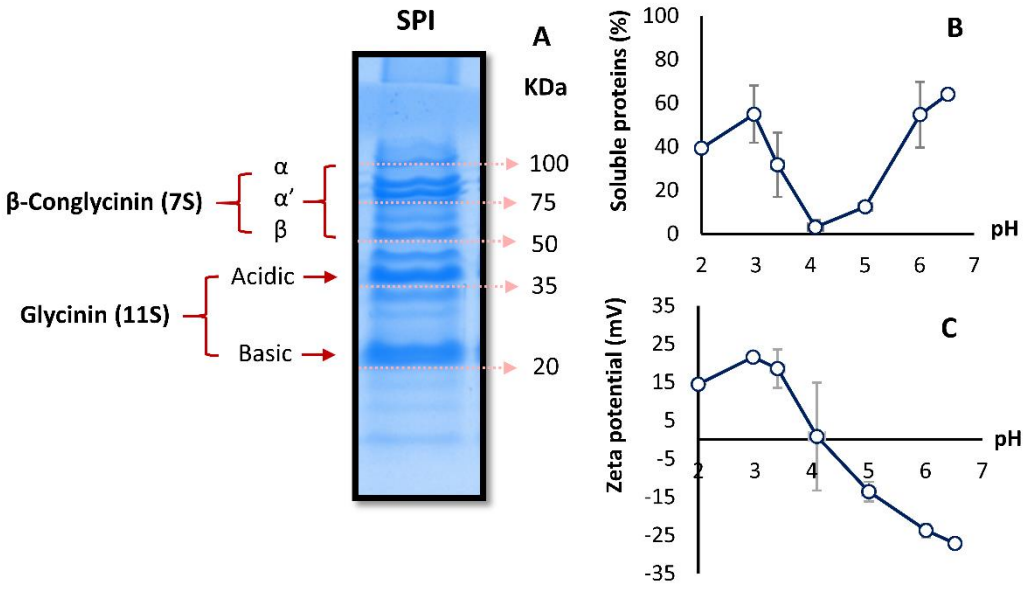


Figure 4.2 Reducing SDS-PAGE pattern of commercial SPI used (conducted by Teresa Wegrzyn) (A), results from protein solubility (B) and ζ-potential profile (C) of SPI as a function of the pH.

[1] SDS-PAGE was performed once. [2] Values of B and C were represented as means ± standard deviations of the two different SPI batches used (n=2).

The solubility profile of SPI over a range of pH can be found in **Figure 4.2 B**. Lower solubility was detected in pH 4.0 and maximal solubility was obtained at pH 7.0. This pH range is in accordance with the literature, more specifically, the maximum

insolubility of β-conglycinin was about pH 4.25−5.25, whereas for glycinin the range was broader, pH 4.25−6.0 (Dias et al., 2003). Similarly, the isoelectric point (pI) of the SPI was approximately 4.0 (**Figure 4.2 C)**

4.2.2 Unpressed SPI gels

The development of silken tofu-like gels and the conditions affecting some of their physical properties were tested. For these types of gels, GDL is the preferred coagulant since the textures obtained were smoother and the procedure was more easily controlled compared to the salt coagulants. The overview of this experimental design can be seen in **Figure 4.3.**

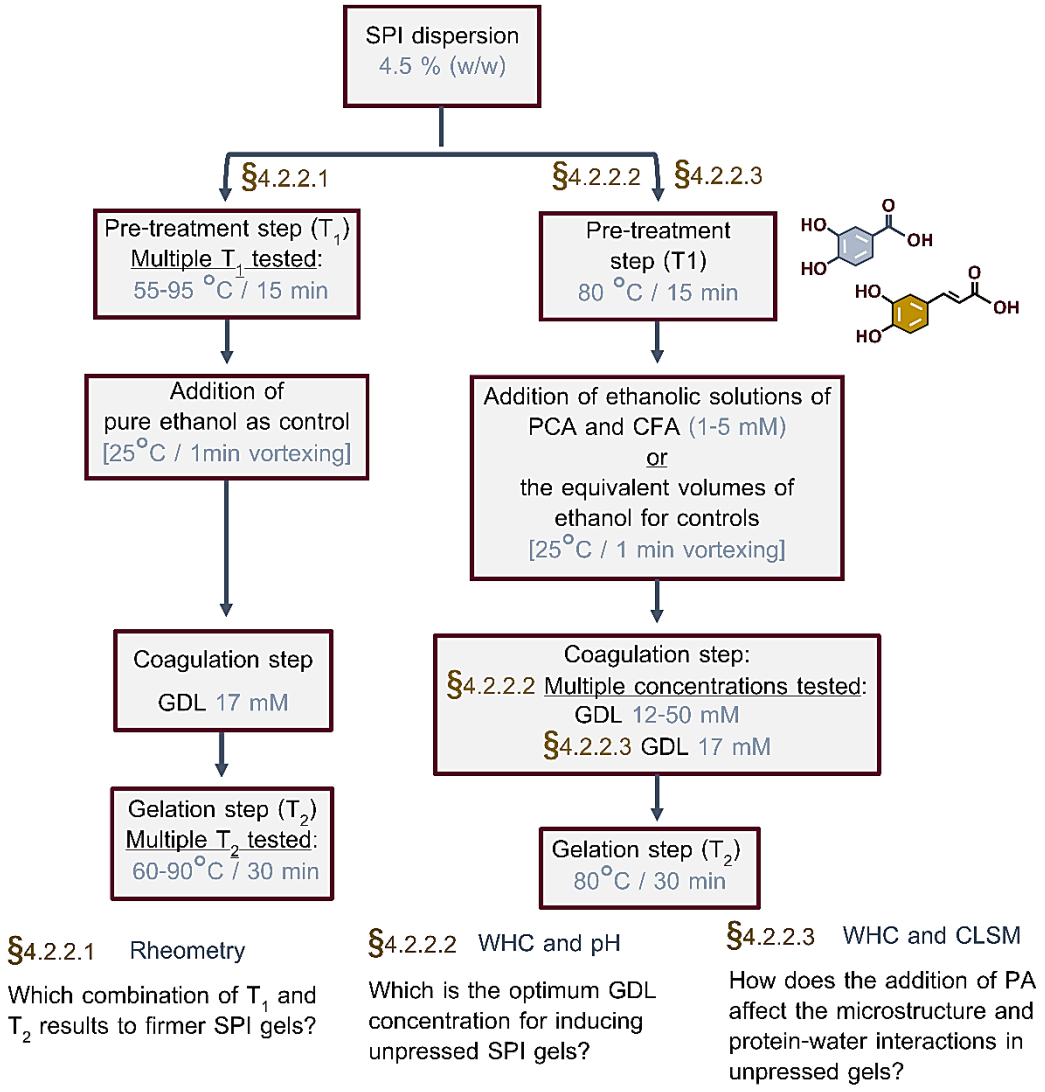


Figure 4.3 Overview of the experimental design of section 4.2.2.

4.2.2.1 Effect of temperature on viscoelastic properties of semi-solid SPI gels

The effect of the pre-gelling (first heating step) and gelling (second heating step) temperature on the solid behaviour of the soy protein gels at the end of the cooling step can be seen in **Figure 4.4** and the temperature profile used can be seen in **section 3.2.4.8**. Heat treatment between 55 to 70 °C for 15 min gave a linear increase on the final G' of the gels. Between 70 to 85 °C the final G' peaked and formed a plateau (700 Pa) and then after 80 °C, G' was rapidly decreased by almost 50 % ($p<0.05$) (**Figure 4.4 A**). The first heating step caused the unfolding of the proteins, exposing their reactive amino acid residues and form a space spanning network (Renkema and van Vliet, 2002).

The G' decline after 80 °C is in contrast to the denaturation temperature of the soy proteins (Td ~ 70 °C and 90 °C for β-conglycinin and glycinin, respectively) (Renkema and van Vliet, 2002, Van Kleef, 1986, Wu et al., 2019). This trend might be explained by the fact that the SPI used was a commercial product and thus already partially denatured. According to Chronakis, commercial protein powders behave differently than the native ones and in some cases, aggregation can occur before protein denaturation (Chronakis, 1996).

The second heating step was conducted after the addition of the coagulant GDL at temperatures between 60 to 90 °C. The final G' after the cooling step can be seen in **Figure 4.4 B**. Although the soy proteins were denatured in all cases, the gels formed between the gelling temperatures 60 and 70 °C were weak and not able to support their own weight (macroscopic observation). Above 80 °C the gels were significantly firmer ($p<0.05$). The latter might signify the involvement of hydrophobic protein-protein bonding which are enhanced at high temperature (Damodaran et al., 2007, van Dijk et al., 2015), similar findings have been reported recently from Wu and co-authors (Wu et al., 2019)

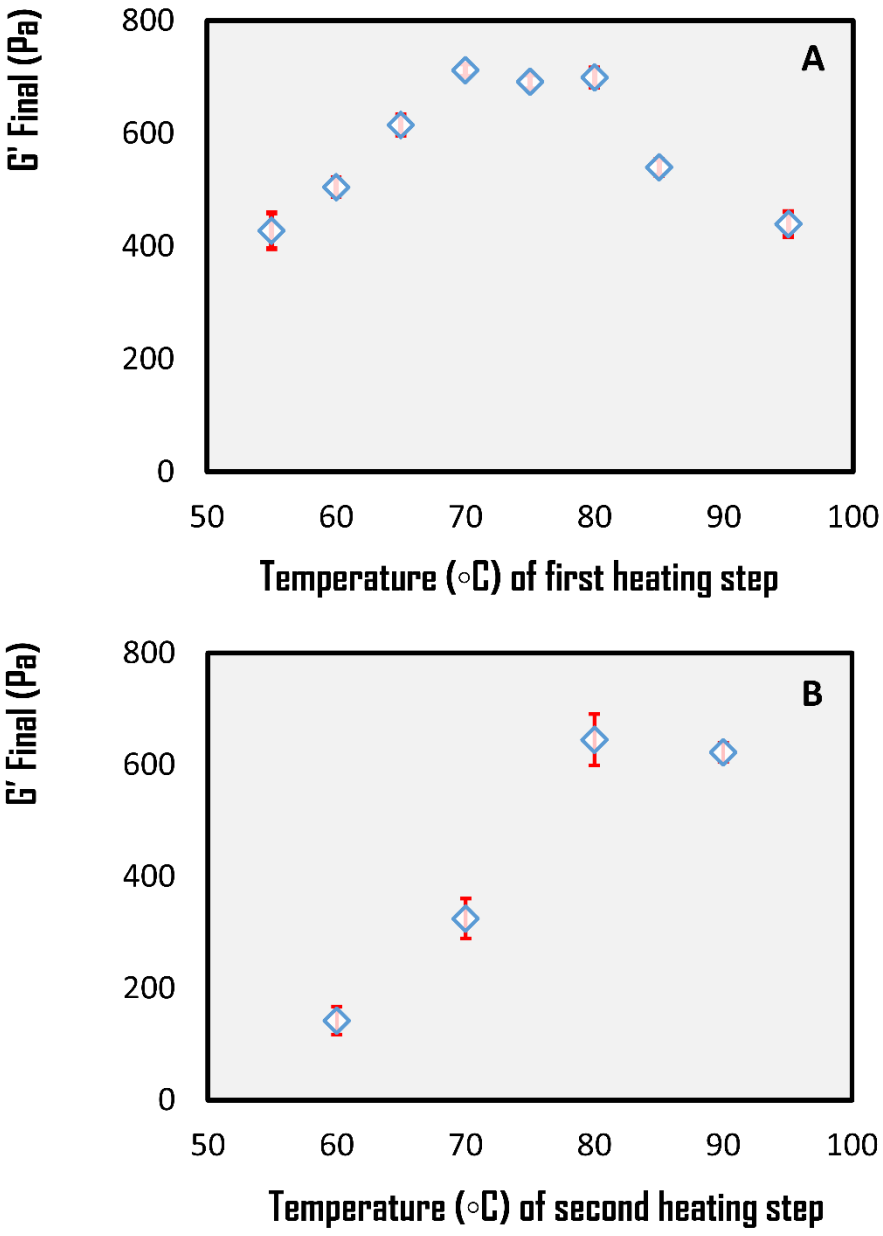


Figure 4.4 Effect of temperature during a two-step heating on soy protein gels elastic behaviour (4.5 % (w/w) SPI gels induced by ~17 mM GDL). Different pre-treatment temperatures (T1) were tested for 15 min with a gelation temperature of 80 °C kept for 30 min (T2) (A) and stable pre-heating temperature at 80 °C for 15 min with various gelling temperatures kept for 30 min (B). The values appeared in the figures correspond to the final G'(Pa) at the end of the cooling step (5 °C).

[1] Values were represented as means ± standard deviations (n=2).

4.2.2.2 Effect of GDL concentration on WHC of gels

As can be seen in **Figure 4.5**, the water-holding capacity (WHC) of the SPI gels changed significantly with the coagulant concentration. At 12 mM GDL, a viscous fluid is formed which retained 100 % of its water. At a concentration of 17 mM, an opaque gel with a considerably lower WHC of 70.0 ± 4.5 % ($p<0.05$) was formed. At higher GDL concentrations (>35 mM) the WHC reduced even further, reaching a plateau at around 35 %. The same tendency is expected with salt coagulants with the only difference that salt-induced gels retain more water, overall. The WHC trend shown in **Figure 4.5** was proportional to the pH changes in the gels ($r = 0.93$, $p<0.01$), which was directly related to the charge density in the mixture.

The results indicated that at GDL concentrations above 25 mM (pH ~ pI) the proteins were unstable due to low charge and might precipitate under agitation, which is desirable only in the case of the firm/ pressed gels. Thus, for a stable silken- tofu like system the optimum concentration is close to 17 mM of GDL.

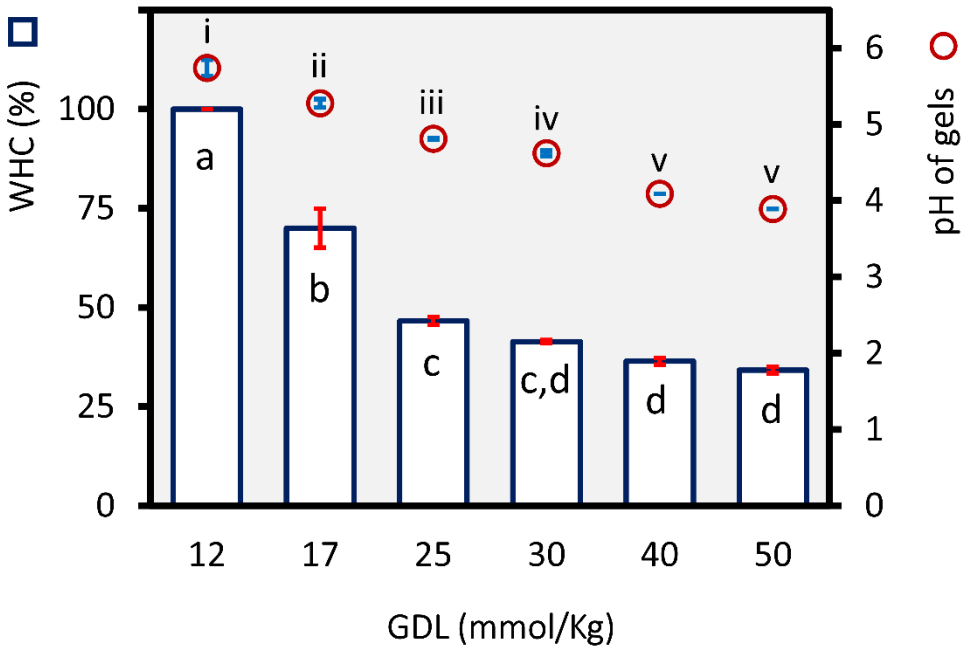


Figure 4.5 Effect of GDL concentration (12-50 mmol/Kg) on pH and the WHC of unpressed control SPI gels (4.5 % w/w).

[1] Values were represented as means ± standard deviations (n = 2). [2] Letters a-d and i-v indicate that values that do not share a letter in the same category are significantly different ($p \leq 0.05$). [3] The WHC and pH of the gels were analysed using a one-way ANOVA with GDL concentration as the main factor and the comparison was performed using the Tukey test.

4.2.2.3 Effect of phenolic acids addition on WHC and gels structure

The addition of phenolic acids significantly affected ($p<0.05$) the WHC of the soy protein gels which was estimated after centrifugation (**section 3.2.4.4**). As can be seen from **Figure 4.6**, the mean WHC of the control sample was 70.7 ± 2.08 %, which was significantly higher than the WHC of the gels with added phenolic acids ($p<0.05$). Both caffeic and protocatechuic acid showed the same trend ($p>0.05$) with the concentration playing a role on the WHC levels. Han et al. (2011) found a significant reduction in the hydration capacity of cheese curd after the addition of various polyphenols (PP). The authors proposed that the PP interact with the hydrophobic amino acid (AA) residues and disrupt the balance between the protein-protein and protein-water bonding and thus, more water can escape from the gel (Han et al., 2011). Similarly, Helal et al. (2015) found a lower moisture content in cheese curd only in the case of added tannic acid (MW ~ 1700).

Confocal microscopy revealed black voids in the gels' microstructure at elevated phenolic acid concentrations (**Figure 4.6**). This phenomenon might be an indication of a disturbance of the protein-protein and protein-water equilibrium. As a response, water was able to escape more easily under external forces. It should be noted that the gels did not show an obvious syneresis. Electron microscopy (SEM) of cheese curd, with incorporated PP revealed a less smooth and less dense curd microstructure in comparison to the control curd (Han et al., 2011). However, the images presented in the study did not show a significant effect on the pore size that could support our hypothesis. A non-destructive and rapid method that could be used to assess porosity is the nuclear magnetic resonance (NMR) relaxation or low-field NMR (Li et al., 2015). Another explanation for the trend shown in **Figure 4.6** is that the phenolic acids reduced the pH of the medium and therefore reduced the electrostatic repulsion between the proteins and allowed them to interact more. Phenols are weak acids and the addition of a carboxyl group on a phenolic structure reduces the pk_a of the molecule even further.

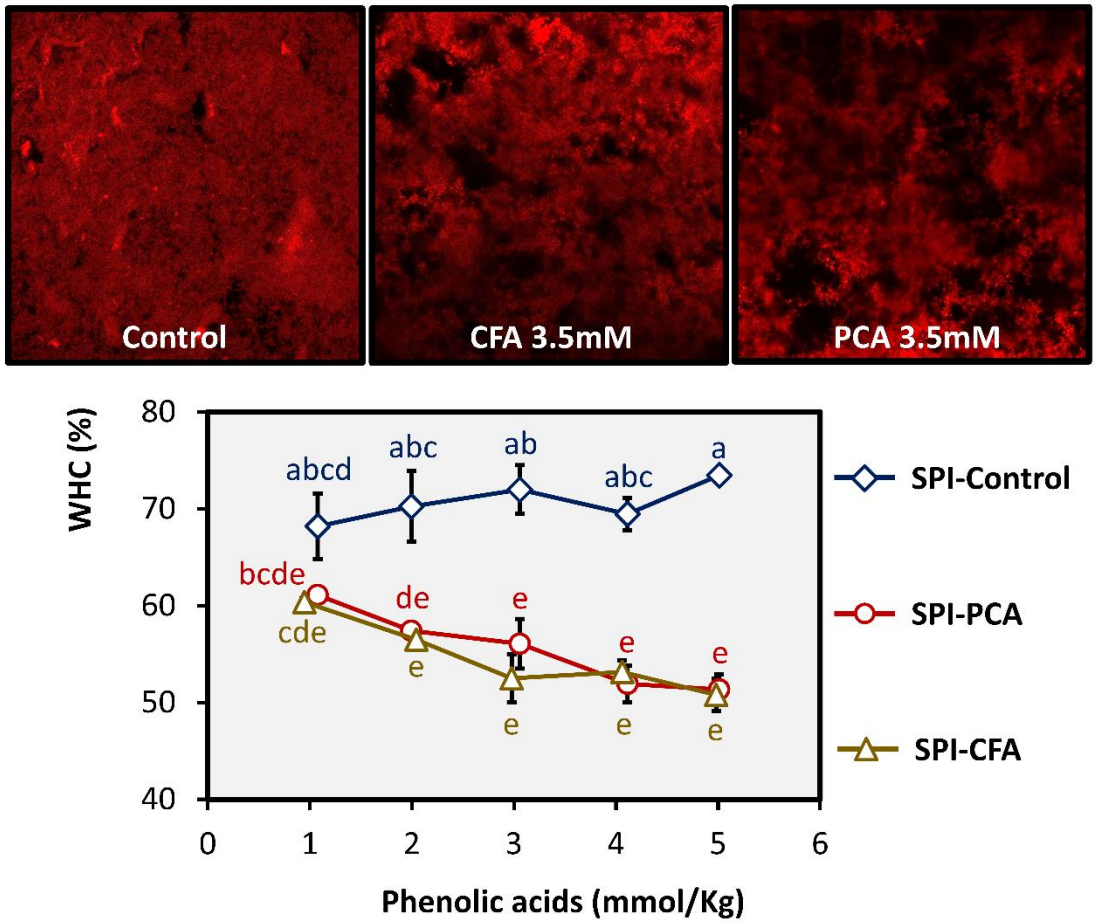


Figure 4.6 Effect of added phenolic acid concentration (1-5 mmol/Kg) on water-holding capacity (WHC %) and microstructure (CLSM) of unpressed 4.5 % w/w SPI-GDL gels (17 mM). The control gels contain different volumes of ethanol equal to the ones with the different volumes of ethanol equal to the ones with the different PA concentrations.

[1] The values represent the mean ± SEM (n=2). [2] Letters a-e indicate that values that do not share a letter are significantly different ($p \leq 0.05$). [3] The WHC of the gels was analysed using the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (control, PCA, CFA) and phenolic acid concentration (1-5 mmol/Kg). The significance of the factor's interaction mean values was assessed with the Tukey test.

4.2.3 Pressed SPI gels

In contrast to unpressed gels, more coagulants can be used for the formation of firm tofu-like gels. The coagulants tested in this study were the salts $MgCl_2$, $MgSO_4$, $CaCl_2$ and the acidifier GDL. To obtain firm SPI gels, a higher concentration of coagulants is needed compared to unpressed gels and phase separation is desirable (**Figure 4.7**).

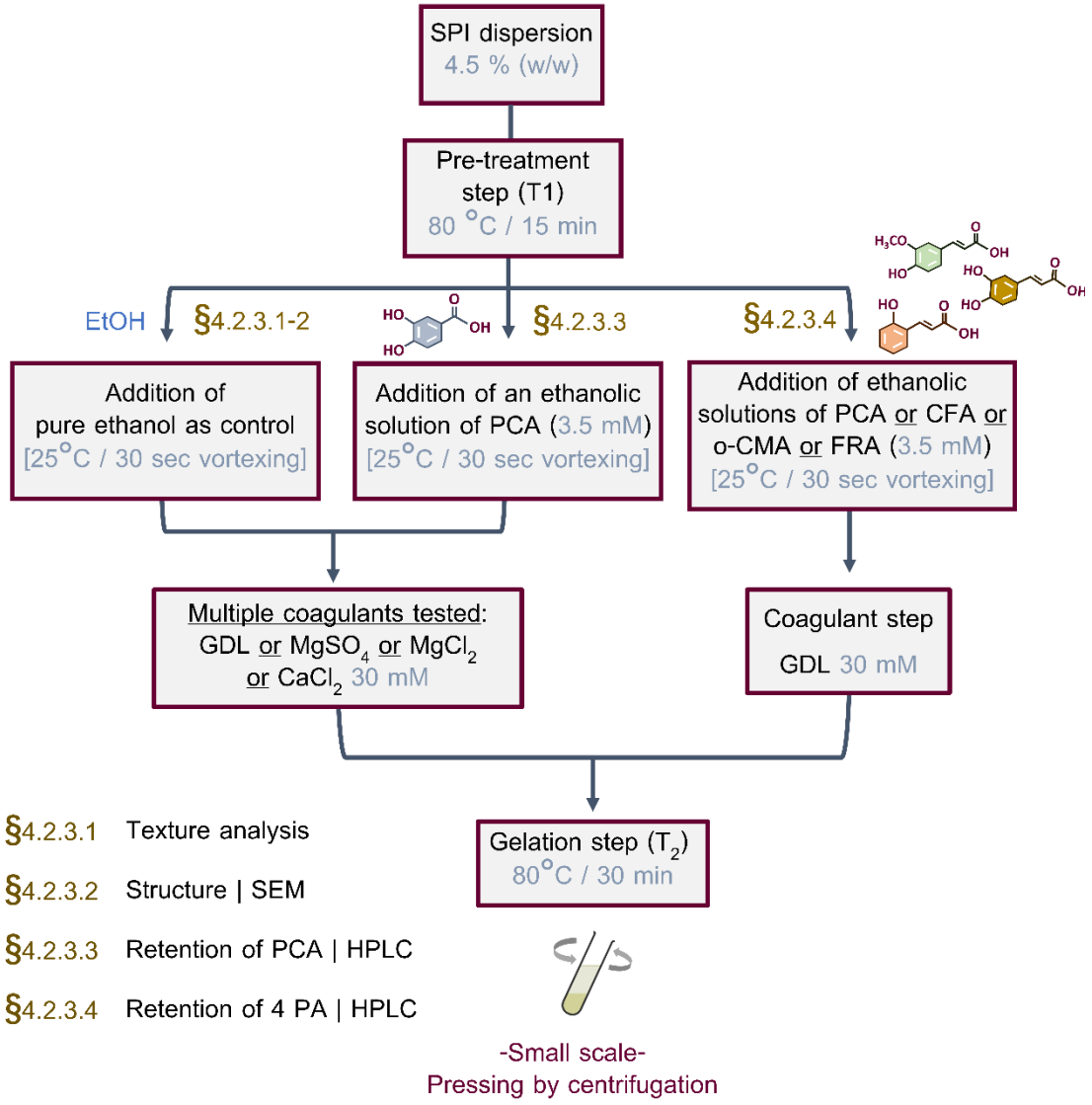


Figure 4.7 Overview of the experimental design of section 4.2.3.

4.2.3.1 Textural characteristics

Texture analysis was performed by measuring fracture stress (kPa), Young's modulus (kPa) and fracture strain (-) of the gels. Both Young's modulus and fracture stress of the gels induced by the salt coagulants were significantly lower than the GDL gels ($p<0.05$). Therefore, GDL gels were the stiffest and strongest, while $MgSO_4$ gels were the softest and weakest among the gels. Even so, salt gels were not significantly different from each other ($p>0.05$) (**Figure 4.8**).

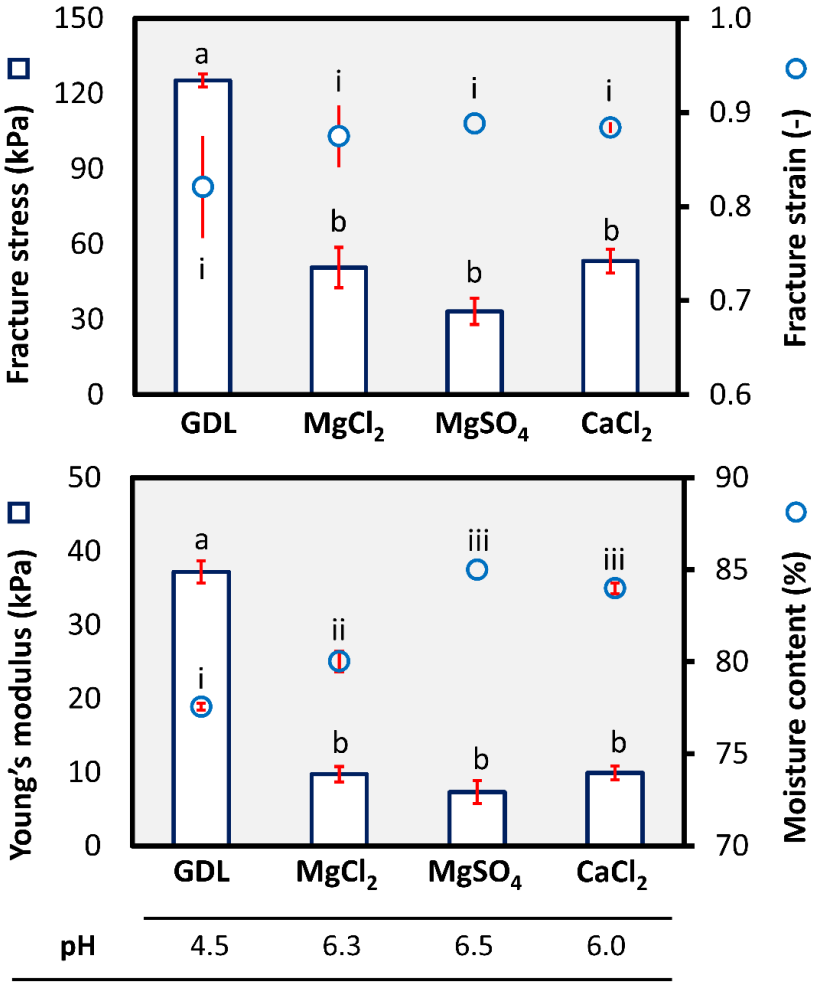


Figure 4.8 Textural characteristics at 80 % of deformation of small-scale pressed control SPI gels induced by different coagulants. The bullet markers correspond to fracture strain on the top and moisture content on the bottom.

[1] Values were represented as means ± standard deviations (n=2). [2] Letters a-b and i-iii indicate that values that do not share a letter in the same category are significantly different ($p \leq 0.05$). [3] The texture properties (fracture stress, fracture strain, Young's modulus) and moisture content of the gels were analysed using a one-way ANOVA with coagulant type (GDL, $MgCl_2$, $MgSO_4$, $CaCl_2$) as the main factor. The comparison was performed using the Tukey test.

Visually, the salt-induced gels were smooth with a spreadable texture, whereas GDL-induced gels were coarse and brittle. These results can be explained by the significant lower moisture content of the GDL gels compared to the salt gels ($p<0.05$). The moisture content of the gels also had a negative correlation with both Young's modulus and fracture stress (r = -0.81, p = 0.186 and -0.84, p = 0.158, respectively). Urbonaite et al. (2014) showed that the higher the stiffness of $MgSO_4$/$MgCl_2$ SPI gels the lower the ability of the gel network to hold water well.

The salt-induced gels retain more water because they consist of divalent cations (Ca^{2+}, Mg^{2+}), which form salt bridges between the polypeptide chains within the protein, leaving water entrapped in the gel. In contrast, monovalent cations (H^+) neutralise negative charges leading to water layer (Zhao et al., 2020) around polypeptides and not within the gel structure.

Nevertheless, fracture strain or extensibility were similar in all the gels ($p>0.05$) and comparable with values reported for mozzarella and mild cheddar cheese (Gunasekaran and Ak, 2002).

Lu et al. (1980) compared the ability of various coagulants to form firm tofu and they found that the GDL-induced tofu was the firmest and the rubberiest. Moreover, Prabhakaran et al. (2006) found that firm tofu produced by $MgSO_4$ was significantly weaker than tofu produced by other salts. Similarly, Li Tay et al. (2006) found that among four salt coagulants the weakest soy protein gels were formed by $MgSO_4$ and the strongest by $CaCl_2$. In contrast, Deman (1986) found that the gel obtained by $MgCl_2$ was harder than GDL, $MgCl_2$ and $MgSO_4$. These differences could be due to the coagulant concentrations used in different studies or more specifically due to SP to coagulant ratio differences.

4.2.3.2 Surface morphology

The surface morphology of gels induced by different coagulants can be seen in **Figure 4.9**. In all cases, a coarse protein network can be observed, which is a result of aggregation induced by a high concentration of the acidifier or the salts (Munialo et al., 2018). Both gelation mechanisms reduced the negative charge by either proton (H^+) or divalent cations (Ca^{2+}, Mg^{2+}) release, therefore particulate protein aggregates are expected.

The microstructures observed at the lower magnification is similar for all gels, yet GDL and especially $MgSO_4$-induced gels had more black voids (pores) compared with the other two gels. The increased porosity indicates a lower hydration capacity for those gels. However, SEM scans only the surface and it is not a reliable method for assessing the porosity. At large magnifications (**Figure 4.9**) more differences can be seen. The aggregates of the salt-induced gels (especially the Mg^{2+} salts) were thick and heterogeneous in size. $MgCl_2$ protein aggregates were the thickest with a more elongated shape. On the contrary, the protein aggregates in GDL induced gels were small, spherical, and homogeneous.

The morphology of the GDL-induced gels can be explained by the fact that GDL needs to be hydrolysed into gluconic acid for the gelation to occur. Kohyama et al. (1995) found that soy proteins gelled immediately with the use of $CaSO_4$, while the gelation with GDL started after 10 min. A slower gelation rate could explain the formation of a fine structure with dehydrated and spherical particles that can undergo larger strain (**Figure 4.8**). On the other hand, salt-induced gels were more hydrated and compact with a lower ability to withhold the stress during the pressing and thus, particulates were fused into a coarse gel structure resulting to a thick and elongated network (**Figure 4.9**).

Deman (1986) compared the microstructure of soybean gels induced by different coagulants using SEM. All the gels were considerably more porous than the microstructure presented in **Figure 4.9**, with GDL induced gels showing a uniform honeycomb-like structure. These large differences can be explained by the drying process followed during the sample preparation. Deman (1986) used freeze-drying (FD) while we used critical point drying (CPD). Both drying techniques cause shrinkage, yet CPD is considered superior for high magnification SEM (above 3000 times) since it can preserve better both the ultrastructure and surface structure (Nordestgaard and Rostgaard, 1985). Although there were some differences in SEM images, other microscopy techniques such as TEM could be more useful for observing the microstructure of the gels.

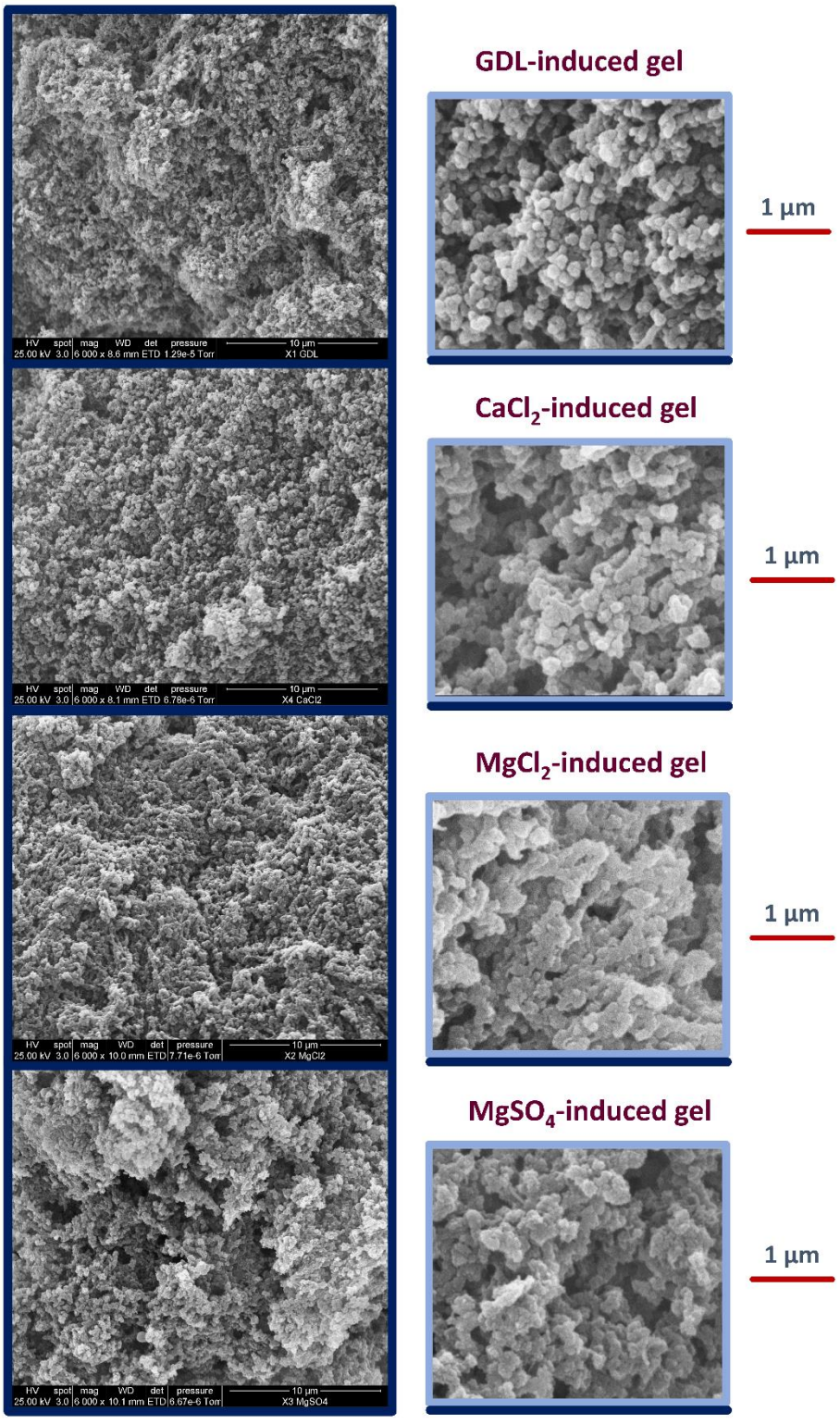


Figure 4.9 Comparison of the surface microstructure (SEM) of small-scale pressed gels induced by four different coagulants: GDL, $CaCl_2$, $MgSO_4$, $MgCl_2$. The left and right images were magnified by 6000x and 40000x times, respectively.

4.2.3.3 Retention of protocatechuic acid in gels

The retention of the bioactive PCA in the SPI gel after the pressing step was higher for the gels induced by GDL ($p<0.05$) and approximately 54 ± 1.4 % of the total amount added before coagulation. All the salt-induced gels gave similar retention percentages ($p>0.05$) that were on average 33.5 % and not significantly different ($p>0.05$) (**Figure 4.10**).

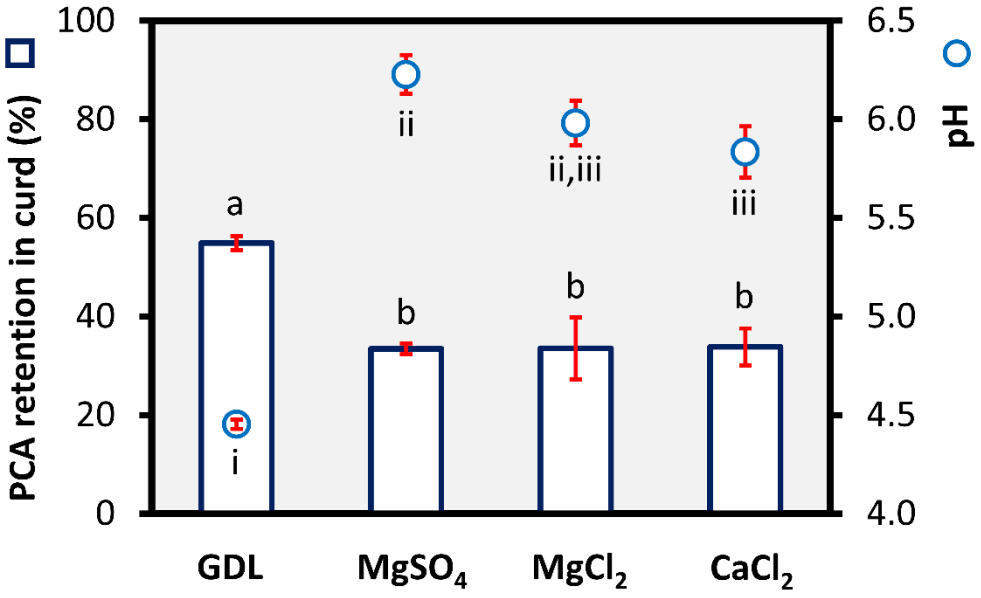


Figure 4.10 Retention of 3.5 mM protocatechuic acid (PCA) in the pellet after centrifugation of gels induced by different coagulants. The bullet markers correspond to the pH of whey.

[1] Values were represented as means ± standard deviations (n=2). [2] Letters a-b and i-iii indicate that values that do not share a letter in the same category are significantly different ($p\leq0.05$). [3] The PCA retention and pH of the gels were analysed using a one-way ANOVA with coagulant type (GDL, $MgCl_2$, $MgSO_4$, $CaCl_2$) as the main factor. The comparison was performed using the Tukey test.

Moreover, the pH of the released whey from the GDL-SPI gel matrix was around 4.5 ± 0.02, which is considerably lower ($p<0.05$) than the pH of the salt-induced gels that ranged between 5.8 and 6.2. The pH of the released whey is an indicator of the gel's pH. It is also, worth mentioning that the released whey from $MgSO_4$-gels had the highest pH (~ 6.2 ± 0.1) among the salt-induced gels, which was significantly higher than the $CaCl_2$-gels' whey ($p<0.05$) (**Figure 4.10**). A strong inverse relationship was found between the pH of the released whey and the retention of the bioactive in the gel ($r = -0.98$, $p = 0.018$).

It has been strongly supported that the retention of phenolics in protein gels depends on the affinity of phenolics with proteins (Giroux et al., 2013, Han et al., 2011, Helal et al., 2015, Lamothe et al., 2016). Phenolics can interact with various proteins, including soy, with both covalent and non-covalent bonds and it is well established by numerous studies (Buitimea-Cantúa et al., 2018, Ferruzzi et al., 2012, Gan et al., 2016b, Kroll et al., 2001, Ozdal et al., 2013, Rawel et al., 2002b, Rawel et al., 2005). Moreover, Hagerman and Butler (1978) showed that proteins with tannins formed insoluble complexes which were favoured at pH values near the isoelectric point of the proteins. The latter could explain the reason that gels induced by GDL had a significantly higher phenolic retention than salt-induced gels ($p<0.05$) (**Figure 4.10**). Indeed, upon the addition of the phenolic acids in the SPI dispersion, the formation of insoluble complexes was observed macroscopically. These complexes disappeared almost immediately after the mixing step (vortexing) which is before the coagulation step. Another explanation of the higher retention in GDL induced gels might be the maximum protein participation in the aggregates at pH close to pI (Puppo et al., 1995, Renkema et al., 2000), which significantly enhanced the strength of the gels (**Figure 4.8**) and might intensify the physical entrapment of the added phenolics in the protein network.

As it was already mentioned, all salt-induced gels retained PCA to a lower extent and to similar levels despite the different salts used ($p>0.05$) (**Figure 4.10**). We hypothesised that the ionised carboxyl group of the phenolic acids (COO-) with a $pK_a \sim 4.5$ might interact with the divalent ions (Ca^{2+}/Mg^{2+} or X^{2+}) to some extent, which is known as metal chelation (Hider et al., 2001) (**Figure 4.11 B**). Consequently, the PA-X^{2+} chelate might escape to the water phase during the pressing step.

As it was mentioned before the addition of the divalent salts shields the negatively charged carboxyl groups on polypeptide chains and enable them to approach each other by forming salt bridges (aggregation) (Zhao et al., 2020). However, due to the neutral pH in the system, the negatively charged phenolic acids might be electrostatically repulsive to the carboxyl groups of the polypeptides or other phenolic acid molecules (**Figure 4.11 C, D**). Although, hydrophobic bonding between phenolics and protein might still be possible, which could explain the residual amount of phenolic acids in the salt-induced gels.

Prabhakaran et al. (2006) studied the effect of coagulants on the isoflavone levels during firm tofu preparation. They found that calcium sulphate was the best coagulant for retaining the native isoflavones within the curd. Although the authors did not use GDL in their study, acetic acid performed equally to $MgCl_2$, but worse than $CaCl_2$ and $MgSO_4$ (Prabhakaran et al., 2006).

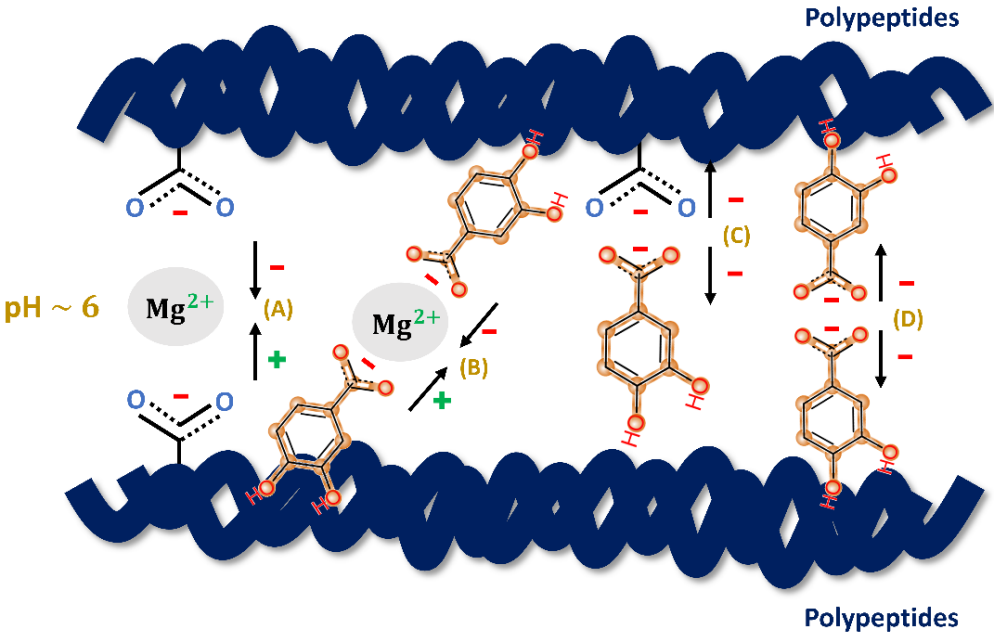


Figure 4.11 Potential explanation for the low retention of PCA observed in salt-induced pressed gels. Salt-bridges formed by divalent ions (A). Metal chelation between phenolic acid and divalent ions (B). Repulsion between the carboxyl groups of phenolic acid and amino acid residues (C) or phenolic acid molecules (D).

4.2.3.4 Retention of phenolic acids in GDL-induced gels

To test the specificity of GDL coagulation in terms of bioactive retention, the GDL induced gels were fortified with three hydrocinnamates (HCA); o-coumaric acid (CMA) caffeic acid (CFA) and ferulic acid (FRA).

As can be seen from **Figure 4.12** the retention of the HCA was between 62 to 73 %, which was significantly better than PCA ($p<0.05$), with CMA having the highest retention of 73.4 ± 1.3 %. It can be seen that the pH of the gel was not a contributing factor for the observed differences ($p>0.05$).

According to Helal et al. (2015), the retention of PP in the cheese curd depends on the following conditions: *a)* phenolics-protein interactions, *b)* the hydrophilicity of

the phenolics, and *c)* the distribution between the solid and liquid fraction of the curd. In the same study, the retention of different PP in the cheese curd varied depending on the structure. The authors found a positive correlation between PP retention and binding affinity with caseins and a negative correlation with the PP hydrophilicity (Helal et al., 2015). Similarly, the native aglycon isoflavones in soybean were more likely to precipitate during the curding process than the glucoside derivatives (Hsia et al., 2016). Both studies indicate that the more hydrophilic the phenolics the more likely to be washed out with whey and water during pressing, which agrees with our results (**Figure 4.12**). PCA was the most hydrophilic and CMA the most hydrophobic among the phenolic acid used with LogP values of 0.86 and 1.80 (Drugbank, 2020, Foodb, 2020), respectively. In contrast, soy protein-phenolic interactions studies have shown a different trend. The more hydrophilic the phenolic acids (two or three -OH groups) the stronger the binding affinity towards β-conglycinin (Rawel et al., 2002b) and thus, the more likely to be retained within the gel.

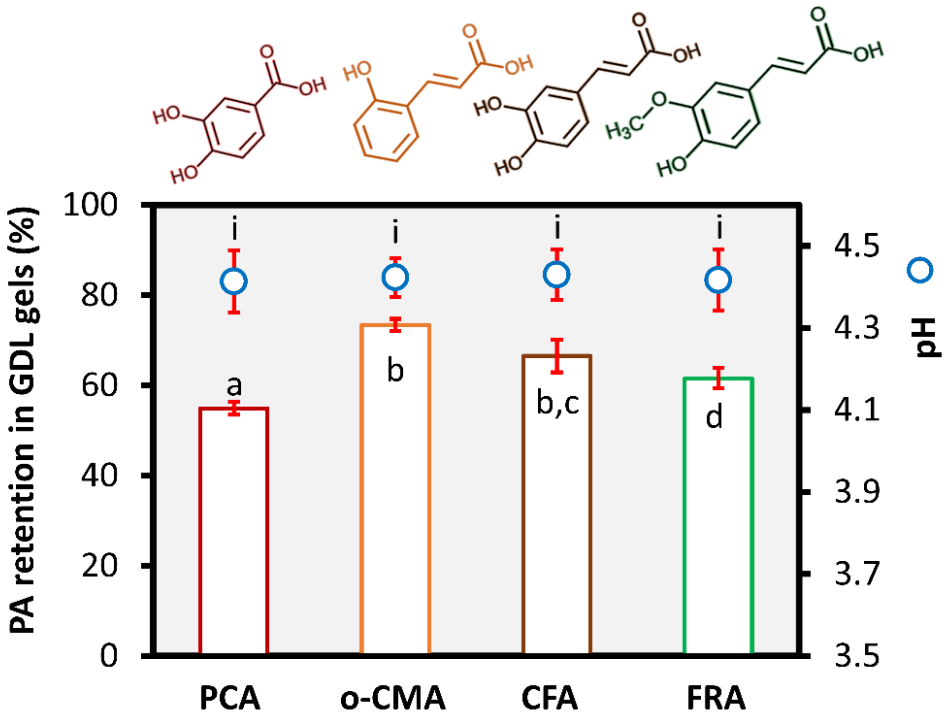


Figure 4.12 Retention of phenolic acids (3.5 mM); protocatechuic (PCA), o-coumaric (CMA), caffeic (CFA), and ferulic acid (FRA) in the pellet after centrifugation of gels induced by GDL. The bullet markers correspond to the pH of whey.

[1] Values were represented as means ± standard deviations (n = 3). [2] Values that do not share a letter in the same category are significantly different ($p \leq 0.05$).

4.3 Conclusions

To produce a silken-tofu model system for our purposes, a concentration of 17 mM GDL and gelation temperatures of 80 ◦C were found as optimum. The addition of phenolic acids increased the pore size of the gels and reduced the water-holding capacity of the gels. The formation of firm-tofu like gels required a higher coagulant concentration (∼30 mM) and both divalent salts and GDL were able to produce gels under pressing. Salt induced gels had significantly different textural characteristics and final pH and overall were less effective in retaining phenolic acids than the ones produced by GDL.

The effect of added bioactives in food products is largely unexplored in the literature. More research on the physicochemical properties of the gels is needed to clarify if the enhancement of soy protein gels with phenolics is possible without adverse effects. GDL and $MgSO_4$ were chosen for further experiments in the next chapters. The reason for selecting those two was that they formed the hardest and the softest gel textures. Therefore, the effect of texture and microstructure could be assessed in the same gel format.

Chapter 5

Soy protein pressed gels: Gelation mechanism affects the *in vitro* proteolysis and bioaccessibility of added phenolic acid

Abstract

In this study, a model system of firm tofu (large scale pressed gel) was prepared to study how the coagulation mechanism - acidification with GDL or coagulation with $MgSO_4$ - affected the physical properties of the gels along with their proteolysis. The two types of gels were also fortified with protocatechuic (PCA) and coumaric acid (CMA) to test whether they can be used as bioactive delivery systems.

Results showed that the coagulation mechanism affected both the macro- and microstructure of the larger scale pressed gels, which is in agreement with **Chapter 4** findings. Transmission electron microscopy revealed that GDL-induced gels were denser while $MgSO_4$ induced gels had thicker aggregates and larger porosity. Although the addition of PCA did not seem to affect the microstructure significantly, CMA induced a dramatic increase in porosity. $MgSO_4$ gels had almost double proteolysis percentages throughout the *in vitro* digestion and showed a significantly higher amino acid bioaccessibility than the GDL gels (essential amino acid bioaccessibility of 56% versus 31 %; $p<0.05$). Lastly, both gel matrices showed a similar phenolic acid release profile, on a percentage basis (~80% for PCA and ~100% for CMA). However, GDL gels delivered significantly higher masses of bioactives under simulated intestinal conditions because they could retain more of the bioactives in the gel after pressing. It was concluded that the coagulation mechanism affects both the macro- and microstructure of the soy protein pressed gels and as a result their protein digestibility. Both pressed gel matrices are promising delivery systems for bioactive phenolic acids

5.1 Introduction

The pressing process during the production of the firm or extra-firm tofu considerably alters the composition of the gel i.e. protein and moisture content (Shurtleff and Aoyagi, 1975) and as a consequence, their physical characteristics. As it was discussed in **Chapter 4**, the type of coagulant; salt or acid significantly affected the textural properties and the retention of added phenolics in firm tofu. It is not clear, however, whether the type of coagulant or the physical characteristics of the soy protein gels can affect the extent of proteolysis and the bioaccessibility of added phenolics. Therefore, this was the main focus of this chapter.

Previous research that studied the effect of different coagulation mechanisms on the *in vitro* proteolysis of soy protein gels, focused on unpressed soy protein gels and did not offer insights on the microstructure of the gels (Rui et al., 2016). Kozu et al. (2014), demonstrated that the gel particles of soft tofu were disintegrated more easily and quickly than the firm tofu during *in vitro* processing. In a recent study, Reynaud et al. (2020) found that soy proteins from a soya juice matrix were more prone to *in vitro* proteolysis than fresh tofu. Although the two soy matrices had a different protein concentration, the authors concluded that the food matrix had a strong influence on protein digestibility (Reynaud et al., 2020). Thus, the physical characteristics of the soy protein gels might affect their protein digestibility. The latter is of great importance for people consuming soy products as a main source of proteins.

Recently, there has been a systematic attempt to answer this question by studying the release (bioaccessibility) of native or added phenolics from various food matrices. Some of the existing food formats that have been fortified with phenolics are mainly dairy products, such as milk (Lamothe et al., 2014, Moser et al., 2014), yoghurt (Chouchouli et al., 2013, Georgakouli et al., 2016, Helal and Tagliazucchi, 2018, Karaaslan et al., 2011, Lamothe et al., 2014, Petrotos et al., 2012), cheese (Giroux et al., 2013, Han et al., 2011, Helal et al., 2015, Lamothe et al., 2016, Lamothe et al., 2014) and ice cream (Çam et al., 2014). The food structure can either facilitate the release of polyphenols or impede it. Some types of soy protein gels have been proposed as good vehicles for modulating the release of riboflavin under *in vitro* digestion conditions (Maltais et al., 2009, Maltais et al., 2010) and it would worthwhile to investigate them further.

To our knowledge, there are no available studies on protein digestibility of pressed soy protein gels or their use for studying phenolic acid bioaccessibility. The study in this chapter had two aims:

- Investigate the impact of two different gelation mechanisms (acidification and salt aggregation) on the physical properties and microstructure of pressed soy protein gels, as well as the effect on proteolysis patterns. In contrast to **Chapter 4**, the gels produced here were more representative to the commercial firm tofu pressing method.

- Explore how the two types of coagulation influence the bioaccessibility of added phenolic acids and thus, the potential of firm tofu as a carrier of biofunctional compounds for health improvement.

5.2 Experimental information

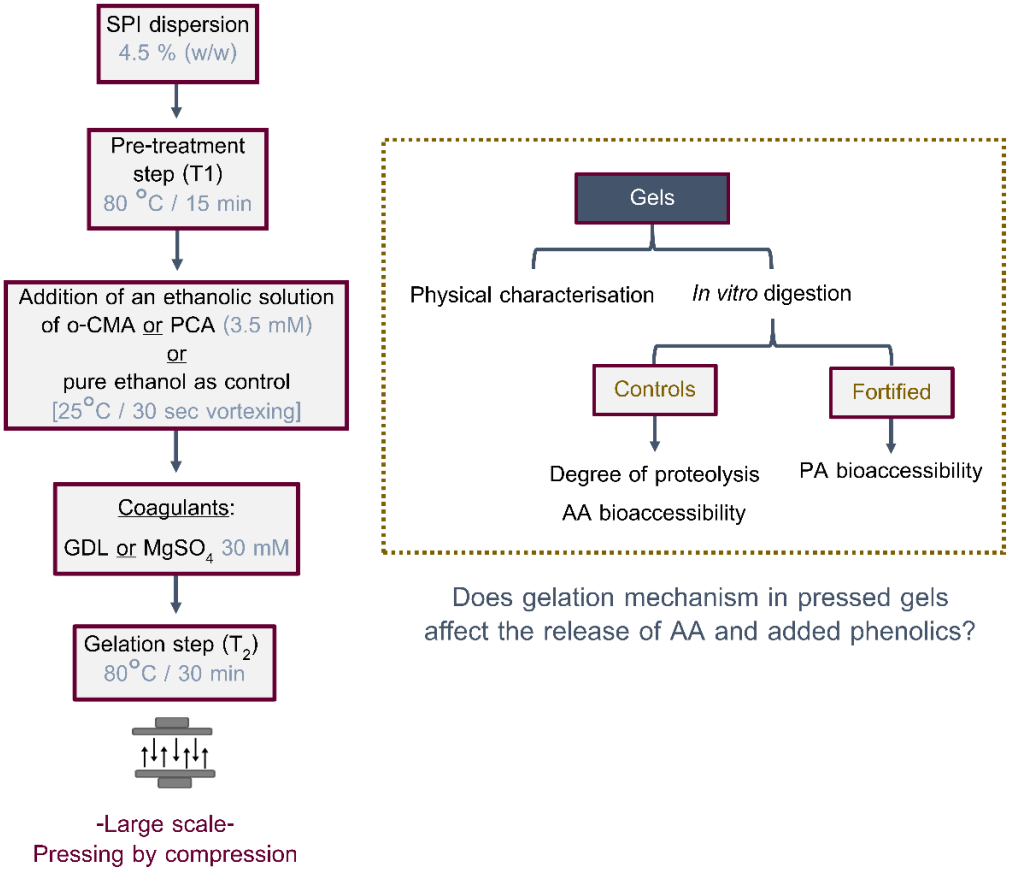


Figure 5.1 Overview of the gelation method and experimental design of Ch. 5.

5.3 Results and discussion

5.3.1 Physical properties of gels

5.3.1.1 Characteristics of fortified and control gels

As can be seen from (**Table 5.1**) the gelation mechanism affected some of the physical characteristics of the gels. Water and whey were expelled during the pressing of the gels, which reduced the moisture content. Whey proteins mainly consist of soluble proteins that do not solidify during the gelling process. Gels produced by GDL had a lower moisture content than the one produced by $MgSO_4$ ($p>0.05$) and thus, lower yield. In other words, there was an inverse relationship between the yield and the moisture content of the gels (**Table 5.1**).

Salt induced gels had a significantly lower protein concentration than the GDL-induced gels ($p<0.05$), which is a consequence of the larger water retention capacity of these gels. Also, the soluble fraction of proteins that drained off with whey was significantly higher ($p<0.05$) than the GDL-induced gels. The protein content and participation in the network can largely affect the stiffness of the final gels (Renkema et al., 2002).

Even though there were large differences in whey pH ($p<0.05$) between the GDL and $MgSO_4$ gels, the net charge of all the wheys was slightly negative (**Table 5.1**). The pH of GDL whey was approximately 4.7, which is close to the isoelectric point of the soy proteins, while the pH of $MgSO_4$ whey was between 6.1 and 6.4. These results could be explained by the DLVO theory (Verwey and Overbeek, 1947, Derjaguin and Landau, 1941). At high ionic strength, the charge is screened, and the electrostatic repulsion is reduced. Both Hermansson and Lee and co-authors found that at $CaCl_2$ concentrations ≤ 0.1 M, the solubility of soy proteins strongly decreased (Hermansson, 1978, Lee et al., 2003). Similar results are expected in the case of $MgSO_4$.

Phenolic acid addition (PCA and CMA) did not alter the characteristics of the gels to a large extent. Han et al. (2011) found a significant reduction in the hydration capacity of cheese curd after the addition of various polyphenols (PP). This phenomenon was explained by the potential hydrophobic interaction of phenolics with milk proteins which, thereby, can disrupt the amino acid side chain interactions and

reduce the quantity of the entrapped water (Han et al., 2011). Nevertheless, the only significant effect observed in our system was the reduction of the whey pH in $MgSO_4$-induced gels, by approximately 0.3 units ($p<0.05$) (**Table 5.1**). This pH change was expected because phenolic acids are weak acids (McMurry, 2011).

The retention of the phenolic acids is the parameter that determines the final concentration of the bioactive in the gels, after pressing. According to **Table 5.1**, CMA showed better retention than PCA in both types of gels, and in the case of GDL induced gels, the difference was significant ($p<0.05$). The difference in phenolic acids retention can be attributed to their polarity. PCA is more water-soluble than CMA and it has a higher affinity towards the water phase (Helal et al., 2015). Preliminary experiments showed that the pressing affects the bioactives' retention. The more the gel is pressed, the less the phenolic acids will be retained (results not shown). Although the trend was the same, the phenolic acid retention values presented here are significantly lower than the ones from **Chapter 4**. The differences can be attributed to the alternative ways used to press the gels; centrifugation (**Chapter 4**) over mould pressing (**current chapter**).

Table 5.1 Physical characteristics of two types of pressed soy protein gels (large scale) induced by GDL and $MgSO_4$ with added phenolic acids: protocatechuic acid (PCA) and coumaric acid (CMA).

	GDL-Induced gels			MgSO4-Induced gels		
Properties	**Control**	**PCA**	**CMA**	**Control**	**PCA**	**CMA**
Moisture (%)	83.61 ± 0.43^{ab}	84.18 ± 0.80^{abc}	83.36 ± 0.34^{a}	86.16 ± 0.74^{bd}	85.24 ± 0.38^{cd}	85.65 ± 0.11^{cd}
Yield (%)	31.89 ± 2.17^{a}	33.14 ± 5.15^{a}	34.10 ± 3.51^{a}	38.70 ± 5.18^{a}	37.13 ± 3.12^{a}	37.87 ± 3.30^{a}
Protein in curd (%)	15.12 ± 0.83^{a}	14.74 ± 0.48^{ab}	15.39 ± 0.38^{a}	13.23 ± 1.05^{bc}	13.24 ± 0.40^{bc}	12.30 ± 0.54^{c}
Soluble protein in whey (%)	0.13 ± 0.02^{a}	0.13 ± 0.02^{a}	0.12 ± 0.00^{a}	0.35 ± 0.03^{b}	0.35 ± 0.05^{b}	0.30 ± 0.05^{b}
Whey (%)	67.00 ± 2.97^{a}	65.89 ± 4.23^{a}	64.49 ± 0.83^{a}	60.89 ± 4.66^{a}	63.85 ± 2.57^{a}	63.28 ± 2.93^{a}
pH of whey (-)	4.75 ± 0.08^{a}	4.72 ± 0.11^{a}	4.81 ± 0.06^{a}	6.44 ± 0.03^{b}	6.06 ± 0.04^{c}	6.09 ± 0.07^{c}
Zeta potential of whey (mV)	-3.36 ± 0.21^{a}	-2.04 ± 0.05^{b}	-2.73 ± 0.04^{cd}	-2.45 ± 0.07^{d}	-2.63 ± 0.03^{d}	-3.08 ± 0.11^{ac}
Retention of PA in curd (%)	n/a	40.16 ± 6.62^{a}	58.76 ± 3.59^{b}	n/a	35.01 ± 6.72^{a}	43.83 ± 6.17^{a}
Protein/ PA ratio (% w/w)	n/a	191 ± 20^{ab}	139 ± 22^{c}	n/a	234 ± 30^{a}	155 ± 4^{bc}

[1] Values were represented as means ± standard deviations (n=3-5). [2] Letters a-d indicate that values that do not share a letter in the same row are significantly different ($p \leq 0.05$). [3] The gels' properties (1st column) were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (control, PCA, CMA) and coagulant mechanism (GDL- and $MgSO_4$-). The significance of the factor's interaction mean values was assessed with the Tukey test

5.3.1.2 Textural properties

Texture analysis was performed by measuring fracture stress (kPa), Young's modulus (kPa) and fracture strain (-) of the gels. According to previous studies, fracture properties are important for understanding the mastication process as well as influencing disintegration during digestion (Kohyama et al., 2008).

The results showed that the gelation mechanism affects fracture stress and Young's modulus significantly ($p<0.05$) (**Figure 5.2 A-B**). On the contrary, all the gel samples regardless of the gelation mechanism or the addition of phenolic bioactives were similar in fracture strain. The only exception was the CMA-$MgSO_4$ gel that gave a slightly higher fracture strain (**Figure 5.2 C**). The last parameter is a measure of gel's elongation at break. The higher the fracture strain value, the more extensible is the gel. Therefore, the brittleness of the different gels is also similar ($1/\varepsilon fr$) (Walstra, 2002).

The effect of different coagulants on the textural properties of soy protein gels has been observed in many studies (Rui et al., 2016, Prabhakaran et al., 2006, Li Tay et al., 2006, Kohyama et al., 1995, Deman, 1986). Rui et al. (2016) found that soy protein gels with different GDL concentrations were significantly harder than $MgCl_2$ and MTGase induced gels. However, there is only a limited number of studies assessing the texture of $MgSO_4$ gels. According to Li Tay et al. (2006), the coagulation power of $MgSO_4$ soy protein gels was the lowest among various salts.

Finally, the addition of PCA and CMA affected the textural properties of both types of gels. PCA reduced the strength and stiffness of the GDL-gels but had the opposite trend in $MgSO_4$ gels. However, CMA affected only the $MgSO_4$ gels in both parameters (**Figure 5.2 A-B**). Also, the pH of the gels can affect the phenolic acid properties, since the pk_a of the -COOH group of PCA and CMA is between 4.0 and 4.3 (Drugbank, 2020, Foodb, 2020). GDL gels had a low pH close to the pk_a of the phenolic acids, which were in their protonated form. On the other hand, the pH of $MgSO_4$ gels was above 6, which means that the phenolic acids were negatively charged. The state of the phenolic acids in the different gels is important because it can determine their reactivity, which might be associated with the effect on the textural properties found in **Figure 5.2**.

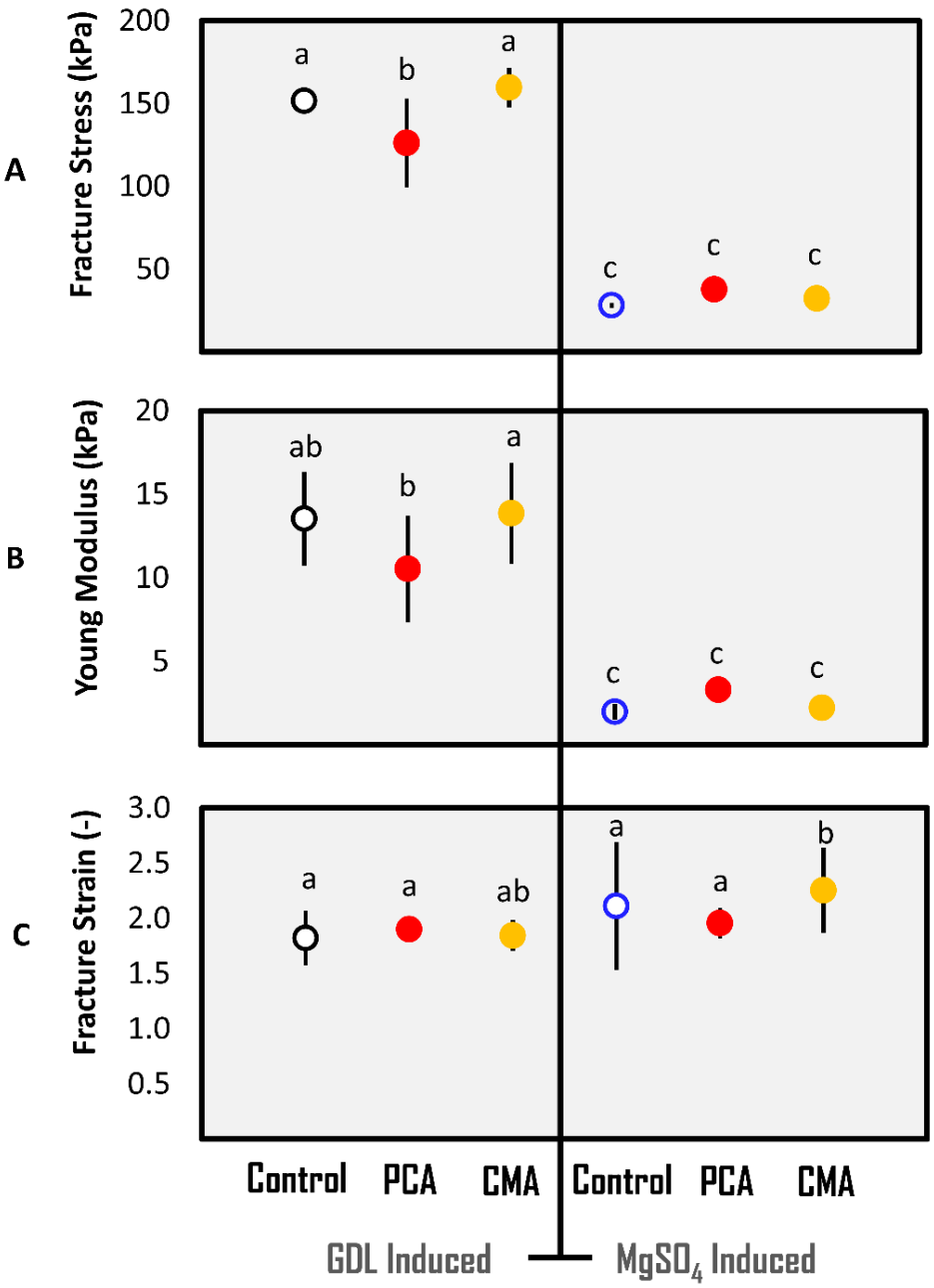


Figure 5.2 Textural properties of pressed soy protein gels induced by GDL and $MgSO_4$ with added phenolic acids: A) Fracture stress (kPa), B) Young's modulus (kPa) and C) Fracture strain, at 80 % of deformation. The red colour bullets indicate the addition of the PCA and the yellow the addition of CMA.

[1] Values were represented as means ± standard deviations (n≥8). [2] Letters a to c indicate that values that do not share a letter in the same graph are significantly different (p≤0.05). [3] The textural properties (fracture stress, Young's modulus, fracture strain) were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (control, PCA, CMA) and coagulant mechanism (GDL- and $MgSO_4$-). The significance of the factor's interaction mean values was assessed with the Tukey test.

5.3.1.3 Microstructural characteristics of the SPI gels

In **Figure 5.3** and **Figure 5.4**, the SEM and TEM micrographs of the gels can be found. The three-dimensional protein network of the gels' surface can be seen with the SEM imaging **(Figure 5.3 A & Figure 5.4 A)**, while the inner structure of the gels is more obvious with TEM imaging **(Figure 5.3 & Figure 5.4 B).** The dark areas of the TEM images correspond to the protein network or aggregates and the white areas to the aqueous phase.

Overall, particulate networks can be seen in all cases, formed through a rapid aggregation that occurring in both gelation mechanisms. However, the protein networks between GDL and $MgSO_4$ gels are significantly different. Although the gels' microstructure consisted of a coarse network of monodisperse spherical particles in both types of gels, the diameter of the particles and the density of the network varied between the different gels. On the one hand, GDL induced gels (both control and PCA) (**Figure 5.3 B1-B2**) were dense with a more "curly" protein network and with many intermediate tiny pores. In contrast, $MgSO_4$ induced gels had larger, thicker aggregates surrounded by large pores (**Figure 5.4 B1-B2**). However, the control GDL gel showed a more porous network on the surface (**Figure 5.3 A1**) than the $MgSO_4$ control gel, which was more compact and denser (**Figure 5.4 A1**). Nevertheless, SEM is not the appropriate method of assessing the porosity since it only gives a view of the samples' surface (Silva et al., 2015). A range of TEM images was used for quantifying the porosity, based on the procedure described in **section 3.2.4.12**.

The addition of PCA and CMA induced changes in both types of gels. Firstly, the addition of PCA increased the density of the $MgSO_4$ gels network and significantly reduced the porosity (**Figure 5.4 B2**). This could explain the small increases that were observed in yield stress and Young's modulus (**Figure 5.2 A, B**). It was found that the trend was the opposite in the GDL gels, following the addition of the PCA. The structure became less dense, but there was no significant change in the porosity (**Figure 5.3 B2**). Similarly, this could explain the reduction in the yield stress and Young's modulus that was observed for this gel.

We hypothesised that the addition of PCA interfered with the protein-protein interactions sites in GDL induced gels which might lower the density of the protein network and consequently the firmness of the gels. However, the effect of PCA was not significant enough to reduce the hydration capacity of the gels (**Table 5.1**). The

addition of CMA induced more dramatic changes to the microstructure of both GDL and $MgSO_4$ gels. Noticeably, the shape and size of the protein aggregates in gels with added CMA was identical, in both types of gels (**Figure 5.3 B3** & **Figure 5.4 B3**). Although the aggregates formed after the addition of CMA were more linear and thinner, which suggest less aggregation, there were no other effects on the gel characteristics.

Finally, crystal formation was observed on the surface of the $MgSO_4$-PCA and $MgSO_4$-CMA gels (**Figure 5.4 A2-3**) and some small evidence on the GDL-CMA gel (**Figure 5.3 A3**). However, the crystal existence was not confirmed with X-ray diffraction experiments (results not shown), which might be an indication of an artefact during the SEM sample preparation.

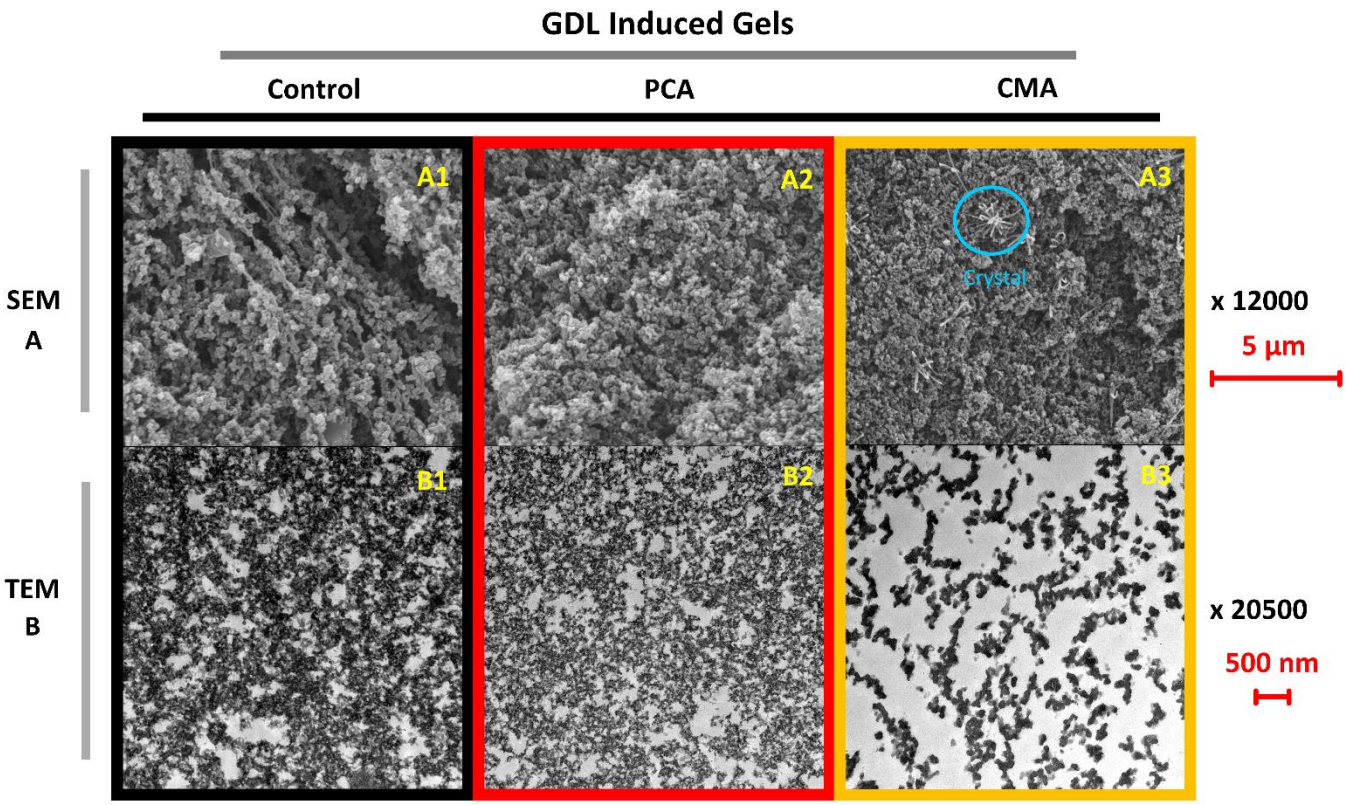


Figure 5.3 Microstructural characteristics of soy protein gels induced by GDL. The SEM micrographs were presented on the A) row and the TEM micrographs on the B) row. GDL induced gels without the addition of bioactives (A1, B1), gels with the addition of 3.5 mM of PCA (A2, B2) and 3.5 mM of CMA (A3, B3).

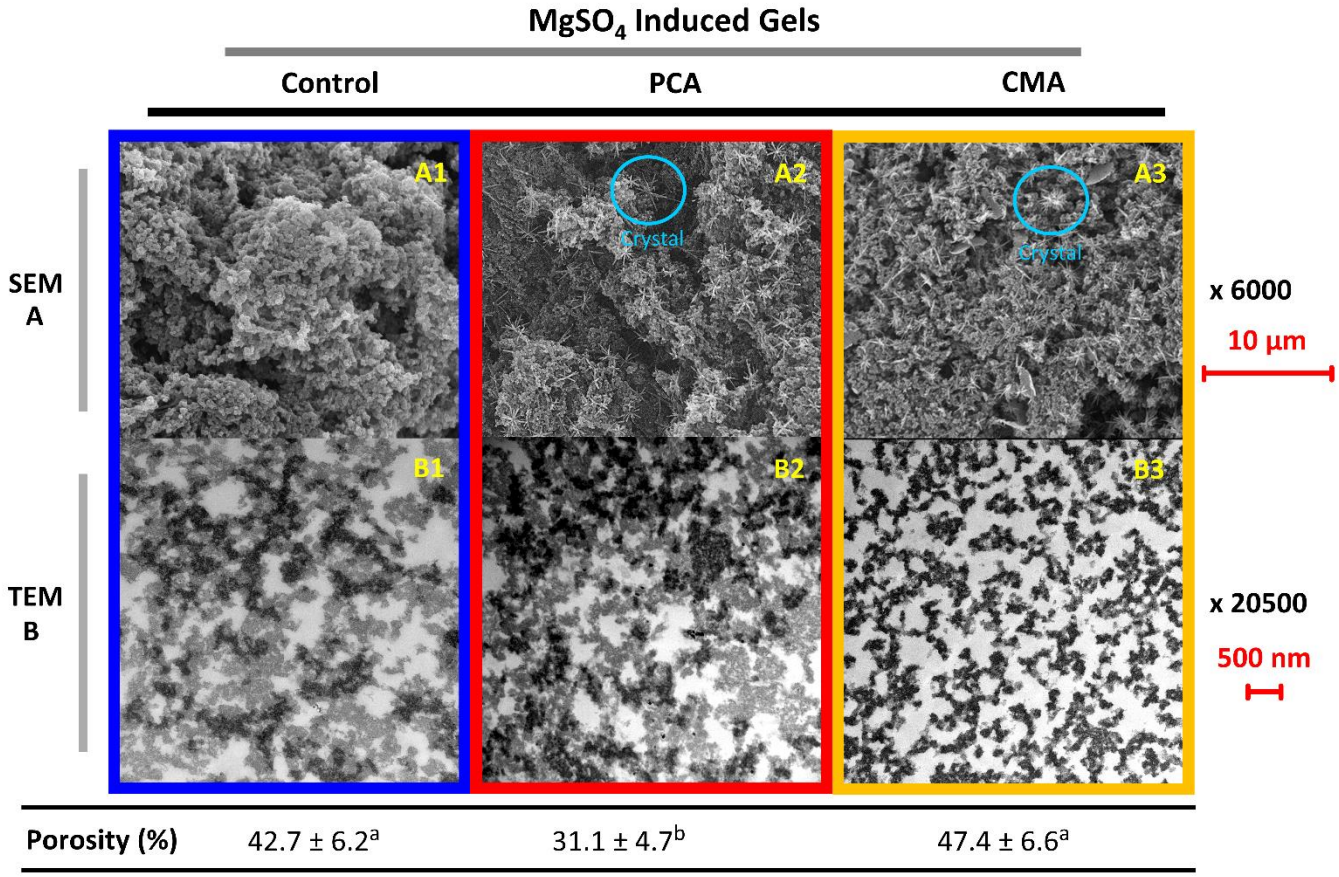


Figure 5.4 Microstructural characteristics of soy protein gels induced by $MgSO_4$. The SEM micrographs were presented on the A) row and the TEM micrographs on the B) row. Gels without the addition of bioactives (A1, B1), gels with the addition of 3.5 mM of PCA (A2, B2) and 3.5 mM of CMA (A3, B3).

5.3.2 Digesta characterisation

5.3.2.1 Degree of proteolysis

Free amino acids and oligopeptides released during *in vitro* gastrointestinal digestion were measured using the o-phthaldialdehyde (OPA) assay (**Figure 5.5**) on the liquid phase of the digesta. The method was conducted only on the control pressed gels (without bioactives) due to adverse reactions between the bioactives and the ABSF inhibitor (**section 3.2.5.4**).

The OPA reagent allows the estimation of the number of primary amino groups released during protein hydrolysis (Church et al., 1985) which correlates with the proportion of amide bonds broken (**section 3.2.6.2**).

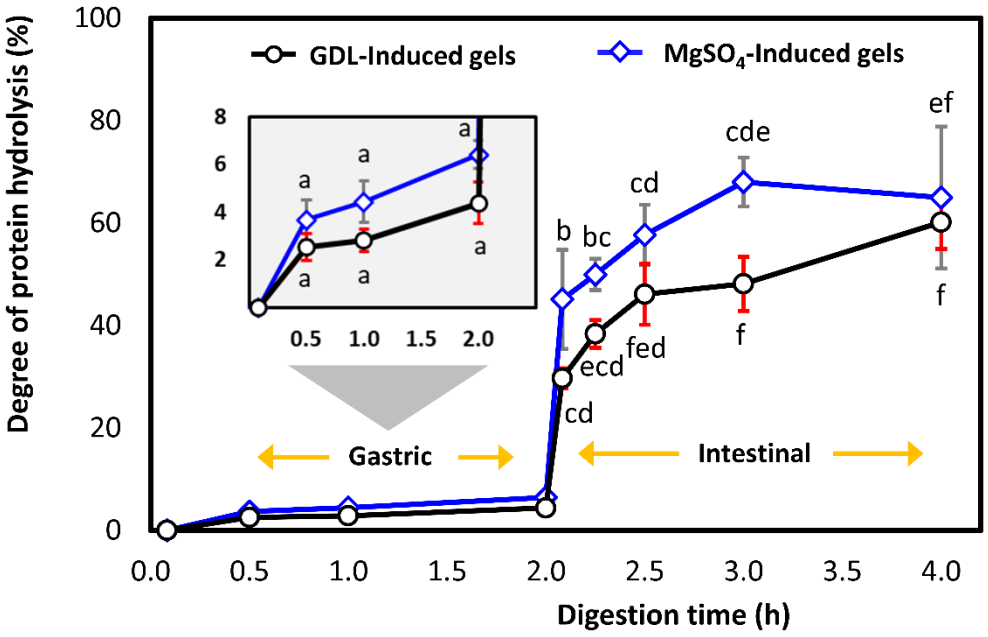


Figure 5.5 Degree of protein hydrolysis (%) of pressed gels (control) gels induced by GDL (○) and $MgSO_4$ (◇) during *in vitro* simulation of digestion.

[1] Values were represented as means ± standard deviations (n =3-5). [2] Letters a and b indicate that values that do not share a letter in the same time point are significantly different ($p \leq 0.05$). [3] The degree of protein hydrolysis was analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of coagulants in control gels (GDL-, $MgSO_4$-) and digestion time (0-4h). The significance of the factor's interaction mean values was assessed with the Tukey test.

In the case of the GDL-induced gel, the amount of free amino acids and peptides liberated by the end of gastric processing was 0.32 ± 0.15 mmol serine

equivalent /g of protein corresponding to 4.4 ± 0.9 % of the total protein hydrolysis. Whereas, $MgSO_4$-induced gels had a degree of proteolysis value of 6.4 ± 0.6 % ($p<0.05$). Pepsin is an endopeptidase that cleaves the proteins internally to smaller polypeptides. It has a higher specificity for hydrophobic/ aromatic amino acids, and it typically digests 10-15 % of dietary proteins in the stomach (Goodman, 2010). Our degree of hydrolysis values at the end of the gastric phase are comparable with the ones reported for fresh tofu using the INFOGEST protocol (DH ~2 to 4 % after 120 min) (Reynaud et al., 2020). The authors used the ninhydrin assay to measure the release of α-amino groups, which could explain the small differences compared to our results (Reynaud et al., 2020).

The addition of the pancreatin juice increased the extent of protein hydrolysis rapidly (**Figure 5.5**). Pancreatic juice contains a mixture of peptidases; both endo and exo-peptidase, with various specificities, that results in the production of smaller peptides and free amino acids. After 5 min of intestinal processing, a large fraction of amide bonds had been broken in both samples corresponding to proteolysis percentages of 29.7 ± 1.9 % for GDL and 45.1 ± 9.6 % for $MgSO_4$-induced gels ($p<0.05$), respectively. After 2 h at intestinal conditions, 60.2 ± 5.2 % and 64.9 ± 13.8 % of protein from GDL and $MgSO_4$ gels ($p>0.05$) had been degraded into oligopeptides and/ or free amino acids. Therefore, a steady increase in the release of the α-amino groups was observed in both types of gels, with $MgSO_4$ gels having higher percentages throughout the *in vitro* processing.

The final degree of proteolysis values (end of the intestinal phase) were different from other articles that studied soy protein gels matrices. Rui et al. (2016) reported DH between 80 to 90 % in soy protein unpressed gels, while Reynaud et al. (2020) reported DH of around 30% in fresh tofu (pressed gel). In general, there is some inconsistency in the degree of proteolysis values reported. Lamothe et al. (2014) compared the protein digestibility of milk, yoghurt, and cheese. Although they found that cheese (pressed product) was more resistant to proteolysis (Lamothe et al., 2014), the reported values of protein digestibility (by TCA method) were very high (~ 90 %). In contrast, other studies on dairy products have reported a degree of hydrolysis below 40 % for pressed cheese, such as mozzarella (Lorieau et al., 2018).

Some of the reasons for this variation are the following; firstly, compositional differences, especially protein content, which can affect the enzyme-to-substrate ratio

(E:S) during *in vitro* digestion. Secondly, the *in vitro* digestion protocol used; in many protocols, the enzyme activities were not estimated. Finally, the extent of enzyme autolytic activity, which is usually ignored.

Our blank *in vitro* digestion trials showed that the pancreatic extract used in this study was susceptible to autolysis (self-digestion) (**Table S 1**). The protease autolytic activity reduces the proteolytic activity of the enzymes (Stewart et al., 2019). In addition, enzyme autolysis releases peptides and amino acids bearing alpha-amino groups, leading to inflation of OPA and amino acid bioaccessibility results (**Figure 5.5, Figure 5.6**). Qiao and co-workers studied the autolytic reaction of pepsin and pancreatic enzymes and have reported important findings (Qiao et al., 2002, Qiao et al., 2005). We believe that the autolytic activity of the digestive enzymes is a very serious issue that needs to be researched further.

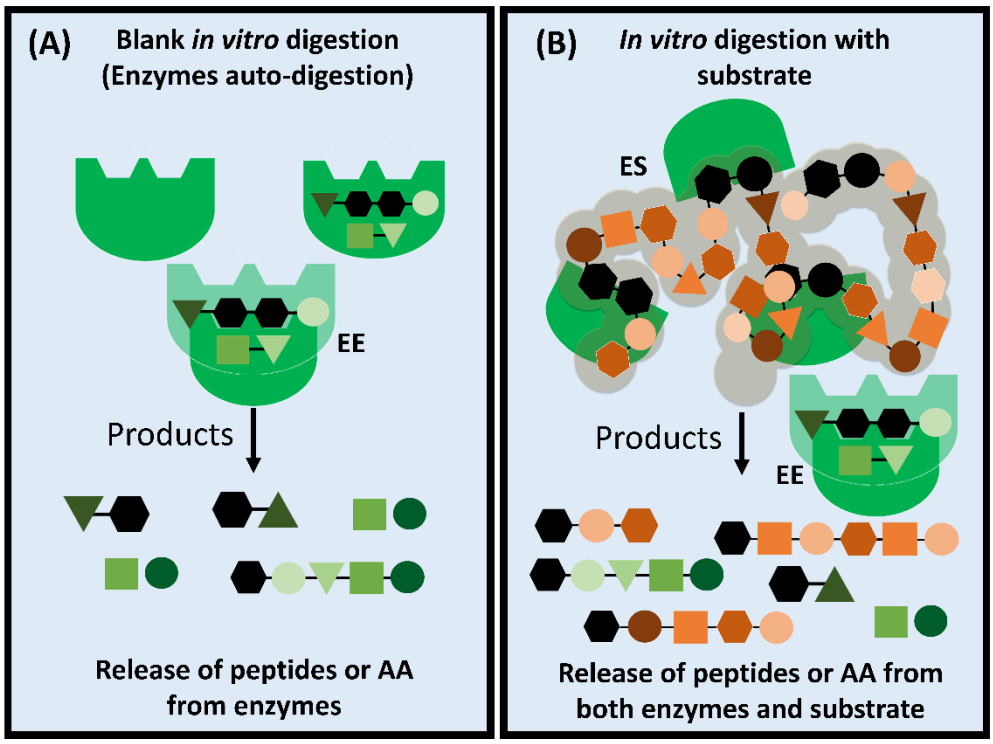


Figure 5.6 Explanation of the protease autolytic activity. Both substrate and enzymes consist of polypeptides (shapes = different AA) that can be hydrolysed during *in vitro* digestion and can cause overestimation of the proteolysis results. Blank *in vitro* digestion and enzyme auto-digestion (A) and *in vitro* digestion with the substrate (B).

5.3.2.2 Free amino acid determination

The amount of free amino acids (FAA) released at the end of the gastric and intestinal processing was measured with o-phthaldialdehyde (OPA) precolumn derivatisation followed by HPLC analysis (**section 3.2.6.3**).

The total amount of FAA released at the end of the gastric phase was negligible (0.71 ± 0.13 % for GDL and 0.81 ± 0.05 % for $MgSO_4$ gels). The overall trend is similar to the degree of proteolysis results **(Figure 5.5).** The release of all amino acids, both total and essential, at the end of the intestinal phase, was considerably higher ($p<0.05$) for the $MgSO_4$ than the GDL coagulated gels, both total (36.4 ± 4.3 %; 21.0 ± 4.6 %) and essential AA (55.6 ± 6.4 %; 31.2 ± 7.3 %), respectively **(Figure 5.7)**. Rui et al. (2016) found a similar trend for the salt-induced ($MgCl_2$) soy protein gels, although the reported values were only slightly different than the GDL-induced gels.

All amino acids were less than 60 % bioaccessible from GDL-induced gels, which was significantly lower than the $MgSO_4$ gels ($p<0.05$). However, the amino acid analysis profile showed a similar trend for both types of gels. Tyrosine, phenylalanine and arginine were the most bioaccessible AAs with percentages between 85 to 81 % for the $MgSO_4$ gels and 58 to 48 % for the GDL gels, respectively **(Figure 5.7)**. The same profile has been reported in soy protein gels before (Rui et al., 2016).

In addition, lysine, leucine, histidine, methionine and isoleucine were moderately bioaccessible with percentages ranging between 76 to 43 % for the $MgSO_4$ gels and 22 to 43 % for the GDL gels, respectively. The negatively charged aspartic and glutamic acids were the least bioaccessible with percentages below 10 % in both types of gels **(Figure 5.7)**. According to in vivo studies, glycine, glutamic acid and aspartic acid are transported as part of small peptides which are hydrolysed further by the action of specific intracellular peptidases. Therefore, the small peptides that the aforementioned amino acids participate cannot be further cleaved under the current *in vitro* digestion conditions (Gray and Cooper, 1971). Another hypothesis of the low Glu/Asp levels is that they might bind to larger materials because their carboxyl group is ionised under the intestinal conditions.

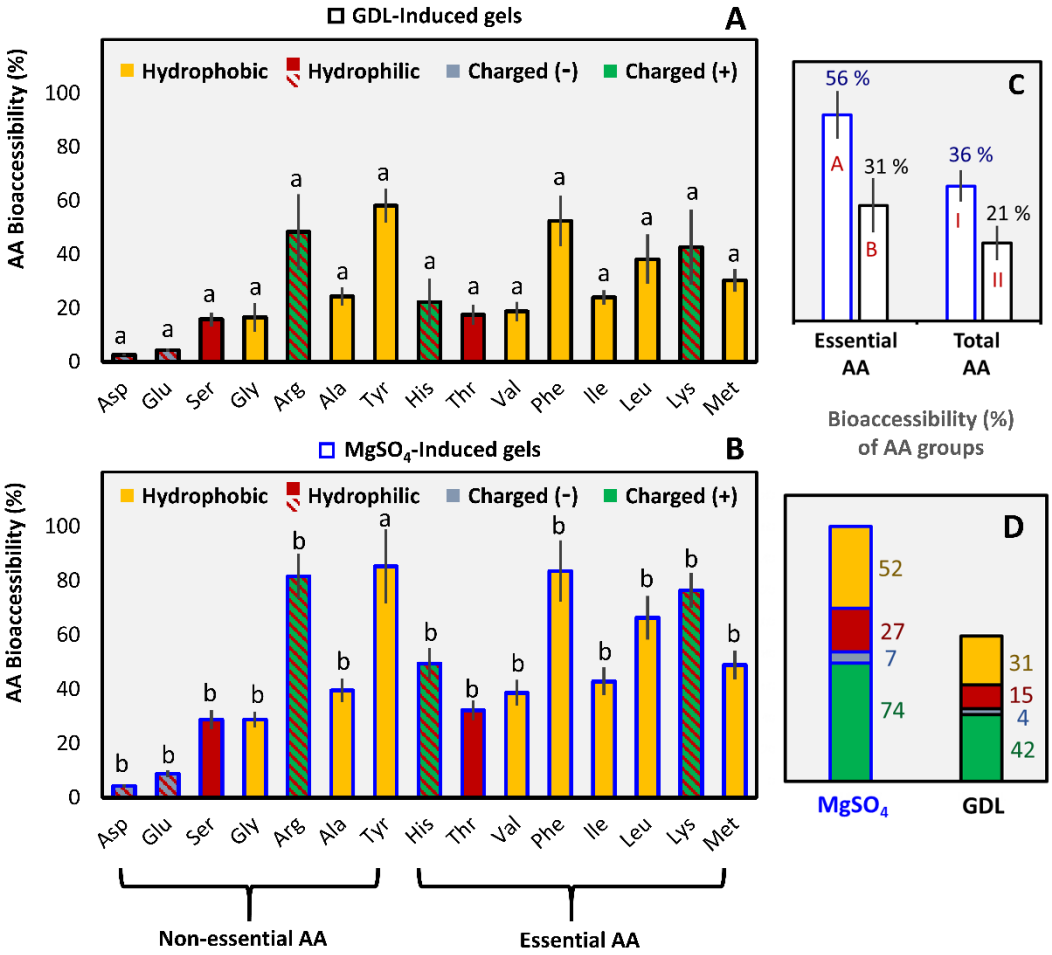


Figure 5.7 Bioaccessibility (%) of total, individual and grouped amino acids at the end of the intestinal processing of the control pressed gels. The black frame around the bars corresponds to GDL-induced gels and the blue to $MgSO_4$-induced gels. Amino acids were grouped according to Petsko and Ringe (2004) and Damodaran et al. (2007).

[1] Values were represented as means ± standard deviations ($n \geq 3$). [2] Letters a-b, A-B and I-II indicate that values that do not share a letter in the same group (AA, EAA, TAA) are significantly different ($p \leq 0.05$). [3] The significant difference of the individual AA (A, B) and grouped AA: EAA and TAA (C) between GDL and $MgSO_4$ induced gels were assessed with the student t-test.

The release of the basic amino acids arginine and lysine was favoured because they are the target of the specific action of both trypsin and carboxypeptidase B (Goodman, 2010). Similarly, tyrosine, phenylalanine and leucine are the cleavage points of both pepsin and chymotrypsin (Goodman, 2010).

Our results suggest that the gelation mechanism can significantly affect the protein digestibility rates, which signifies the importance of the gel's physical properties. $MgSO_4$ gels had a significantly softer texture, larger porosity and a less convoluted protein network, which could increase the accessibility to the digestive enzymes and therefore render them more prone to proteolysis.

Finally, a high concentration of free amino acids was found in our blank digestion trials. The concentration of some amino acids in the blank digestions was equal or higher than in the gel samples (**Table S 1**). Our results confirmed the autolytic reaction of the pancreatic enzymes, mentioned in section **5.3.2.1** showed that the extent of the autolytic reaction is different depending on the presence or absence of substrate, which is in contradiction with the findings of Qiao et al. (2005) and indicates that further research is needed.

5.3.2.3 *In vitro* bioaccessibility of phenolic acids

The bioaccessibility of phenolic acids during *in vitro* digestion was illustrated in two ways; the concentration of bioactives detected in the liquid fraction of digesta (**A**) and the bioaccessibility (%) based on the mass remaining within the gel after pressing (**B**). Although the bioaccessibility profiles of PCA and CMA from both $MgSO_4$ and GDL-induced gels were not significantly different on a percentage basis ($p>0.05$) (**Figure 5.8 B**), GDL gels were superior ($p<0.05$) (**Figure 5.8 A)**, because they retained more of the bioactives in the gel and therefore released a greater mass of CMA and PCA. This statement is very important because it shows that the structure and texture of the gels do not significantly affect the relative (normalised) release rate of the two phenolic acids ($p>0.05$). Comparison of **Figure 5.5** and **Figure 5.8 A, B** showed that the degree of proteolysis of the gels did not affect the bioaccessibility of the bioactives which was also confirmed by blank digestion experiments (results not shown). The trend observed in **Figure 5.8 A** is a result of physical phenomena involved during gelation as a result of the coagulant mechanism (indirect effect). In summary, GDL coagulation produced a low yield of gel with high retention of phenolic acids, whereas $MgSO_4$ gave higher gel yields but lower retention of phenolic acids (**Figure 5.8 C-D; Figure S 1).**

The most significant difference between the different coagulants is that $MgSO_4$-induced gels tend to result in a higher bioactive release at the end of the oral phase (**Figure 5.8 A, B,** t=0.08 h**)**. This could be due to differences in the acidity and the protein content of the gel matrices. $MgSO_4$ gels have significantly higher pH than the GDL gels (**Table 5.1**), and after the addition of the simulated salivary fluid (SSF), the pH was not re-adjusted to 7 due to the short duration to the oral phase. Therefore,

GDL gels had a slightly acidic pH (~ 5) in the salivary phase with more phenolic acid molecules being in the protonated form, which makes them less water-soluble (Helal et al., 2015) and more likely to be entrapped to the protein network. A dependency of the bioactive release/ bioaccessibility with the pH condition of the media is an indication of potential interactions between the bioactives with proteins or peptides.

Overall, the release profiles of the phenolic acids were significantly different ($p<0.05$). The release of PCA was faster than the CMA from the end of the oral processing. Around 90 % of the PCA was initially released, slowly reducing to 80 % where it remained relatively constant with a slight increase during the intestinal phase ($p>0.05$). The modulation of CMA release was more gradual than PCA during the oral and gastric phase, but they increased dramatically ($p<0.05$). The release percentage of CMA reached almost 100 % and remained stable throughout intestinal processing. The phenolic structure effect on the release will be discussed with more details in **section 6.3.4** that more phenolic acid structures were screened.

In a previous study investigating a cheddar like cheese with incorporated tea PP, researchers found that even though the levels of total PP were low in stomach conditions, they gradually increased during the first 40 min of the intestinal processing (Lamothe et al., 2016). Unfortunately, the authors did not characterise the tea extract and they only measured the total polyphenols with a photometric method (Lamothe et al., 2016). Several studies have demonstrated significant losses in endogenous phenolic acids during *in vitro* digestion of pomegranate products (Mosele et al., 2015), whole grapes (Tagliazucchi et al., 2010), and broccoli (Vallejo et al., 2004). Therefore, our results signify that pressed soy protein gels are promising food systems for the delivery of phenolic acids. More research is needed, however, to determine the exact features responsible for the protective effect of soy protein gels on phenolic acids observed here.

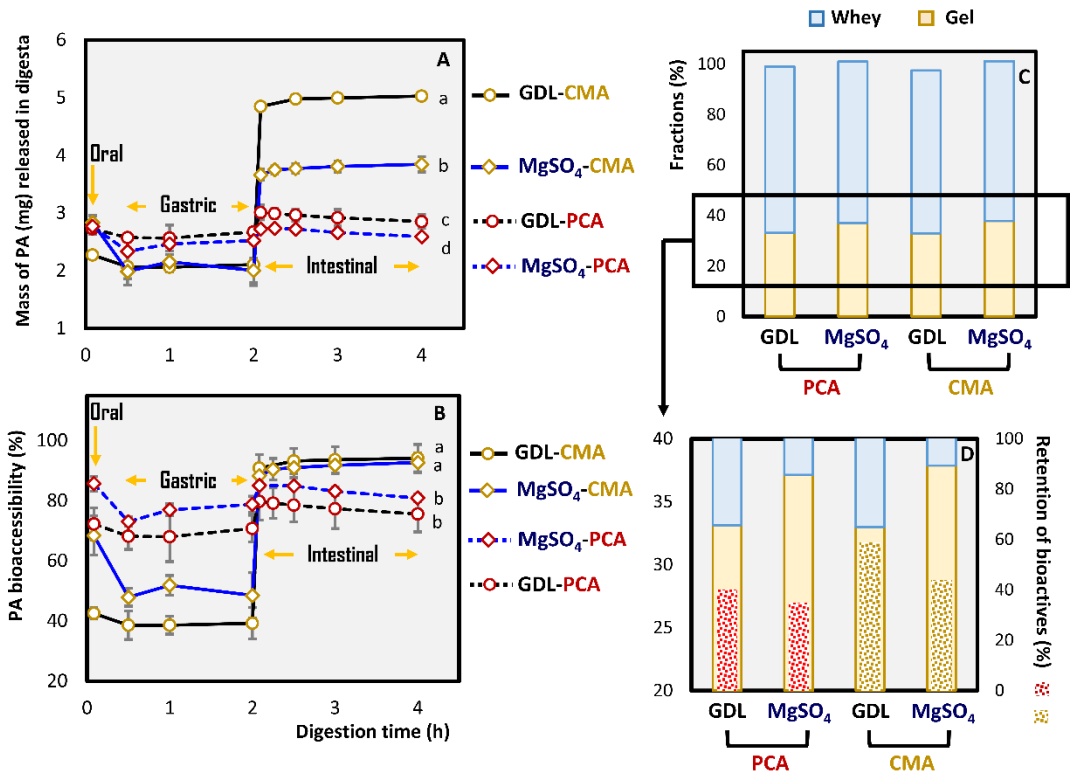


Figure 5.8 Release of added phenolic acids (PA); CMA and PCA from GDL and $MgSO_4$ induced pressed gels during *in vitro* digestion. Mass of bioactives released (mg) (A) and release percentage normalised with the mass of bioactives remained in the gel after pressing. Fraction (%) of gel and whey serum after pressing (C) and magnification of image C including the PA retention (%) in the gel after pressing (D).

[1] Values were represented as means ± standard deviations (n≥3). [2] Letters a-d indicate that values that do not share a letter in the same graph are significantly different ($p \leq 0.05$). [3] End bioaccessibility (%) and mass (mg) of PA were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (PCA, CMA) and coagulant mechanism (GDL- and $MgSO_4$-). The significance of the factor's interaction mean values was assessed with the Tukey test.

5.4 Conclusions

The two types of gels were similar in composition, but they had significant differences both in texture and microstructure. $MgSO_4$-induced gels were more porous with larger aggregates, but they had significantly lower firmness than the GDL-induced gels, which might be the reason for their higher protein digestibility. The release of the bioactives on a percentage basis was similar for both gel matrices, but GDL-induced gels delivered larger masses of bioactives in the intestinal phase because they could retain more of the phenolics (indirect effect). Our results suggested that the coagulation mechanism affected both the proteolysis of the soy protein gels and the bioaccessibility of added phenolic acids.

Chapter 6
Bioaccessibility of added phenolic acids from unpressed soy protein gels

Abstract

Soy protein unpressed gels with incorporated bioactive phenolic were prepared using glucono-δ-lactone (GDL) as an acidifier or magnesium sulphate ($MgSO_4$) as a salt coagulant. This study aimed to explore how the addition of phenolic acids affects the rheological properties and microstructure of the gels and secondly, the effect of the coagulation mechanism and phenolic acid structure on the bioaccessibility of the bioactives. The addition of phenolic acids in acidified gels doubled their elastic modulus (G') ($p<0.05$), without showing the same effect on salt-induced gels. Also, the addition of coumaric acid in both types of gels induced the formation of thicker and longer aggregates. Nevertheless, the bioaccessibility of phenolic acids was not affected by the gel matrix ($p>0.05$) but it was dependent on the phenolic acid structure. The concentration of phenolic acids with two or three hydroxyl groups, such as gallic acid and caffeic acid, decreased significantly during intestinal conditions ($p<0.05$).Whereas, methylation of one hydroxyl group (ferulic acid and vanillic acid) reduced the losses to a great extent ($p<0.05$).

6.1 Introduction

In this chapter, we selected SPI unpressed gels as a food matrix for studying the release of added phenolic acids. This type of soy protein gel represents an existing food format, the silken tofu, which is a popular soy product in Asian countries. Soy is a source of high-quality protein and it is commonly used as an animal protein substitute for vegetarians (Rizzo and Baroni, 2018a). Only a few studies have focused on the use of soy protein gels as a delivery system for bioactives. Maltais and co-authors encapsulated riboflavin into different types of tableted SPI cold-set hydrogels; filamentous and particulate (Maltais et al., 2009, Maltais et al., 2010). They studied the effect of riboflavin on the mechanical properties of gels and its release profile during gastrointestinal processing. Filamentous gels delayed the release of the bioactive during intestinal conditions, in contrast, particulate gels gave more rapid release (Maltais et al., 2009). In general, protein gelation can be modulated with environmental conditions, such as pH and salt type or concentration, leading to a range of different microstructures and physical properties.

This work aimed to study how adding two structurally similar phenolic acids into soy protein gels, affect the rheological and microstructural characteristics of the gels. Secondly, we studied how the physical and chemical interactions between the phenolic acids and soy proteins affected the bioaccessibility of the phenolic acids, during *in vitro* digestion. Two different coagulation mechanisms were tested to compare different textures of the same types of gels. Finally, the relationship between phenolic acid structure and release profile and some factors that could affect them were also investigated.

6.2 Experimental information

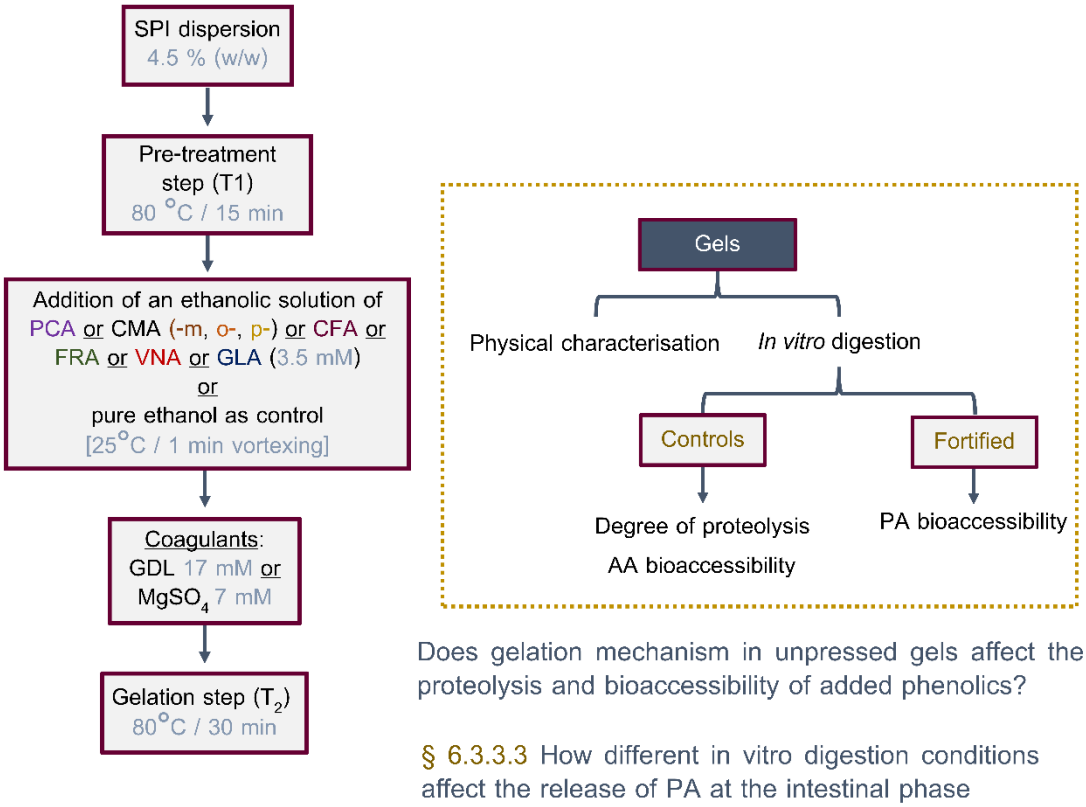


Figure 6.1 Overview of the experimental design of Ch 6.

6.3 Results and discussion

6.3.1 Characterisation of unpressed gels induced by different coagulants

6.3.1.1 Composition

The composition of all the gels was similar, with protein content around 4.3 % and moisture content of approximately 95 %. Although the conditions were identical in the mixtures, the gels induced by $MgSO_4$ contained a lower concentration of coagulant than the GDL gels (7 and 17 mM respectively). Beyond a certain coagulant concentration of $MgSO_4$, phase separation occurred which is not desirable for silken-like tofu gels. Therefore, the coagulant concentrations selected were based on the

minimum amount needed to give self-supported gels without phase separation. Lower coagulant concentrations gave weaker gels that collapsed under their own weight. The pH of the gels varied depending on the coagulant and phenolic acid addition (Table 6.1).

Table 6.1 Characteristics of soy protein unpressed gels with added phenolic acids: protocatechuic acid (PCA) and coumaric acid (CMA).

	GDL-Induced gels			$MgSO_4$-Induced gels		
Properties	**Control**	**PCA**	**CMA**	**Control**	**PCA**	**CMA**
pH of gels	5.65 ± 0.15^{a}	5.44 ± 0.16^{b}	5.46 ± 0.11^{ab}	6.67 ± 0.07^{c}	6.32 ± 0.07^{d}	6.25 ± 0.02^{d}
Gel surface hydrophobicity BPB (µg)	49.0 ± 1.2^{a}	50.1 ± 0.1^{a}	50.6 ± 1.2^{a}	49.9 ± 0.4^{a}	50.0 ± 1.4^{a}	48.8 ± 1.4^{a}

[1] Values were represented as means ± standard deviations (n≥3). [2] Values that do not share a letter in the same row are significantly different (p≤0.05). [3] pH and hydrophobicity were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (control, PCA, CMA) and coagulant mechanism (GDL- and $MgSO_4$-). The significance of the factor's interaction mean values was assessed with the Tukey test.

As expected, GDL-induced gels were more acidic than the $MgSO_4$ gels (p≤0.05). GDL acidifies the mixture by spontaneous hydrolysis to gluconic acid. In contrast, $MgSO_4$ did not affect the pH of the mixture significantly (SPI dispersion pH is around 7.0). However, the addition of both phenolic acids reduced the pH to some extent in both GDL and $MgSO_4$ gels (p≤0.05), which was expected because of their acidic nature (McMurry, 2011). The surface hydrophobicity showed no significant differences (p≥0.05).

6.3.1.2 Small-deformation viscoelastic properties

The formation of SPI gels, during the second heating step and after the addition of the two coagulants, GDL and $MgSO_4$ were observed with rheometry by recording the continuous changes in the viscoelastic properties of the SPI mixtures at small deformation. The changes in G′ during the heating-cooling cycles are shown in **Figure 6.2**, with specific points of interest summarised in **Table 6.2**.

However, upon cooling from 80°C to 5°C, the G' increased at a faster rate until it reached a saturation point at 5°C, which is typical behaviour for soy protein gels

(Catsimpoolas and Meyer, 1970). The decrease in temperature diminishes the entropy of the system, which leads to short-range non-covalent protein-protein interactions such as hydrogen bonding (Chronakis, 1996). The starting G' of the control $MgSO_4$ mixture was slightly higher than the ones induced by GDL. This difference in the starting G' between GDL and $MgSO_4$ mixture is reasonable, and it has been observed before with other divalent salts (Kohyama et al., 1995).

Table 6.2 Rheological properties of the unpressed gels with added phenolic acids: protocatechuic acid (PCA) and coumaric acid (CMA).

	GDL-Induced gels			$MgSO_4$-Induced gels		
Properties	Control	PCA	CMA	Control	PCA	CMA
G'_{80} (Pa)	113.0 ± 14.2^a	205.0 ± 19.3^b	235.6 ± 20.5^b	31.5 ± 0.8^c	90.5 ± 17.9^a	98.3 ± 34.7^a
G'_{Fin} (Pa)	1275.0 ± 91.1^a	2326.7 ± 173.9^b	2620.0 ± 253.6^b	289.0 ± 11.4^c	256.6 ± 33.1^c	279.0 ± 122.1^c
G''_{Fin} (Pa)	217.0 ± 18.4^a	408.0 ± 27.5^b	479.3 ± 49.5^c	52.6 ± 3.4^d	56.9 ± 9.4^d	66.7 ± 31.4^d
Tan δ_{fin} (-)	0.170 ± 0.003^a	0.176 ± 0.002^a	0.183 ± 0.002^a	0.182 ± 0.006^a	0.221 ± 0.012^b	0.237 ± 0.010^b

[1] Values were represented as means ± standard deviations (n≥3). [2] Values that do not share a letter in the same row are significantly different (p≤0.05). [3] Rheological properties (G'80, G'Fin, G''Fin, Tan δFin) were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (control, PCA, CMA) and coagulant mechanism (GDL- and $MgSO_4$-). The significance of the factor's interaction mean values was assessed with the Tukey test.

The phenomenon can be easily explained by the nature of GDL, which is a lactone that hydrolyses to gluconic acid that reduces the pH of the solution (Pocker and Green, 1973) and thus induces gelation. The hydrolysis rate is temperature-dependent (Kohyama et al., 1995) and hydrolysis occurs gradually, allowing smoother textures to be formed, this is the reason that GDL is the preferred coagulant for the production of silken tofu.

Although the SPI-$MgSO_4$ control mixture had a similar pattern to the SPI-GDL gels, the $MgSO_4$ mixtures containing phenolic acids had a considerably higher initial G' ($p<0.05$). Finally, all the $MgSO_4$-induced gels had a significantly lower ($p<0.05$) final G' than the GDL-induced gels and therefore were much weaker (**Figure 6.2**). The addition of the bioactives PCA and CMA affected the rheological properties of the gels significantly, especially the gels induced by GDL ($p<0.05$). The final G' of the GDL gels was doubled after the addition of the phenolic acids, with CMA-GDL mixtures giving slightly higher G'_{Fin}.

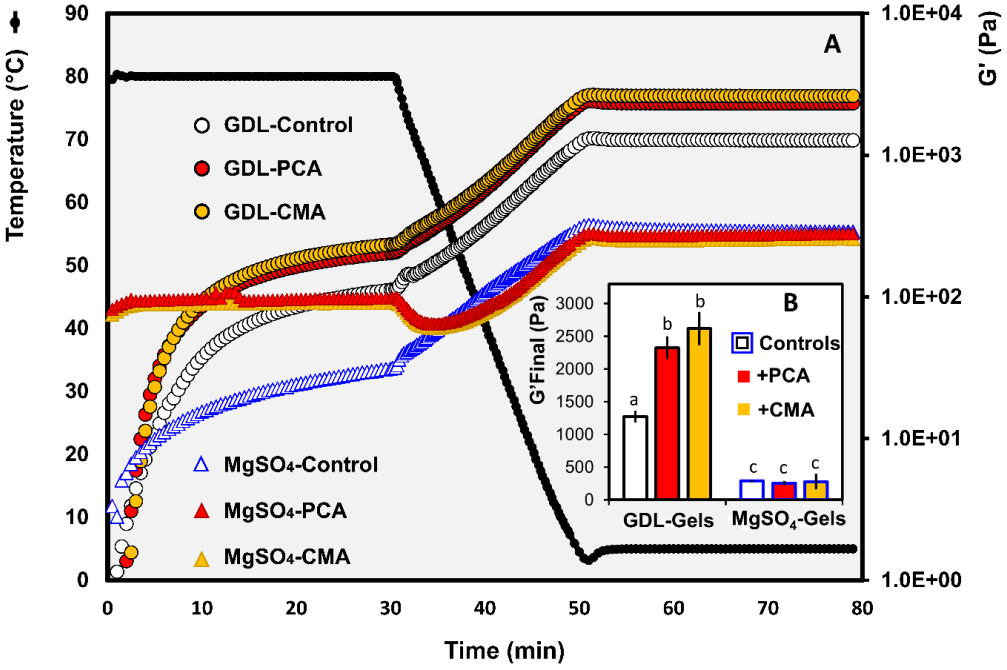


Figure 6.2 Changes in the small-deformation viscoelastic properties and final storage modulus of the unpressed gels induced by GDL and $MgSO_4$ (17 and 7 mM respectively). The white filled lines/ bars indicate the control gels, the red filled lines/ bars indicate the addition of PCA and the yellow filled lines/ bars the addition of CMA.

[1] Values were represented as means ± standard deviations (n≥3). [2] The letters a-c indicate that values in the same graph are significantly different (p≤0.05) (**B**).

Previous studies showed that the addition of mono- or polyphenols could enhance the mechanical properties of gelatin gels (Zhang et al., 2010, Wu et al., 2001, Strauss and Gibson, 2004) or films (Le et al., 2018, Cao et al., 2007). The mechanism that was proposed was either cross-linking through quinone formation under oxidising conditions or via hydrogen bonding, based on the fact that phenols are excellent hydrogen donors (Wu et al., 2001, Cao et al., 2007). In the presence of oxygen, and especially at alkaline conditions, phenolics are susceptible to oxidation (Cilliers and Singleton, 1989), as such, we believe that is less likely the formation of oxidative products in our system. Thus, the hydrogen bonding between phenolic acids and the amino acids residues of the soy protein is more likely. Phenolic acids can form hydrogen bonds through both the carboxyl group (-COOH) and the hydroxyl group (-OH) of the phenolic ring. A recent study found that the greater the number of the -OH groups on the phenolic acid ring the greater the binding affinity towards the β-conglycinin (Gan et al., 2016b).

Surprisingly, CMA that has only one hydroxyl (-OH) group was a slightly better enhancer than PCA, which has two -OH, however, the difference was not significant ($p>0.05$). Perhaps in some PCA molecules, the neighbouring -OH groups form intramolecular-hydrogen bonding that limits their ability to crosslink (Wu et al., 2001).

In the case of $MgSO_4$ gels with added PCA or CMA, the starting G' was substantially higher ($p<0.05$) and remained constant until the end of the heating step (**Figure 6.2**). It is unclear if this occurred due to the reduction of the pH, induced by the phenolic acids (**Table 6.2**). However, during the cooling phase, a slight decrease with a subsequent increase in the G' was observed. Finally, the G' stabilised to values very close to the ones found in the control $MgSO_4$ gels ($p>0.05$). This suggests that the phenolic acids could not enhance the gelling network in the presence of $MgSO_4$ as it was observed with GDL gels. In addition, the final tan δ of the enhanced gels was significantly larger than the control sample ($p<0.05$) (**Table 6.2)**, which shows a more viscous behaviour.

As mentioned before, the pH of the $MgSO_4$ gels was between 6.2 to 6.7, where most of the carboxyl groups are dissociated (-COO-) and able to interact with the divalent salt (Mg^{2+}). Similarly, the carboxyl groups of the phenolic acids ($pK_a \sim 4.5$) are dissociated too. The latter could increase an electrostatic repulsion between the phenolics and protein's sites. Also, it could limit the hydrogen bonding between proteins and phenolics because only the -OH groups can participate at pH ~ 6.5. Our hypothesis is supported by the fact that the final elasticity of the fortified $MgSO_4$ gels was slightly but significantly reduced ($p>0.05$). Low temperatures strengthen hydrogen bonds (Damodaran et al., 2007)..

6.3.1.3 Microstructure of gels

The inner microstructure of the gels can be found in the TEM micrographs in **Figure 6.3**. The dark areas correspond to the protein network or aggregates and the white areas to the aqueous phase.

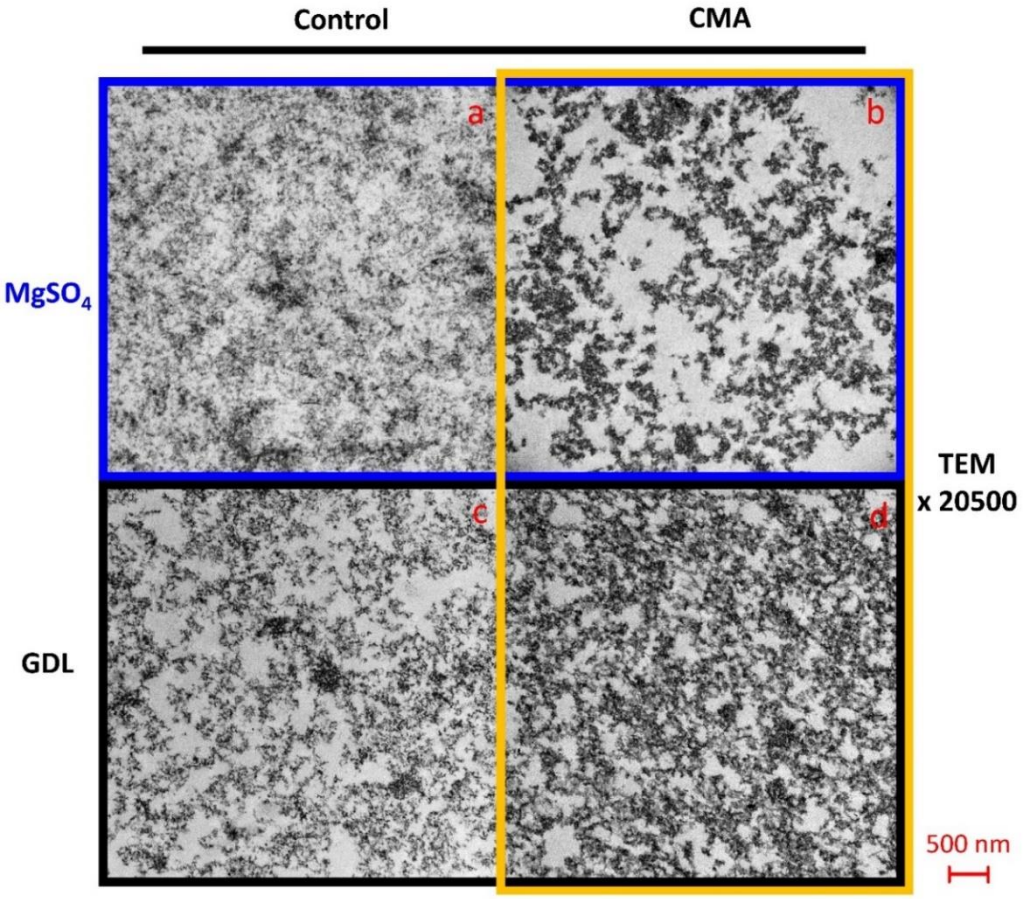


Figure 6.3 Microstructural characteristics of unpressed gels induced by GDL and $MgSO_4$. $MgSO_4$ induced control gel (a) and with the addition of 3.5 mM CMA (b). GDL-induced gels control (c) and with the addition of 3.5 mM CMA (d).

A convoluted, thin protein network can be observed in both GDL and $MgSO_4$ control gels. In both cases the amount of coagulant added was low (17 mM and 7 mM respectively). Therefore, the net charge was negative in both cases (**Table 6.1**), thus there was electrostatic repulsion to some extent. However, the repulsion was not large enough to form fine-stranded networks. The addition of CMA induced significant changes in the microstructure of both GDL and $MgSO_4$ gels (**Figure 6.3 b-d**). The aggregates became thicker and longer. Although this phenomenon was more pronounced in GDL-CMA gels (**Figure 6.3 d**), the network was irregular, and the areas

fluctuated in density and thus gel's porosity. In contrast, $MgSO_4$-CMA gels had a more regular network and the micrograph presented in **Figure 6.3 b** is a representative one. The increase in thickness and aggregates size after the addition of the CMA could also explain the increase in elastic modulus in GDL gels presented in **Figure 6.2**. However, it is unclear why the microstructural changes did not affect the elasticity of the $MgSO_4$ gels.

6.3.2 Effect of the coagulant on proteolysis and amino acid bioaccessibility

Free amino acids and oligopeptides released during *in vitro* gastrointestinal digestion were measured using the o-phthaldialdehyde (OPA) assay **(Figure 6.4 A**) on the liquid phase of the digesta. The method was conducted only on the control pressed gels (without bioactives) due to adverse reactions between the bioactives and the ABSF inhibitor (**section 3.2.5.4**).

The degree of protein hydrolysis during the intestinal phase was higher (>100 %) than the total amount of amino acids released after acid hydrolysis of the samples (**section 3.2.6.1**). The results indicated extensive enzyme autolysis in the unpressed gels and thus, contamination of the digesta with peptides or amino acids produced by the pancreatic enzymes. It was hypothesised that the enzyme autolysis phenomenon was more intense in the unpressed gels because their protein (substrate) concentration was ~ 4 times lower compared to pressed gels which correspond to a higher enzyme to substrate (E:S) ratio. It is worth mentioning that correction of the results with blank digestions as it has been proposed before (Qiao et al., 2002, Qiao et al., 2005, Brodkorb et al., 2019) did not lead to reasonable results since the blank digestions proteolysis values were close to the sample values. Although it seems that the contamination problem was observed only in the OPA results, FAA analysis of blank *in vitro* digestions resulted in significantly high concentrations (**Table S 2**).

Despite the limitations, some observations can be noted. Firstly, $MgSO_4$-induced gel was more prone to digestion than the GDL-induced gels, which is in agreement with the pressed gels proteolysis results from **Chapter 5 (Figure 5.5)**. Similarly, the bioaccessibility of the essential amino acid is slightly higher in $MgSO_4$

than in GDL induced gels ($p<0.05$) (**Figure 6.4 B**). Therefore, there is a strong positive relationship between the texture of the gels and the degree of protein hydrolysis.

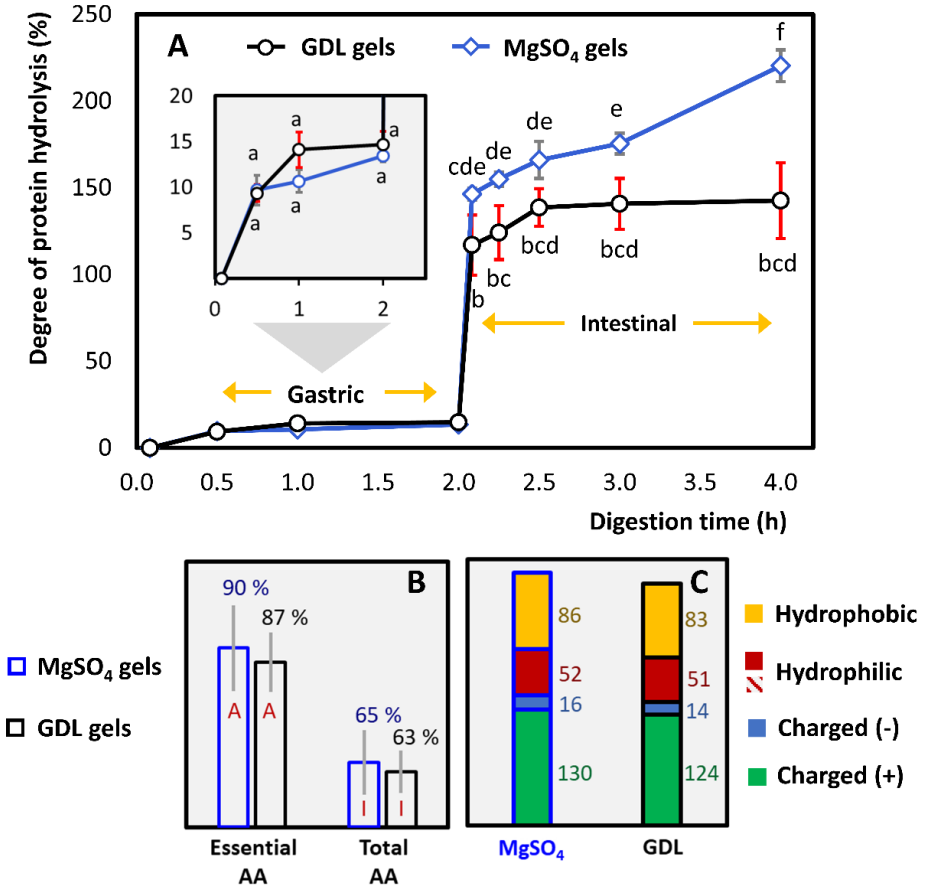


Figure 6.4 Degree of proteolysis (%) (A) and amino acid (AA) bioaccessibility: EAA, TAA (B) and grouped AA (C) of unpressed gels (controls) induced by GDL (black) and $MgSO_4$ (blue) during *in vitro* simulation of digestion.

[1] Values were represented as means ± standard deviations (n=3-5). [2] The letters a-f and A or I indicate that values that do not share a letter in the same group (DH, EAA, TAA) are significantly different ($p \leq 0.05$). [3] The degree of protein hydrolysis was analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of coagulants in control gels (GDL-, $MgSO_4$-) and digestion time (0-4h). The significance of the factor's interaction mean values was assessed with the Tukey test. The significant difference of the grouped AA: EAA and TAA (B) between GDL and MgSO4 induced gels were assessed with the student t-test.

6.3.3 Effect of the coagulant on phenolic acid bioaccessibility

The bioaccessibility profile of the CMA and PCA from $MgSO_4$ and GDL-induced gels during *in vitro* digestion can be found in **Figure 6.5**. The coagulants used and thus, the texture of the gels, had a negligible effect on the phenolic acid release. However, CMA and PCA gave different bioaccessibility patterns. A similar trend was found in the pressed SPI gels (**Chapter 5**), even though the general bioaccessibility profile was distinct **(Figure 5.8 B)**.

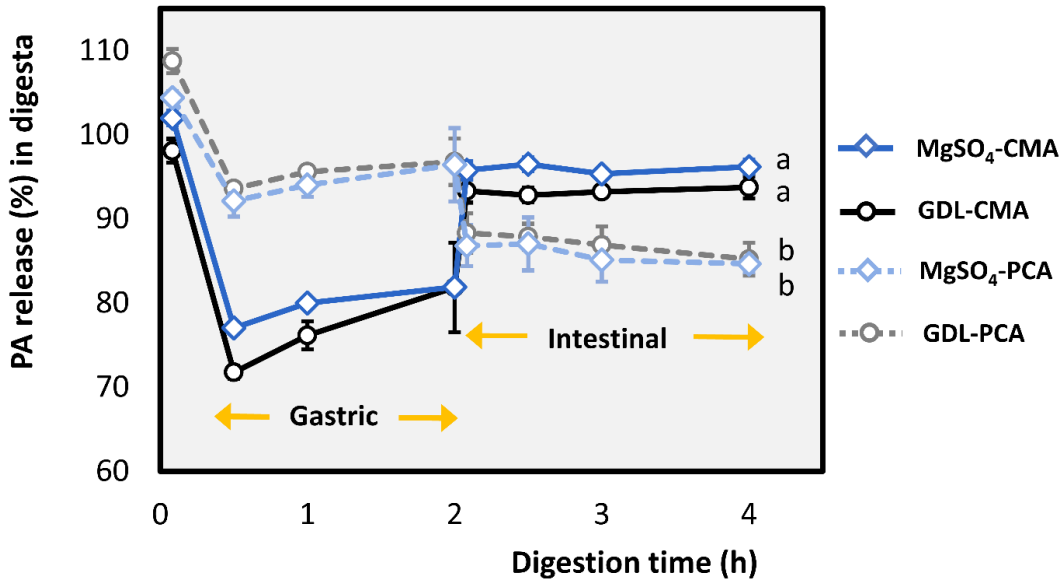


Figure 6.5 Bioaccessibility (%) of PCA and CMA (full and dashed line, respectively) from unpressed gels during *in vitro* digestion. The black and grey lines correspond to GDL-induced gels and the blue to $MgSO_4$-induced gels.

[1] Values were represented as means ± standard deviations (n=3). [2] Values that do not share a letter are significantly different ($p \leq 0.05$). [3] End bioaccessibility (%) of PA were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the added bioactive (PCA, CMA) and coagulant mechanism (GDL- and $MgSO_4$-). The significance of the factor's interaction mean values was assessed with the Tukey test.

A noticeable phenomenon is that both phenolic acids from both gel matrices were released rapidly and completely (≥ 95%) at the end of the oral incubation (pH ~ 7). Therefore, the neutral pH favours the bioaccessibility of the phenolic acids from the gel matrix. This tendency was not pronounced in the oral incubation of the pressed gels, studied in **Chapter 5** (**Figure 5.7 A, B**). The latter can be explained by the higher protein content and thus, the higher buffering capacity (Mennah-Govela et al., 2019) of the pressed protein gels. The high buffering capacity of the pressed gels enabled

them to maintain a pH < 6 despite the addition of the neutral pH salivary fluid. In the gastric and intestinal phase, the pH was adjusted and checked before and during the incubations and thus, it remained at the desirable levels in all gel samples (pH ~3 and ~7, respectively).

The levels of both phenolics reduced rapidly at the beginning of the gastric phase ($p<0.05$) and then they gradually increased reaching approximately 90 % and 80 % for PCA and CMA respectively, until the end of the gastric incubation (**Figure 6.5)**. In the intestinal phase, the bioaccessibility levels of CMA increased significantly ($p<0.05$) reaching approximately 95 %. In contrast, PCA levels dropped by 10 % ($p<0.05$) and reached bioaccessibility levels of 85 %.

The rheological analysis showed significant differences in the viscoelastic properties of these gels. **Figure 6.2** denoted that PCA and CMA were strengthening the protein network of the GDL-induced gels while weakening the $MgSO_4$ gels. Hence, textural differences to the scale presented here are not significant to affect the bioaccessibility of phenolic acids during digestion conditions. Although the bioactives released rapidly from the unpressed gel matrices, the bioaccessible levels of both PCA and CMA are very high, and it is still a promising system for delivering phenolic acids to the intestine. However, a low release of phenolics during oral and gastric phase would be more desirable, because it minimises potential losses before reaching the intestine, thus the release behaviour of the pressed gels (**Chapter 5**) is more preferred than the one of the unpressed gels.

6.3.4 Effect of phenolic acid structure on bioaccessibility from unpressed gels

6.3.4.1 Phenolic acid structures

Representative structures from both phenolic acid categories; hydroxycinnamic (C_6-C_3) and hydroxybenzoic (C_6-C_1) acids were used in this study (**Figure 6.6**).

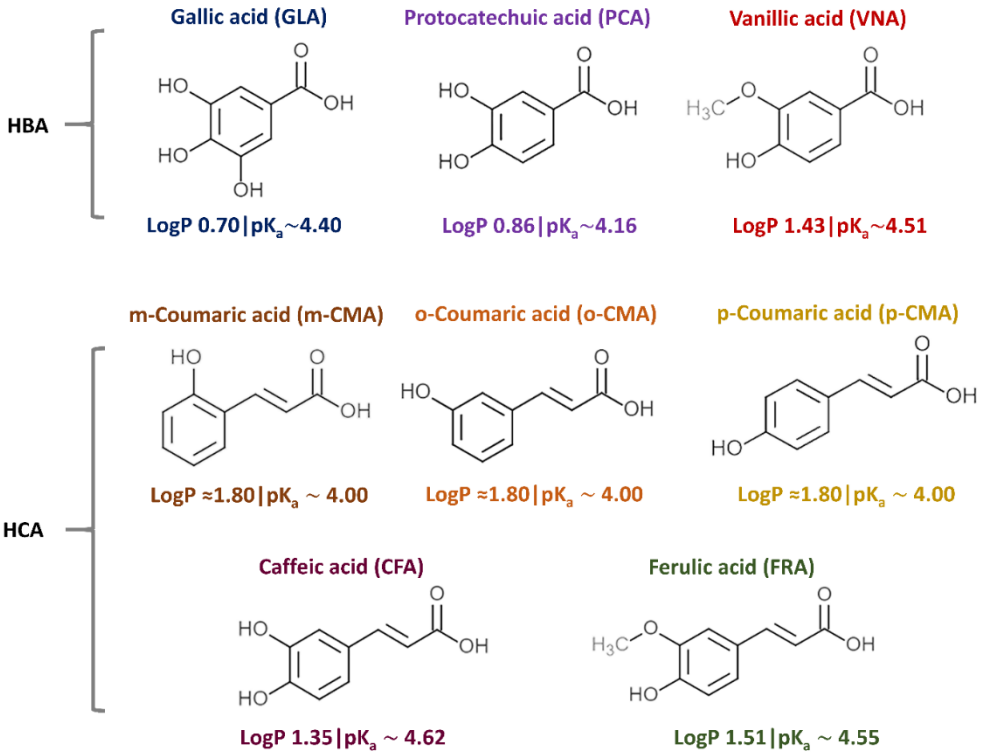


Figure 6.6 Phenolic acid structures, LogP and pKa (25 °C) values from (DrugBank, 2020, PubChem, 2020, Foodb, 2020). Hydroxybenzoic acids (HBAs); vanillic acid (VNA), protocatechuic acid (PCA) and gallic acid (GLA). Hydroxycinnamic acids (HCAs); ferulic acid (FRA), p-, m-, o- coumaric acids (CMA) and caffeic acid (CFA).

The presence of the propenoic acid (CH=CH-COOH) group is the distinct feature of the cinnamic acid derivatives which results in slightly lower hydrophilicity. A value that demonstrates the lipophilicity of a molecule is the partition coefficient (P) expressed as LogP (Lindsley, 2010), the lower the value the higher the affinity for the aqueous phase. It is known that the hydroxyl groups (-OH) attached to the aromatic ring can increase the hydrophilicity of a molecule. Therefore, gallic and protocatechuic

acids are the most hydrophilic, while coumaric acids are the most lipophilic among the phenolic acids tested (**Figure 6.6**).

The use of a homologous series allowed to test the relationship between the release profile and the chemical structure. The features tested are the following: *a)* presence of propenoic acid, *b)* the number of hydroxyl groups on the benzene ring, *c)* methylation of hydroxyl groups and *d)* position of the hydroxyl group.

6.3.4.2 Phenolic acid structure and release profile relationship

The peak of the release for all the phenolic acids was at the end of the oral incubation (pH 7) and varied depending on the structure, with caffeic acid having the lowest value (87 ± 2.4 %) (**Figure 6.7**). Apparently, neutral pH favours the bioaccessibility of phenolic acids. Unfortunately, most of the studies present only the end release values from the gastric and intestinal phases thus, we cannot compare our oral phase results.

After the oral phase, the concentration detected in the liquid fraction of the digesta was reduced at the early stages of gastric incubation (pH 3). At acidic pH, phenolics are less soluble due to protonation in -OH and -COOH groups (Helal et al., 2015). It has been stated that at acidic pH, porcine pepsin can interact with tannins which results in precipitation (Hagerman and Butler, 1978). Similarly, Helal and Tagliazucchi (2018) found that pepsin was able to interact with cinnamon polyphenols, under gastric conditions, resulting in the precipitation of complexes. Other studies, however, have demonstrated an increase or no substantial effect on polyphenol release during gastric incubation (Helal et al., 2015, Bermúdez-Soto et al., 2007). Later, at the intestinal phase, the bioactive levels either significantly increased ($p<0.05$), in the case of FRA and o-, p-, m-CMA) or significantly reduced ($p<0.05$) such as PCA, GLA, and CFA. An exception was the VNA, the levels of which remained constant during this transition (**Figure 6.7**). The reduction during the pancreatic phase has been observed in several studies, (Bermúdez-Soto et al., 2007, Çam et al., 2014, Helal et al., 2015, Tagliazucchi et al., 2010, Record and Lane, 2001, Vallejo et al., 2004, Gayoso et al., 2016) and has been described as degradation due to alkaline conditions.

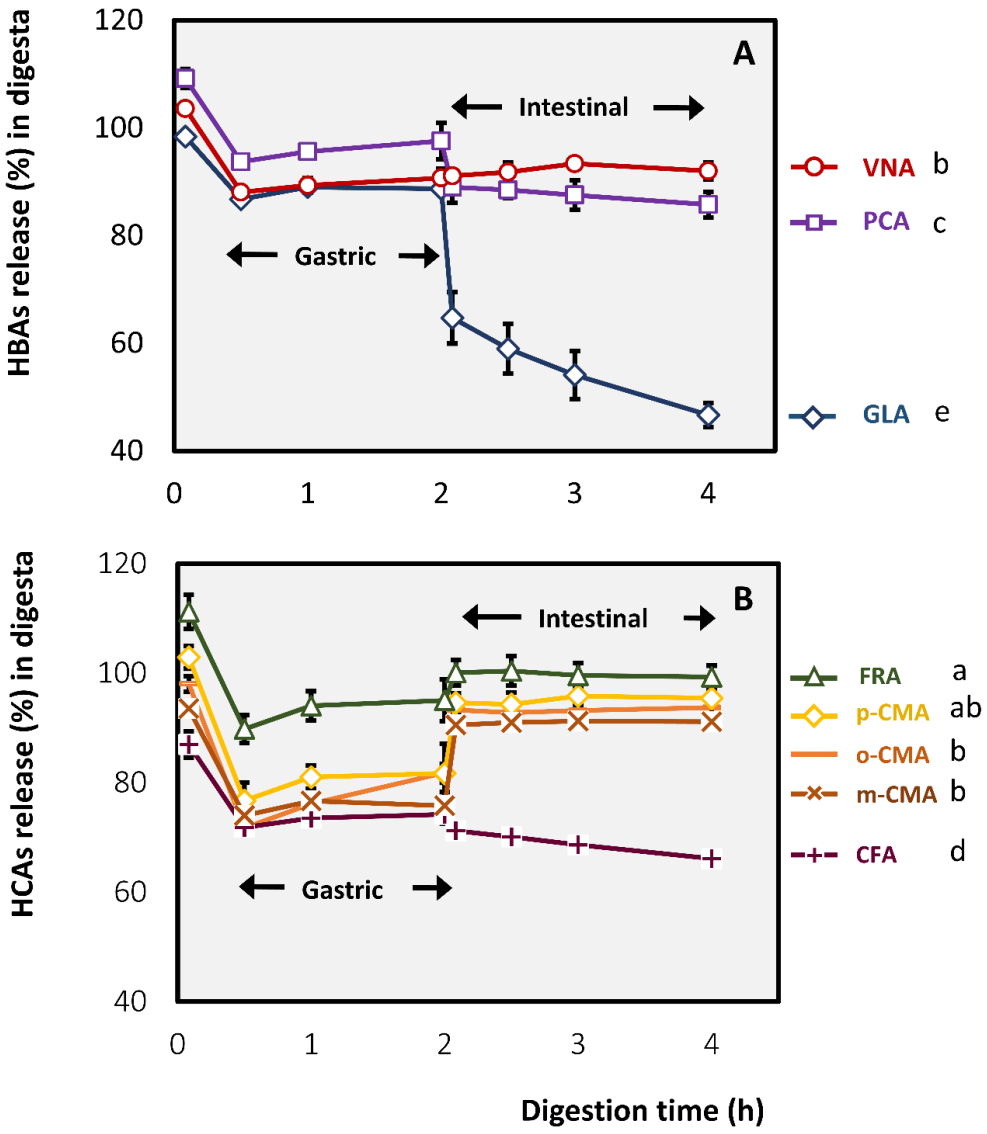


Figure 6.7 Bioaccessibility (%) of different phenolic acids structures during *in vitro* digestion; HBAs (A) and HCAs (B).

[1] Values were represented as means ± standard deviations (n=3). [2] Values that do not share a letter are significantly different ($p \leq 0.05$). 3 The bioaccessibility of PA at the end of the gastric (t=2h) and intestinal (t=4h) phase was analysed using a one-way ANOVA with the associated time points (2 and 4h) as the main factor and the comparison was performed using the Tukey test.

Our kinetic experiments gave two important insights. Firstly, the increase or decrease in release (%) at the intestinal phase is rapid, since it was detected from the first five minutes of the intestinal incubation. If this phenomenon is only an effect of the pH 7, then similar results would be expected at the oral incubation (pH 7). Thus, it appears to result from both the change in pH and the *in vitro* digestion history of the sample.

Also, if the degradation that was implied in the other studies was oxidation, a more gradual reduction of the phenolics levels would be expected. Gallic acid gave a very interesting release pattern (**Figure 6.7 A**), with an initial rapid drop and an additional gradual reduction throughout the intestinal incubation. The gradual decline might suggest oxidation. Thus, the question remains, why were some structures more rapidly affected during the *in vitro* digestion transitions?

From **Figure 6.6** and **Figure 6.7** we can observe some important structural features that affect the bioaccessibility of phenolics in the intestinal phase. Firstly, a comparison of VNA and FRA showed that the phenolic acid with the longer carbon backbone (FRA) had a higher bioaccessibility ($p<0.05$). Whereas a contradictory trend was found between PCA and CFA, thus it is unclear whether the presence of the propionic group can affect the bioaccessibility of phenolics. Secondly, it was found that the more the hydroxyl groups (-OH) attached to the benzene ring, the less bioaccessible the bioactive was in the intestinal phase (i.e. GLA < CFA < PCA) ($p<0.05$). Also, methylation of the -OH group attached to the C_3 of the benzene ring resulted in a significant increase of the intestinal phase bioaccessibility (i.e. VNA vs PCA and FRA vs CFA) ($p<0.05$). Furthermore, a comparison of the bioaccessibility concentrations at the intestinal phase between CMA isomers (o-, p-, m-) did not reveal any significant differences ($p>0.05$). Thus, the position of the -OH group did not make any difference. Finally, a correlation assessment between the logP values and the end bioaccessibility phenolic acids values showed a medium negative correlation between lipophilicity and gastric bioaccessibility ($r = -0.53$, $p = 0.177$), while a strong positive correlation between lipophilicity and intestinal bioaccessibility ($r = 0.73$, $p = 0.038$). Thus, the more hydrophilic the phenolic acid the higher the bioaccessibility in the gastric and the lower in the intestinal phase. Nevertheless, the number of -OH groups attached to the benzene ring appears to have a higher impact on the bioaccessibility of the phenolic acids.

Some of our findings are correlated well with the findings of Friedman and Jürgens (2000) who studied the stability of different phenolics in different pH (3-11). The authors found that the UV-Vis spectrum of CFA and its derivatives dramatically changed with increasing pH and these changes were irreversible. In another study, Fulcrand et al. (1994) detected oxidative products of CFA at acidic conditions (pH 3-5), although the rate of oxidative reaction increased drastically over pH 5. At alkaline

pH, a non-enzymatic oxidative reaction of CFA can be progressed rapidly (within minutes) (Cilliers and Singleton, 1989, Fulcrand et al., 1994, Hapiot et al., 1996). Thus, the low bioaccessibility rates of CFA during our incubations might suggest oxidation (**Figure 6.7 B**). According to Cilliers and Singleton (1989), the oxidation products of CFA do not absorb at 325 nm (max for cinnamate derivatives) but at 200 nm, which might explain why the new peaks did not appear during HPLC analysis (**section 3.2.4.5**).

Similarly, GLA that has 3 -OH attached to the phenolic ring was also unstable at high pH. In contrast, FRA with a single -OH free was stable at high pH and for more than 24 hours (Friedman and Jürgens, 2000). The authors suggested that the molecules with two or more -OH attached to the phenolic ring are more susceptible to chemical transformations at high pH. These transformations can be due to the formation of unstable quinone intermediates which may eventually oxidize in the presence of oxygen to degradation products (Friedman and Jürgens, 2000). Another important finding of the same study was the irreversibility of the induced changes in some phenolics. Although the phenomenon was tested from alkaline conditions (pH 8-11) to neutral (pH 7), it shows that pH incubations can cause permanent changes to the phenolics, and this might explain our results.

Apart from the GLA and CFA, the rest of the phenolic acid were bioaccessible by more than 80 %, which is a very high percentage and indicated that these compounds could be used as antioxidants in soy protein food formulations.

6.3.4.3 Possible explanations for the observed losses

As a static *in vitro* digestion protocol was used in these studies, there was no emptying of the digesta as would be the case in the dynamic protocols. Therefore, the losses of some PA observed in the intestinal phase might be an indication of interaction or degradation phenomena in the system.

Some of the scenarios tested that could further explain the losses were the following; a) binding of phenolic acids with the released amino acids or peptides, b) binding with pancreatic enzymes, c) binding between phenolics and bile acids, and d) o-quinone formation as a result of oxidation. Other possibilities that were not tested are the following: e) metal chelation between phenolic acids and digestive fluid salts, f) pH reversibility effect at digestion conditions.

6.3.4.3.1 Did phenolic acids bound to amino acids at the intestinal phase?

Numerous studies have shown that phenolic compounds can interact with food proteins such as whey and soy and alter their digestion properties (Gan et al., 2016b, Kroll et al., 2001, Rawel et al., 2002b, Rawel et al., 2005, Świeca et al., 2018). It was found that lysine, tryptophan and sulphur-containing AA such as cysteine and methionine can interact with phenolics such as chlorogenic acid and quercetin and thus, limit the absorption of free amino acids (FAA) under *in vitro* and *in vivo* digestion. However, no significant differences in the FAA levels between the control and the phenolic acid-fortified gels were detected in our study ($p>0.05$) (**Table 6.3)**. In most of these studies, food proteins were derivatized with phenolics under oxidative conditions (pH 9) that favour the formation of covalent bonding, which is irreversible (Kroll et al., 2001, Rawel et al., 2002b, Rawel et al., 2005). The conditions used in our study, however, justify only noncovalent interactions, which are reversible under digestion and therefore, do not inhibit protein digestibility. Another study was in agreement with our statement. The authors showed no effect on soy protein *in vitro* digestion after noncovalent interaction with phenolic acids (Gan et al., 2016b).

Table 6.3 Free amino acid composition (mmol of amino acids/ g of protein) of unpressed soy protein gels induced by GDL with added PCA and CMA, at the end of the intestinal *in vitro* digestion.

AA (mmol/g of protein)	GDL-Induced gels		
	Control	+PCA	+CMA
Aspartic acid	0.17 ± 0.02[a]	0.18 ± 0.03[a]	0.18 ± 0.01[a]
Glutamic acid	0.44 ± 0.05[a]	0.47 ± 0.07[a]	0.48 ± 0.05[a]
Asparagine	0.26 ± 0.04[a]	0.27 ± 0.05[a]	0.28 ± 0.03[a]
Serine	0.50 ± 0.08[a]	0.51 ± 0.09[a]	0.52 ± 0.05[a]
Glutamine	1.61 ± 0.23[a]	1.66 ± 0.27[a]	1.67 ± 0.13[a]
Histidine	0.18 ± 0.03[a]	0.18 ± 0.03[a]	0.19 ± 0.02[a]
Glycine	0.58 ± 0.09[a]	0.61 ± 0.11[a]	0.59 ± 0.05[a]
Threonine	0.32 ± 0.05[a]	0.33 ± 0.06[a]	0.34 ± 0.03[a]
Arginine	0.84 ± 0.12[a]	0.88 ± 0.15[a]	0.88 ± 0.08[a]
Alanine	0.63 ± 0.09[a]	0.65 ± 0.12[a]	0.66 ± 0.06[a]
Tyrosine	0.52 ± 0.06[a]	0.54 ± 0.09[a]	0.55 ± 0.06[a]
Cysteine	0.17 ± 0.02[a]	0.17 ± 0.02[a]	0.19 ± 0.02[a]
Valine	0.41 ± 0.05[a]	0.41 ± 0.07[a]	0.42 ± 0.04[a]
Tryptophan	0.11 ± 0.01[a]	0.12 ± 0.02[a]	0.11 ± 0.01[a]
Methionine	0.14 ± 0.01[a]	0.15 ± 0.02[a]	0.15 ± 0.01[a]
Phenylalanine	0.48 ± 0.06[a]	0.51 ± 0.09[a]	0.51 ± 0.05[a]
*Proline	n/d	n/d	n/d
Isoleucine	0.32 ± 0.05[a]	0.33 ± 0.06[a]	0.34 ± 0.03[a]
Leucine	0.99 ± 0.14[a]	1.03 ± 0.21[a]	1.05 ± 0.11[a]
Lysine	1.00 ± 0.20[a]	1.08 ± 0.32[a]	1.01 ± 0.10[a]
Total Essential	**3.96 ± 0.60[a]**	**4.15 ± 0.88[a]**	**4.12 ± 0.36[a]**
Total	**9.69 ± 1.41[a]**	**10.09 ± 1.85[a]**	**10.13 ± 0.88[a]**

[1] Values were represented as means ± standard deviations (n=3). [2] Values are not significantly different ($p \leq 0.05$). The bioaccessibility of FAA at the end of the intestinal phase (t=4h) was analysed using a one-way ANOVA with the type of GDL- gels (control, PCA, CMA) as the main factor and the comparison was performed using the Tukey test.

6.3.4.3.2 Did phenolic acids interact with digestive enzymes?

The second scenario was tested by blank digestions of gels with added PCA and CMA, in the absence of digestive enzymes and bile (**Figure 6.8)**.

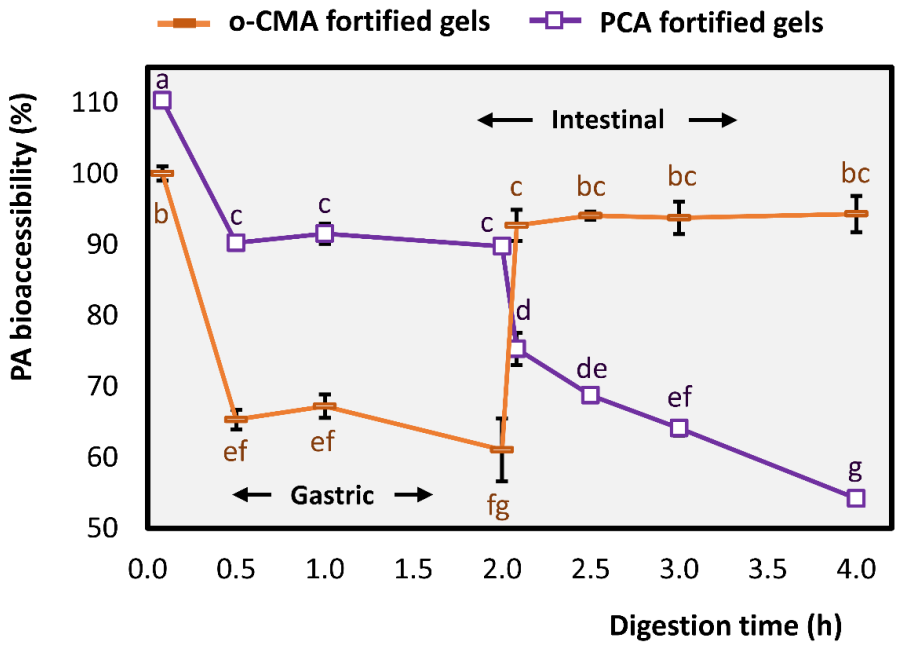


Figure 6.8 Release of CMA and PCA from unpressed GDL-induced soy protein gels, during *in vitro* blank digestion (pepsin, pancreatin and bile salts were excluded).

1 Values were represented as means ± standard deviations (n=2). [2] Letters a and b indicate that values that do not share a letter in the same time point are significantly different ($p \leq 0.05$). [3] The PA bioaccessibility was analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the added bioactives (PCA, CMA) and digestion time (0-4h). The significance of the factor's interaction mean values was assessed with the Tukey test.

The exclusion of pepsin reduced the release of both PCA and CMA during the gastric phase ($p<0.05$), while the exclusion of pancreatic enzymes (and not bile) significantly reduced the release of PCA ($p<0.05$). The latter might indicate a protective effect of the pancreatic mixture on the PCA. It is unclear, however, if this is an effect of the digestive enzymes themselves or other substances that might be present in the commercial pancreatic mixture. In any case, this experiment demonstrated that the phenolics used did not bind to the digestive enzymes.

It was found a similar release profile with **Figure 6.5** which showed that the pH during *in vitro* digestion is responsible for this observed pattern, regardless the presence of the digestive enzymes. A similar trend was observed with blank digestion

trials of pressed gels (results not shown), which indicate that the release profile of the phenolic acids depends on the gel matrix and the pH conditions.

6.3.4.3.3 Does flushing with nitrogen during *in vitro* digestion affect the bioaccessibility of phenolic acids?

The effect of oxygen exclusion and bile during *in vitro* digestion of gels with added PCA and GLA was also tested (**Figure 6.9**). Bermúdez-Soto et al. (2007) suggested that flushing with nitrogen (N_2) for 10 min, after the addition of enzymes, enhanced the recovery of anthocyanins by 50 %. According to the authors, this resulted in reduced levels of oxygen during digestion and therefore gave a more realistic representation of gastric and intestinal conditions (Bermúdez-Soto et al., 2007, He et al., 1999). A similar approach was tested here, although the N_2 flushing lasted for 4 minutes due to practical limitations. Interestingly, GLA showed a 50 % increase in the intestinal phase after N_2 flushing ($p<0.05$), whereas PCA was not affected (**Figure 6.9**). Hence, the gradual decline of GLA during intestinal incubation is indeed, an effect of oxidation as we hypothesised in **section 6.3.4.2**, which is most probably a result of the 3 -OH groups attached to the aromatic ring. But it is still unclear why there is a rapid reduction in the bioaccessibility of PCA and GLA during the transition from gastric to intestine conditions.

It was recently suggested that tea polyphenols (catechins) can form complexes with taurocholic acid (present in bile extract) which might result in a decrease in cholesterol absorption (Ogawa et al., 2016). On the other hand, Yang et al. (2018) demonstrated that bile acids enhanced greatly the bioaccessibility of polyphenols present in kale. Despite all these, our results did not indicate either enhancement of the phenolic acid levels or molecular interaction with bile acids (**Figure 6.9**).

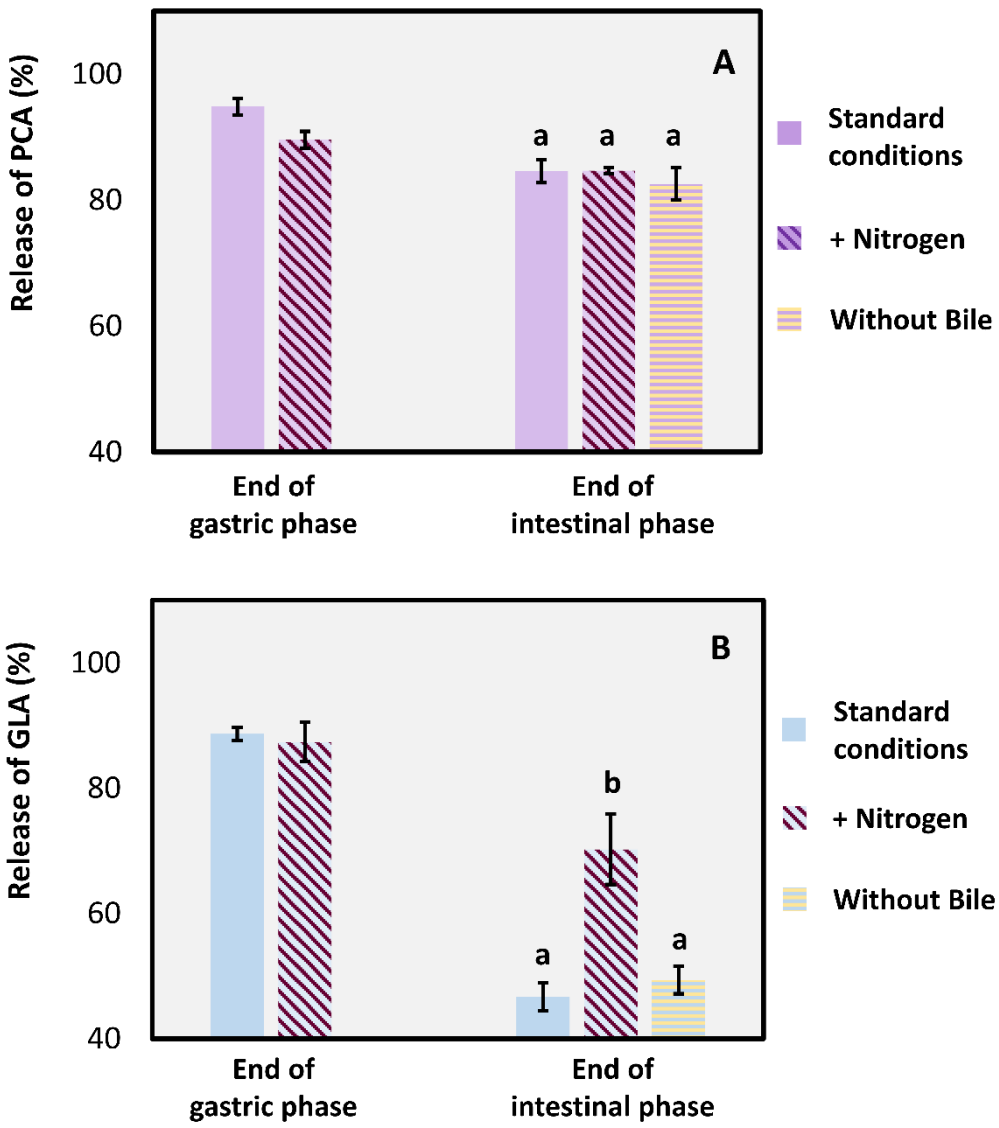


Figure 6.9 Bioaccessibility (%) of PCA (top) and GLA (bottom) at the end of gastric and intestinal *in vitro* digestion. The standard conditions (as presented in Figure 6.7) are represented with the solid filled bars, the nitrogen flushing incubations with the diagonally striped bars and the incubations with the absence of bile salts with horizontal stripes.

[1] Values were represented as means ± standard deviations (n=3). [2] The letters a and b indicate that the values within the same graph are significantly different ($p \leq 0.05$). [3] The bioaccessibility of PCA and GLA at the end of the gastric (t=2h) and intestinal (t=4h) phase was analysed using a one-way ANOVA with the associated time points (2 and 4h) as the main factor and the comparison was performed using the Tukey test.

6.4 Conclusions

The objective of this study was to assess the effect of phenolic acids on the physical properties of soft soy protein gels and to measure the stability and bioaccessibility of the bioactives during *in vitro* digestion. PCA and CMA both induced significant changes in the rheological properties of GDL gels, as well as microstructural changes in both GDL and $MgSO_4$ gels. However, the general bioaccessibility profile and levels of phenolics from the different gel matrices were similar and it would seem significantly affected by the pH conditions of the *in vitro* digestion regardless of the presence of the digestive enzymes.

In experiments with a homologous series of hydroxybenzoic and hydoxycinnamic acids, minor changes in the phenolic acid structure resulted in significant differences in their bioaccessibility profile. Overall, the bioaccessibility rates of most of the phenolic acids were above 80 %, which indicates that unpressed soy protein gels could be a good delivery matrix for phenolics.

Phenolic acids with two or more -OH groups such as protocatechuic acid, caffeic acid and gallic acid were sensitive to intestinal conditions, with caffeic and gallic acid having the lowest bioaccessibilities (< 60 %). Our experiments suggested that gallic acid was susceptible to oxidation during the *in vitro* intestinal conditions and that flushing with nitrogen could prevent this phenomenon, although this was not the case for the protocatechuic acid.

Chapter 7
Effect of gel composition on phenolic acid release

Abstract

This chapter aimed to identify some of the compositional features of the SPI gels that might affect the bioaccessibility of the phenolic acids and protein hydrolysis during *in vitro* digestion of soy protein gels.

Results showed that an increase in gels acidity reduced the concentration of bioactives released in the oral phase ($p<0.05$). Grinding of the gels with salivary fluids in a coffee grinder, which is a commonly used technique used to mimic mastication, resulted in significantly higher PCA levels released in both gastric and intestinal phase, compared to non-ground gels. The latter suggests that gel structure is a physical barrier to the bioaccessibility of bioactives. Another factor that was tested, was the protein content of the gels. The higher the protein content, the lower the bioaccessibility of the bioactives in the oral and gastric phase, which slightly reduced the losses in the intestinal phase. These results were attributed to the buffering capacity and denser microstructure of these gels with higher protein content. Moreover, when pressed gels were diluted 2 and 3 times before *in vitro* digestion, the bioaccessibility of CMA increased greatly, which was a direct effect of the changes in the gel-to-simulated fluid ratio. Similarly, proteolysis results showed that diluted gel samples were more prone to protein digestion, which can be attributed to an increased enzyme-to-substrate ratio. Finally, the pressing or straining step used for the production of firm tofu increased the concentration of the bioactives in the gels and thus, larger amounts than the unpressed gels were detected at the end of intestinal incubations.

7.1 Introduction

In the previous chapters, it was shown that the bioaccessibility profile of coumaric acid and protocatechuic acid differ depending on the soy protein gel matrix. In unpressed gels, both phenolic acids were rapidly released during the oral phase, whereas in pressed gels their release was more gradual and was completed in the intestinal phase. Although the mixtures used for producing the SPI gels have the same composition, the pressing step significantly alters the protein content of the pressed gels. Zhang and Vardhanabhuti (2014) found that whey protein aggregates formed at protein concentrations between 3 to 5 % w/w were digested at faster rates than those formed at higher protein concentrations (7 to 9 % w/w). The authors assumed that the smaller size of the low protein content aggregates might be responsible for the higher degradation rate, which might increase the accessibility of pepsin (Zhang and Vardhanabhuti, 2014). More recent studies found that the protein concentration of whey and casein gels affected the pepsin diffusivity and thus their proteolysis rate (Thévenot et al., 2017, Luo et al., 2017). One more difference between the pressed and unpressed gels used in this research is the coagulant concentration. In the case of the pressed gels where protein precipitation is desirable, a high coagulant (GDL and $MgSO_4$) concentration was needed (~ 30 mM). Rui et al. (2016), found that an increase in the GDL concentration slightly increased the proteolysis percentage of unpressed soy protein gels.

It is not clear, however, how the increase in the protein and coagulant concentration can affect the bioaccessibility of added bioactives. Therefore, this chapter aimed to investigate aspects of gel composition that might affect phenolic acid bioaccessibility and the extent of proteolysis in soy protein gels.

7.2 Experimental design

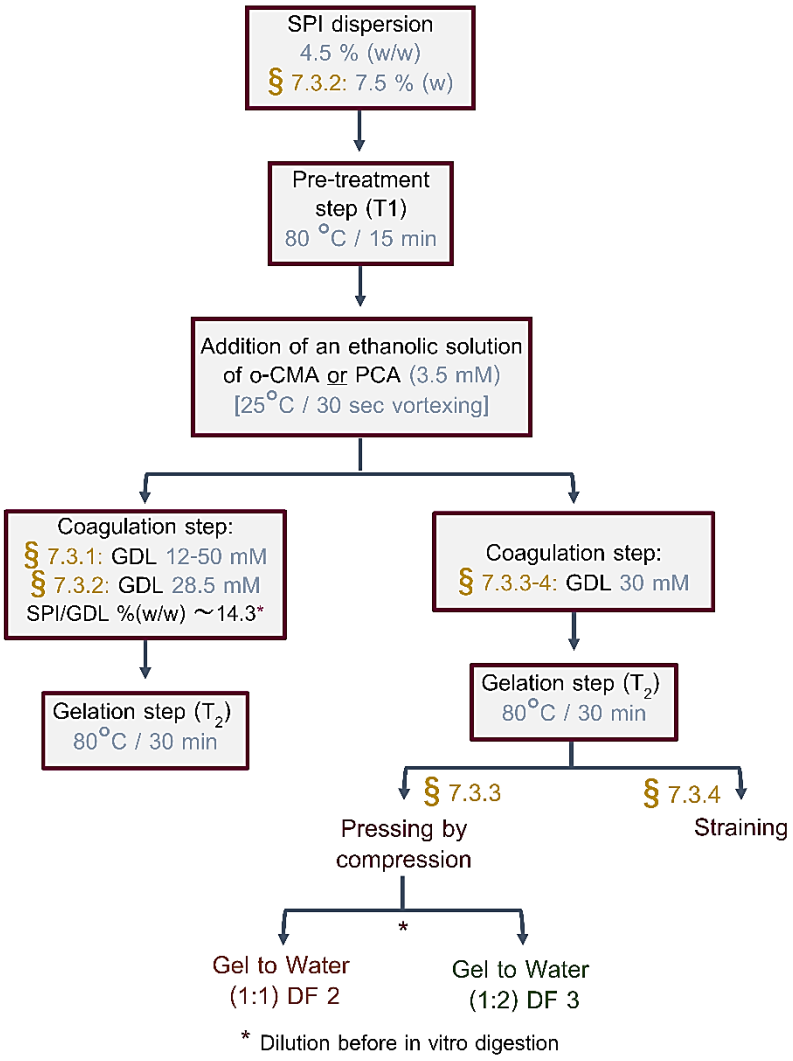


Figure 7.1 Gelation conditions of the different sections of Ch. 7.

7.3 Results and discussion

7.3.1 Effect of the GDL concentration and oral processing on the bioaccessibility of phenolics

The impact of coagulant concentration was tested using GDL and its impact on protocatechuic acid (PCA) release during *in vitro* digestion can be seen in **Figure 7.2**. The gels were either ground in a coffee grinder or gently mixed (non-ground) with the simulated salivary fluid (SSF) during the oral processing step. A high-speed grinder is a fully destructive technique that allowed studying the effect of composition without the interference of the gel structure. The higher the GDL concentration the higher the acidity of the gels (**Figure 4.5**) and, as a result, the lower the net charge of the gels, which increases the overall firmness (results not shown). Also, at a pH close to the pI of the proteins the interaction of phenolics with proteins are favoured (Hagerman and Butler, 1978).

Results from **Figure 7.2 A** showed a clear trend; the higher the GDL concentration, the lower the PCA bioaccessibility in the oral phase, regardless the type of oral processing experienced; $r = -0.98$, $p<0.001$ for ground and $r = -1.00$, $p<0.001$ non-ground boluses, respectively. At a GDL concentration of 12 mM, the PCA amount released was on average 103.4 ± 1.4 % while at 50 mM the bioaccessibility reduced significantly to 83.6 ± 0.8 % ($p<0.05$). The trend was a clear effect of the gel's composition (acidity) since there is no significant difference between ground and non-ground mixtures ($p >0.05$).

Although the pH of the simulated salivary fluid is neutral, the final pH of the mixture depends on the buffering capacity or acidity of each gel sample. During mastication, the food converts rapidly into a bolus and thus, a pH adjustment at 7 would not be realistic. Therefore, the pH of the bolus was inversely proportional to GDL concentration ($r = -0.99$, $p<0.001$). Many *in vitro* phenolic delivery studies either skip the oral phase or measure the bioaccessibility of bioactives only at the end of the gastric and intestinal phase. Our results show that the conditions in the oral phase can affect the bioaccessibility of the phenolics and it should be considered.

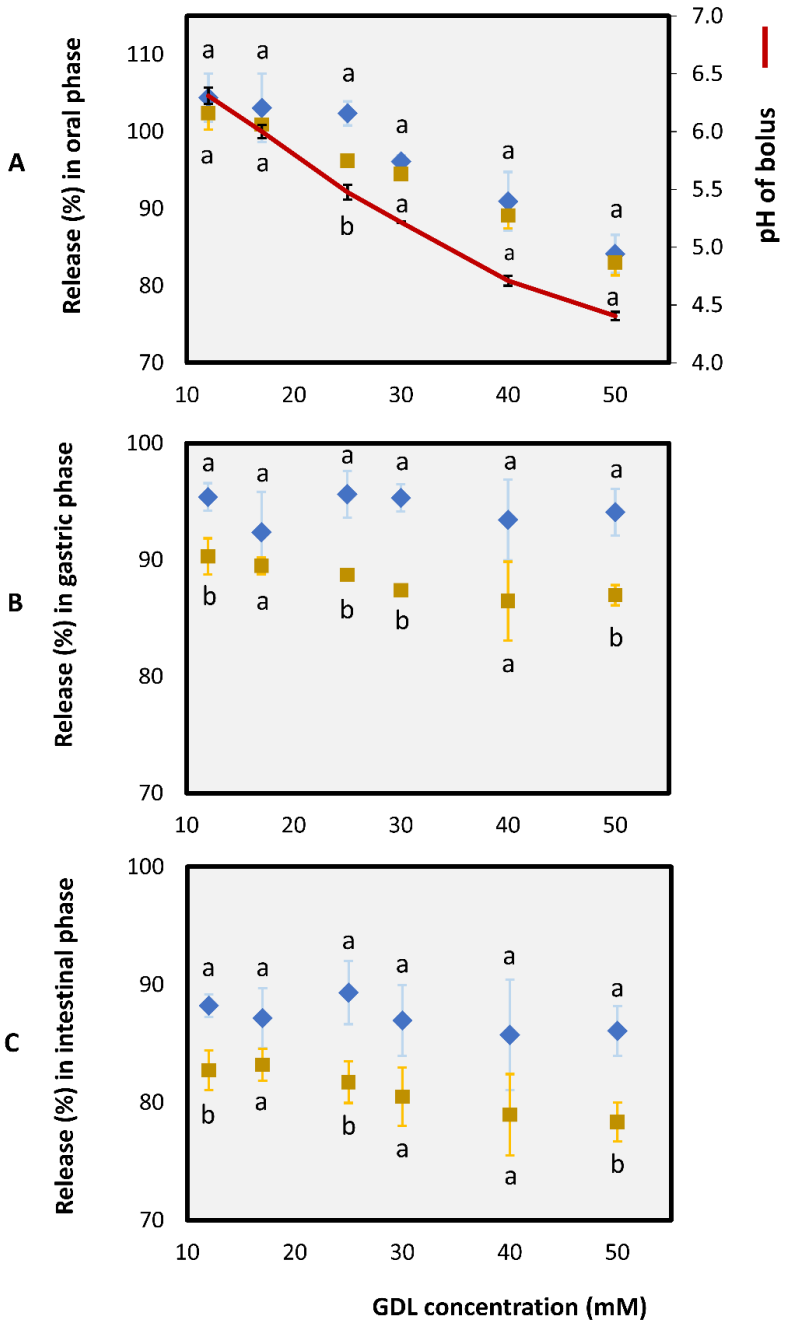


Figure 7.2 Bioaccessibility of PCA from SPI gels (4.5 % w/w) induced by different GDL concentrations (10-50 mM). Comparison of different oral processing (ground ◆ versus non-ground ■ boluses) during the different stages of *in vitro* digestion: Oral (A), gastric (B), intestinal (C).

[1] Values were represented as means ± standard deviations (n=3). [2] Values with a different letter within the same GDL concentration group are significantly different ($p \leq 0.05$). [3] The significant difference between ground and non-ground values for each GDL concentration were assessed with the student t-test.

Gels induced by high GDL concentrations were macroscopically firmer and some small gel particles could be observed during the oral and gastric phase of the

non-ground mixtures. The gel fragments may inhibit the ability of PCA to be completely released from the matrix due to the smaller surface area. Recent studies have shown that the larger the surface area of the food particles during oral processing, the higher the bioaccessibility of added bioactives such as capsaicinoids after mastication (Luo et al., 2019, Luo et al., 2020).

During the gastric conditions, the boluses' pH was adjusted to ~ 2.9 and a more distinct trend can be seen in **Figure 7.2 B**. Overall, the ground mixtures showed a higher PCA bioaccessibility than the non-ground by ~ 3 to 8 %, which was significant in some cases ($p<0.05$). The latter indicated that the existence of gel fragments can inhibit the bioaccessibility of the phenolic acids. In addition, the PCA bioaccessibility was inversely proportional to the GDL concentration ($r = -0.92$, $p = 0.084$) in the non-ground mixtures, whereas in ground mixtures there was no correlation ($r = -0.15$, $p = 0.78$).

Finally, the levels of PCA released during the intestinal phase (pH ~ 7) from all the gel matrices was reduced, by ~ 8 % for both ground and non-ground mixtures, respectively. As it was discussed in **Chapter 6** a decline in phenolic bioaccessibility during the intestinal phase is a common phenomenon. From **Figure 7.2 C**, it can be seen that PCA bioaccessibility from non-ground mixtures was lower than the ground mixtures, which ranged from between ~ 5 to 9 % and was statistically significant in some cases ($p <0.05$). As it was observed in the gastric phase, there was an inverse relationship between GDL concentration and bioaccessibility of PCA in the intestinal phase, which was stronger for non-ground gels ($r = -0.97$, $p < 0.01$ versus $r = -0.68$, $p = 0.13$).

The tendency of non-ground boluses towards lower levels of PCA bioaccessibility during digestion indicates that the gel structure may limit the PCA bioaccessibility to some extent. A reasonable scenario would be that protein digestion was facilitated in the ground boluses, which would imply a lower extent of protein/peptide – PCA interactions in the gastric phase and thus a higher bioaccessibility in the intestinal phase. In contrast to our hypothesis, non-ground boluses were more susceptible to proteolysis than the ground ones (**Figure 7.3**). Also, the proteolysis of non-ground mixtures showed a descending order with increasing GDL concentration ($r = -0.97$, $p = 0.13$). Nevertheless, the degree of protein hydrolysis

at the end of the intestinal incubation did not show any statistically significant differences between ground and non-ground mixtures.

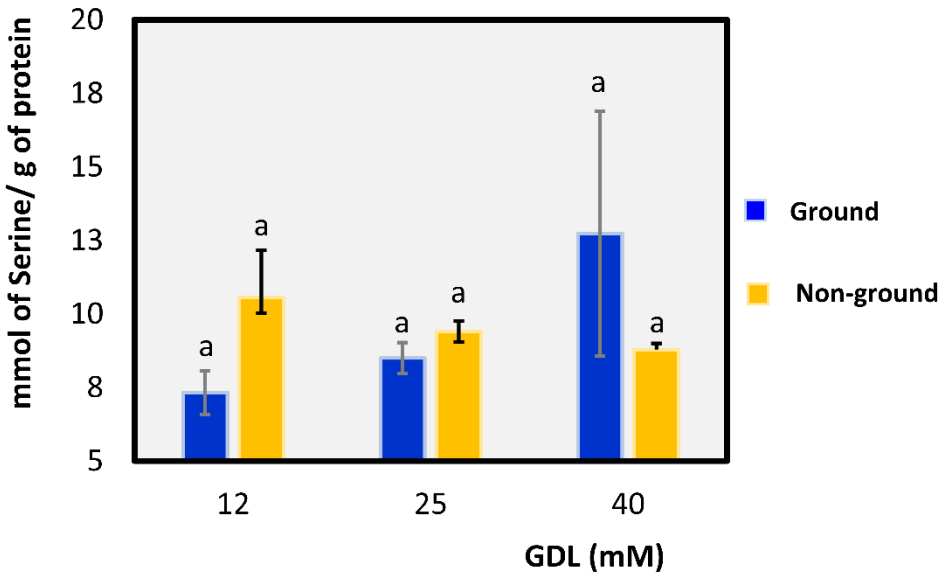


Figure 7.3 Concentration of released peptides, expressed as mmol Serine per gram of protein equivalents, of GDL induced gels at the end of the intestinal phase. concentration. Comparison of different oral processing (grinding with blue versus non-ground with yellow) and different GDL concentrations.

[1] Values were represented as means ± standard deviations (n = 2). [2] The letters a to b indicate that the values are significantly different ($p \leq 0.05$). [3] The mmol of Ser/ g of protein for ground and non-ground gels were analysed using a one-way ANOVA with the GDL concentration (12-40 mM) as the main factor and the comparison was performed using the Tukey test.

7.3.2 How does the protein content of the unpressed gels affect the microstructure and release profile of bioactives?

An increase in the protein content of the unpressed gels induced what looked to be firmer gel textures. The maximum protein content that could be achieved with the commercial SPI used was around 7.5 % (w/w). At higher concentrations than this, a viscous dispersion with clumps of undissolved powder was formed that was challenging to use.

The microstructure of the gels formed by 7.5 % (w/w) protein content was significantly different to the ones formed by 4.5 % (w/w). A more interconnected protein network with larger particle aggregate size (441 ± 76 nm) can be seen in **Figure**

7.4 c. In contrast, 4.5 % (w/w) control gels did not show a clear aggregate protein network and the protein strands were very thin and short in length (**Figure 7.4 a**).

The addition of o-coumaric acid (CMA) resulted in a denser network with a clear aggregate formation in the case of 4.5 % (w/w) gels (**Figure 7.4 b**). The 7.5 % (w/w) gels with added CMA had a more distinct microstructure, which was very dense with more interconnection points and aggregate clumps than previously seen (**Figure 7.4 d**). Analysis of numerous images of the 7.5 % (w/w) -CMA gels showed a variation in the microstructure, although the large aggregates were identified in most of the images. In any case, other studies have suggested an increase in the microstructure density with increased protein content in dairy matrices, which is in agreement with our findings (Thévenot et al., 2017, Luo et al., 2017).

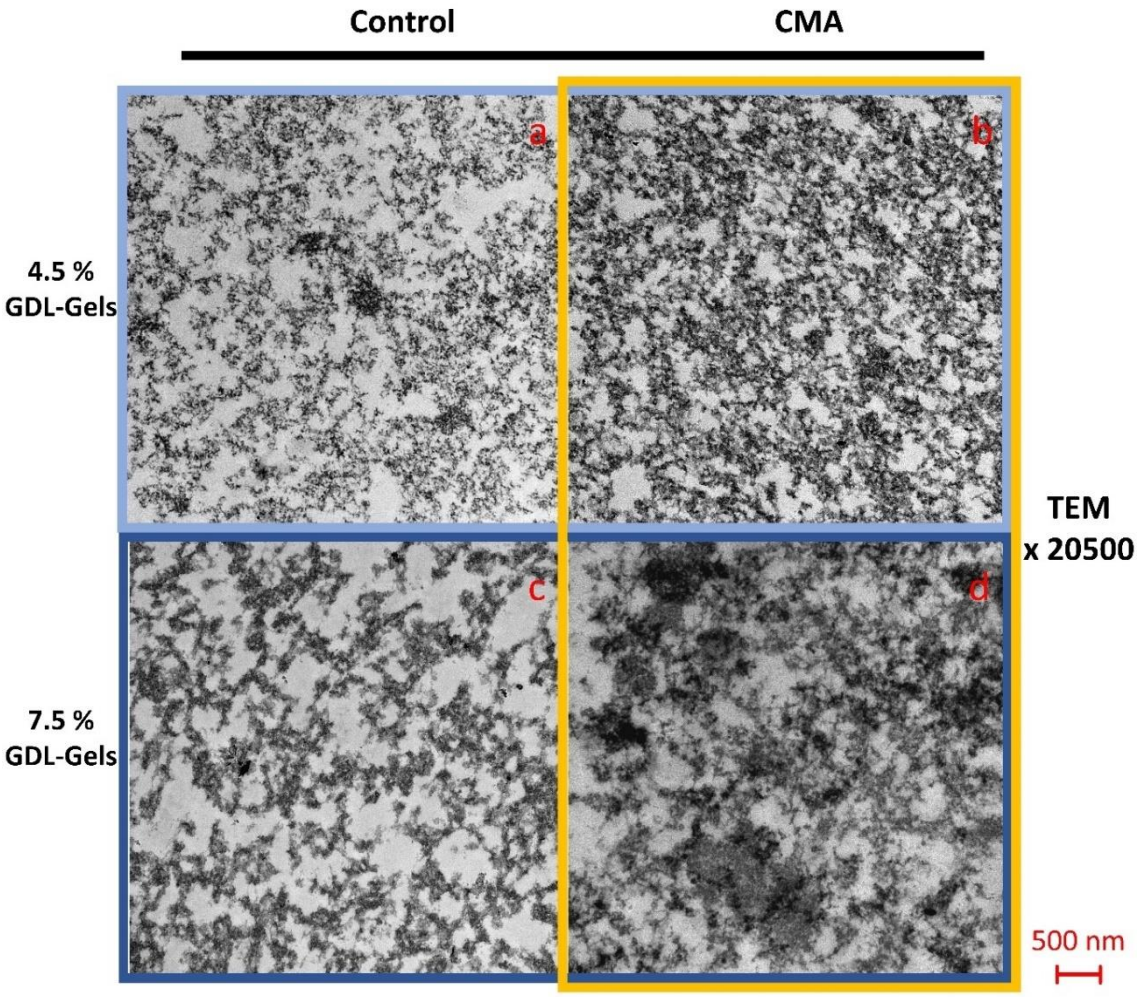


Figure 7.4 Microstructural characteristics of SPI gels enhanced with CMA, induced by GDL with different protein content: Control gel with 4.5 % w/w SP (a) and gel with 4.5 % w/w SP and 3.5 mM CMA (b), control gel with 7.5 % w/w SP (c) and gel with 7.5 % w/w SP and 3.5 mM CMA (d).

Gelation with increased protein content had a similar general bioaccessibility pattern compared with the gels with lower protein content (**Figure 7.5 A**). As described in **Chapter 6**, the release profile of the unpressed gels had three main characteristics: ***a)*** High bioactive release at the end of the oral phase, ***b)*** a significant reduction at gastric conditions and ***c)*** a significant increase or reduction of the phenolic acid levels depending on the chemical structure.

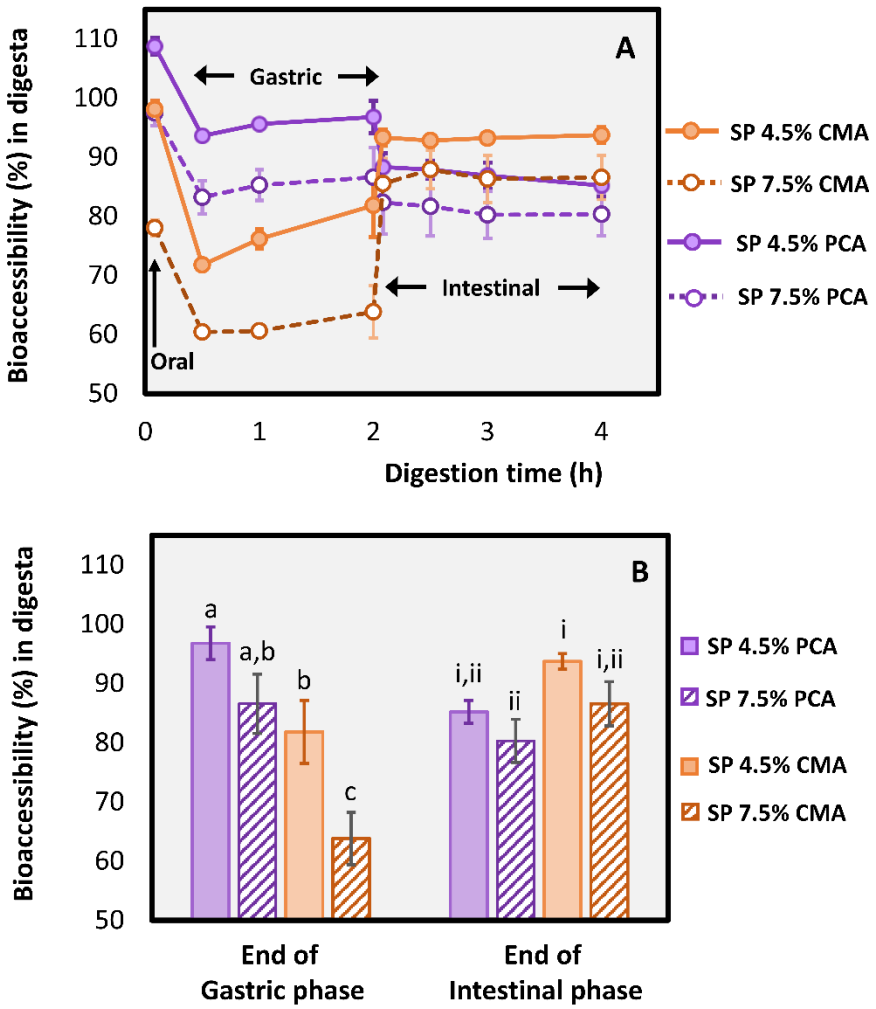


Figure 7.5 Bioaccessibility of PCA and CMA from soy protein unpressed gels induced by different protein content (4.5 and 7.5 % (w/w)) but same SP to GDL ratio (13.4). Phenolic acid bioaccessibility profile (A) and end digestion values (B).

[1] Values were represented as means ± standard deviations (n ≥ 2). [2] The letters a-c and i-ii indicate that the values within the same group are significantly different ($p \leq 0.05$). [3] The bioaccessibility of PCA and CMA at the end of the gastric (t=2h) and intestinal (t=4h) phase were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the added bioactive (PCA, CMA) and the protein content (4.5 % w/w and 7.5 % w/w). The significance of the factor's interaction mean values was assessed with the Tukey test.

The higher protein content (7.5 % w/w) in unpressed gels resulted in reduced phenolic acid bioaccessibility, during the *in vitro* gastrointestinal digestion (**Figure 7.5 B**). Specifically, the amount of PCA and CMA that were released from the 7.5 % (w/w) soy protein gel matrices, at the end of the oral phase, were lower by ∼11 % and ∼ 16 %, respectively, compared with 4.5 % (w/w) gels. This reduction was due to the higher buffering capacity of the 7.5 % (w/w) gels (Mennah-Govela et al., 2019) and the denser gel microstructure (**Figure 7.4**). Similarly, the bioaccessibility concentrations of both phenolics from 7.5 % (w/w) gels were lower than 4.5 % (w/w) gels throughout the gastric phase by ∼10 % for PCA and 22 % for CMA ($p<0.05$).

Studies have shown that denser dairy protein gel microstructures induced by 20 % (w/w) protein content might hinder the pepsin diffusivity and thus their proteolysis (Thévenot et al., 2017, Luo et al., 2017). Also, higher protein content lowers the enzyme-to-substrate (E:S) ratio, which might also reduce the rate of proteolysis. Therefore, undigested proteins or peptides might have a protective effect on the bioactives.

At the intestinal conditions (pH ∼ 7), the bioaccessibility of PCA was reduced by ∼ 12 % and 7 % from the 4.5 and 7.5 % (w/w) gels, respectively, compared with the gastric phase. Thus, 7.5 % (w/w) gels seemed to slightly prevent the PCA losses in the intestinal phase ($p>0.05$). Whereas, CMA bioaccessibility was significantly increased at the intestinal conditions, by ∼ 13 and 26 % from the 4.5 and 7.5 % (w/w) gels, respectively (**Figure 7.5 B**).

Therefore, the lower the bioaccessibility of the bioactives in the gastric phase, the lower the losses of the PCA and the higher the bioaccessibility of CMA in the intestinal phase. Although the bioactives' general bioaccessibility profile seemed similar, there were significant differences in the final bioactive levels. Consequently, protein content affected the modulation of bioactive bioaccessibility under *in vitro* digestion conditions.

7.3.3 Effect of gel-to-simulated fluid ratio during *in vitro* digestion: Pressed gels case

As it has been mentioned before, the pressed and the unpressed gels varied significantly in their composition due to the changes induced by the pressing. The protein concentration of the pressed gels was more than 3-fold higher than the unpressed gels and thus the enzyme-to-substrate ratio (E:S) of these two gels was different during the *in vitro* digestion. The E:S was calculated according to **Table S 3, Table S 4** and **Table S 5 (Appendix 1)**.

This section aimed to evaluate if the different E:S ratios during *in vitro* digestion are responsible for the phenolic acid bioaccessibility differences found between pressed and unpressed gels. To achieve this, the pressed gels were diluted with water in different dilution factors (DF 2 and 3) before oral processing, in order to lower the protein concentration before the incubations. The pressed gels with DF 3 had an E:S ratio equal to the unpressed gels (**Figure 7.6)**.

Bioaccessibility results from the previous chapters showed that the unpressed gels did not prevent the release of bioactives at the oral and gastric phase. In contrast, bioactives released more gradually from pressed gels. From **Figure 7.6 A**, it can be seen that the bioaccessibility of CMA was affected significantly by the change in the E:S ratio during the gastric phase. An increase in the E:S ratio from 0.12 (undiluted pressed gels, DF 1) to 0.24 (DF 2) and 0.36 (DF 3), increased the CMA bioaccessibility in the gastric phase by 36 % and 48 %, respectively ($p<0.05$).

Therefore, the bioaccessibility levels and the pattern of CMA from the DF 3 and the unpressed gels digesta was similar and not significantly different ($p>0.05$). During the intestinal phase, the bioaccessibility levels on a percentage basis were similar for all the gels (**Figure 7.6A**).

Nevertheless, it is not certain that the bioaccessibility of CMA from diluted gels was an effect of the E:S ratio (physical entrapment) or the protein concentration by itself (chemical binding). As it was mentioned before, the E:S ratio was adjusted by diluting the pressed gels with water before the incubations, thus the solvent to gel ratio was altered, which might facilitate the extraction of the phenolics. It has been suggested that an *in vitro* digestion model acts as a polyphenol extractor (Tagliazucchi et al., 2010) and thus, factors such as temperature, type of solvent and solvent to solid

ratio can affect the outcome (Pinelo et al., 2006). Moreover, we know from the previous chapter that the phenolic acids are released at neutral pH with or without digestive enzymes (**Figure 7.6 B**).

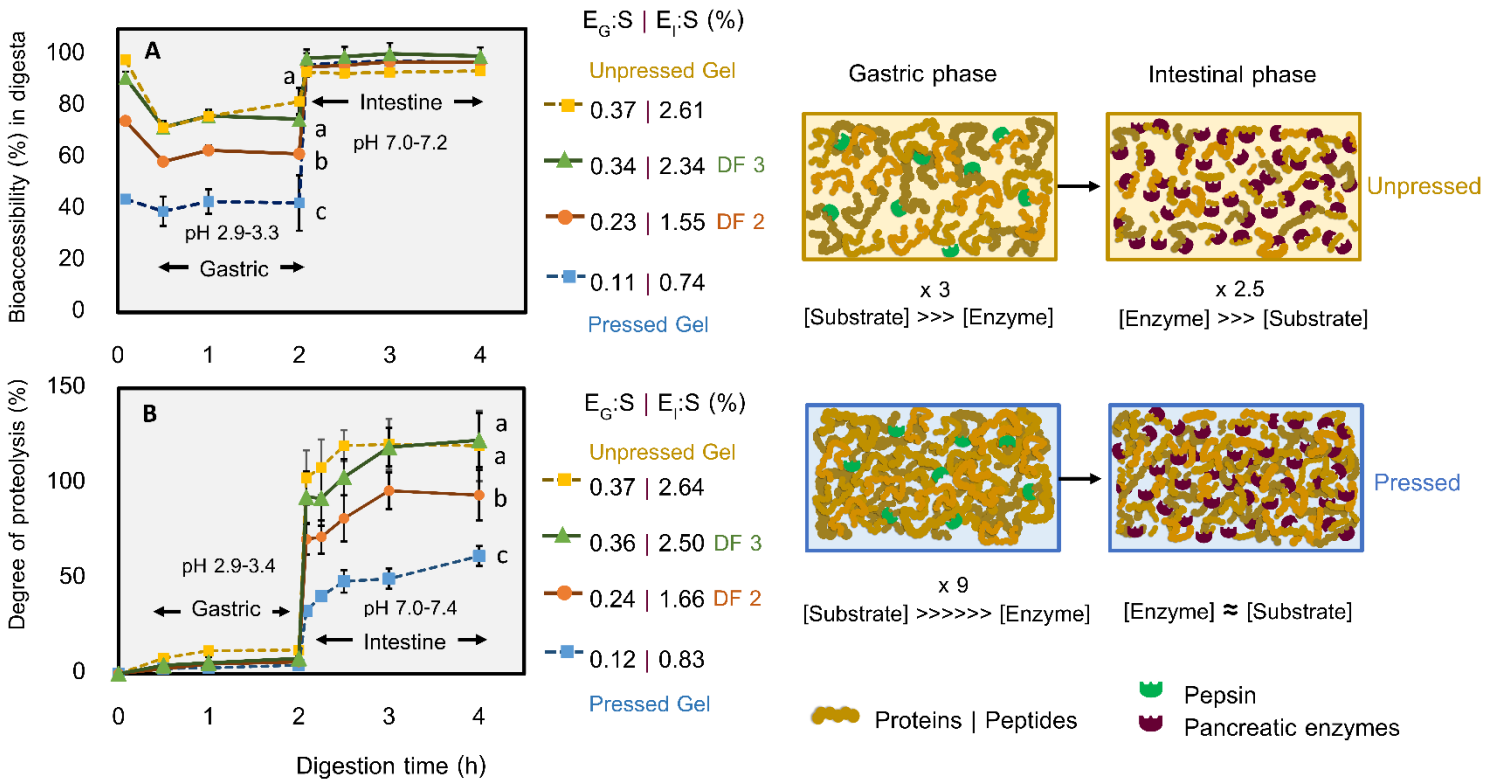


Figure 7.6 Bioaccessibility profile of CMA from pressed SPI-CMA-GDL gels. (A) and the degree of proteolysis (%) of control (SPI-GDL) pressed gels (B). Comparison of undiluted (DF 1) pressed gels with diluted (DF 2 and 3) and unpressed gels, under *in vitro* gastrointestinal conditions. Illustration of E:S ratio in the incubations of pressed and unpressed gels (right).

[1] Values were represented as means ± standard deviations (n=2-5). [2] Letters a and b indicate that values that do not share a letter in the same time point are significantly different ($p \leq 0.05$).[3] The bioaccessibility of CMA at the end of the gastric (t=2h) (A) and intestinal (t=4h) (B) phase were analysed with one-way ANOVA with the type of gels (unpressed, DF 3, DF 2, pressed) as the main factor and the comparison was performed using the Tukey test.

In the case of the pressed gels, the concentration expressed as % (w/w) of pancreatic enzymes and protein is almost equal (E:S = 0.8), while in the case of the unpressed gels (or DF 3 gels) the amount of enzyme is ~2.5 times higher than the protein (E:S = 2.6). As a consequence, the rate of protein hydrolysis was faster as the E:S ratio was increasing and even more rapid for unpressed and DF 3 gels (**Figure 7.6 B**). Studies have shown that a higher substrate concentration (or lower E:S) decreased the rate of hydrolysis in various sources, such as whey proteins (Gonzàlez-Tello et al., 1994) and rapeseed protein isolates (Chabanon et al., 2007). Moreover, Zhang and Vardhanabhuti (2014) found that whey protein aggregates with a protein content between 1 to 5 % w/w were fully digested within 30 min, while those with a protein content of 7 and 9 % w/w remained undigested even after 2 hours (Zhang and Vardhanabhuti, 2014). Thus, the protein content or protease concentration (activity) in the *in vitro* digestion protocols can affect the proteolysis results, which is in agreement with Reynaud et al. (2020).

7.3.4 Effect of pressing on CMA bioaccessibility

To evaluate the effect of the pressing procedure on the bioactive release, a gel was subjected to straining instead of pressing. Even though the straining process resulted in water removal, it was not sufficient to produce a gel with identical composition to pressed gels. The yield of the strained gel was almost double than that of the pressed one, 62.6 ± 4.1 and 32.9 ± 3.5 % respectively. Also, the protein content of the gels tested was significantly different ($p < 0.05$), 4.3 ± 1.5, 10.5 ± 1.2 and 15.1 ± 0.8 % for unpressed, strained and pressed gels, respectively. Moreover, the retention of CMA in the gel was also different because it was dependant on the pressing magnitude. As a result, the larger the force exerted on the gel, the higher the phenolic acid losses. More specifically, the addition of 3.5 mM CMA resulted in the following retention percentages, 100.0 ± 0.0, 79.1 ± 2.5 and 58.8 ± 3.6 % for unpressed, strained and pressed gels, respectively (**Figure 7.7 C, Figure S 1, Figure S 2**).

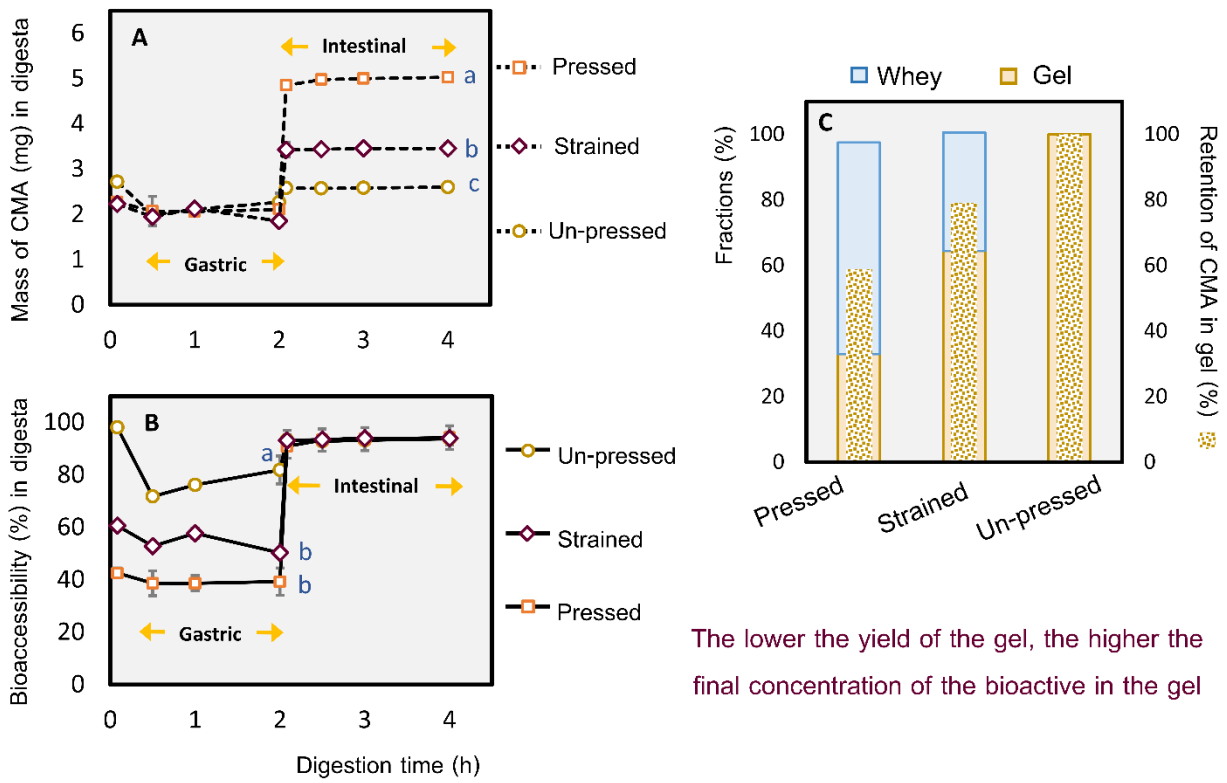


Figure 7.7 Effect of gel pressing on the bioaccessibility of added CMA from GDL induced gels during *in vitro* digestion (unpressed, strained and pressed). Release percentage normalised with the mass of CMA remained in the gel after pressing (A) and mass of CMA released (mg) (B).

1 Values were represented as means ± standard deviations (n=2). [2] Letters a and b indicate that values that do not share a letter in the same time point are significantly different ($p \leq 0.05$). [3] The bioaccessibility of CMA at the end of the intestinal (t=4h) (A) and gastric (t=2h) (B) phase were analysed with one-way ANOVA with the type of gels (unpressed, strained and pressed) as the main factor and the comparison was performed using the Tukey test.

From **Figure 7.7 A** can be seen that the pressing step did not affect the general bioaccessibility pattern of the CMA. The level of CMA bioaccessibility was significantly different between the unpressed and the pressed/ strained gels ($p<0.05$). Also, the main site for complete release was the intestine for all the different gel matrices. The difference was the CMA concentration contained in the gel, which varied based on the yield of the gel and the retention of CMA after strain or pressing **(Figure 7.7 C)**. For instance, the unpressed gels did not show any phase separation and thus the CMA concentration (% w/w) added remained constant (**Figure S 2**). On the other hand, strained and pressed gels were formed by protein precipitation and as a result, the amount of CMA found in the final gel after pressing was inversely proportional to the amount of the gel produced (yield %).

An interesting fact was that the amounts (masses) of CMA released during the gastric phase were identical for all the different gels ($p>0.05$) (**Figure 7.7 B**). Nevertheless, the 2.07 ± 0.21 mg of CMA corresponded to different release percentages after considering the total CMA mass remained in the final gels. The different ways of accounting for CMA release, i.e. percentage vs. mass, explain the different bioaccessibility patterns in **Figure 7.7 A, B** during the gastric and intestinal phase.

7.4 Conclusions

The objective of this study was to assess if the coagulant and protein concentration are responsible for the bioaccessibility differences between pressed and unpressed gels observed in the previous chapters.

Results showed that a higher coagulant concentration (GDL) increased the acidity of the gels during the oral phase which reduced the bioaccessibility of phenolics significantly. It was also found that the grinding process commonly used to mimic the mastication of the bolus, facilitated the bioaccessibility of phenolics. Despite the observed effects, coagulant concentration was not the factor responsible for the bioaccessibility profile differences of the pressed and unpressed gels.

On the other hand, altering the protein content of the gels either during the gel production or during the *in vitro* digestion incubations (by dilution) affected the enzyme to substrate ratio of the digestion reaction, which is responsible for the differences of the pressed and unpressed gels.

Chapter 8

Discussion and concluding remarks

8.1 General discussion

Naturally, phenolics are poorly bioavailable because they are esterified with glucose, organic acids or bound in the fibre matrix of the plants (Bento-Silva et al., 2019, Bohn, 2014b). The use of enzymes such as esterases and cellulases have been suggested as an effective strategy to increase the free form of phenolics and thus their bioavailability (Bento-Silva et al., 2019). The approach in this research was to probe the physical and chemical phenomena limiting the bioaccessibility of phenolics by embedding them in protein gels of varying structure and texture and using a range of structurally related phenolics with different chemical properties. This has direct relevance to the release of endogenous soy polyphenolics in tofu and broader relevance for the development of protein-based functional foods designed to deliver health-enhancing bioactive compounds. Tabletted soy protein hydrogels have been successfully used for modulating the release of riboflavin (Maltais et al., 2009, Maltais et al., 2010). The authors found that gel microstructure was the factor that determined the release profile of the bioactive (Maltais et al., 2009). In this research, we used soy proteins in gelled formats that are analogous to existing food formats (firm and silken tofu).

The challenge in this approach is to protect the bioactives until they reach their target in the human body, which is the intestinal phase (Kahle et al., 2005). Phenolics are sensitive to enzymatic and chemical oxidation, however, food structure could protect bioactives by physically entrapping them within a matrix and delivering them to a targeted site.

One of the challenges of adding bioactives to soy protein gels was maintaining the physical stability of the gels. For the unpressed gels, the addition of phenolic acids (1-5 mM) significantly reduced the water holding capacity (**Figure 4.6**), which could be explained by disruption of the balance between the protein-protein and protein-water interactions in the system (Han et al., 2011). Also, in **Chapter 6**, acidified unpressed gels with added phenolics were significantly ($p<0.05$) firmer than the controls (**Figure 6.2**. The changes of the physical properties of the unpressed gels suggested the formation of non-covalent protein – phenolics interactions, such as hydrogen bonding (Zhang et al., 2010, Wu et al., 2001, Strauss and Gibson, 2004). In contrast, the textural properties of the pressed gels, regardless of the coagulation

mechanism, were not affected significantly by the addition of the phenolics (**Figure 5.2**).

Another objective of this research was to gain a more fundamental understanding of whether there is a relationship between soy protein gels physical properties and *in vitro* proteolysis. To study this, softer and firmer textures of pressed and unpressed gels, with similar composition were developed using different types of coagulants such as $MgSO_4$ and GDL. Gels induced by $MgSO_4$ were significantly softer than the GDL-induced gels. A relationship between the firmness, microstructure and the proteolysis results was observed (**Figure 8.1**).

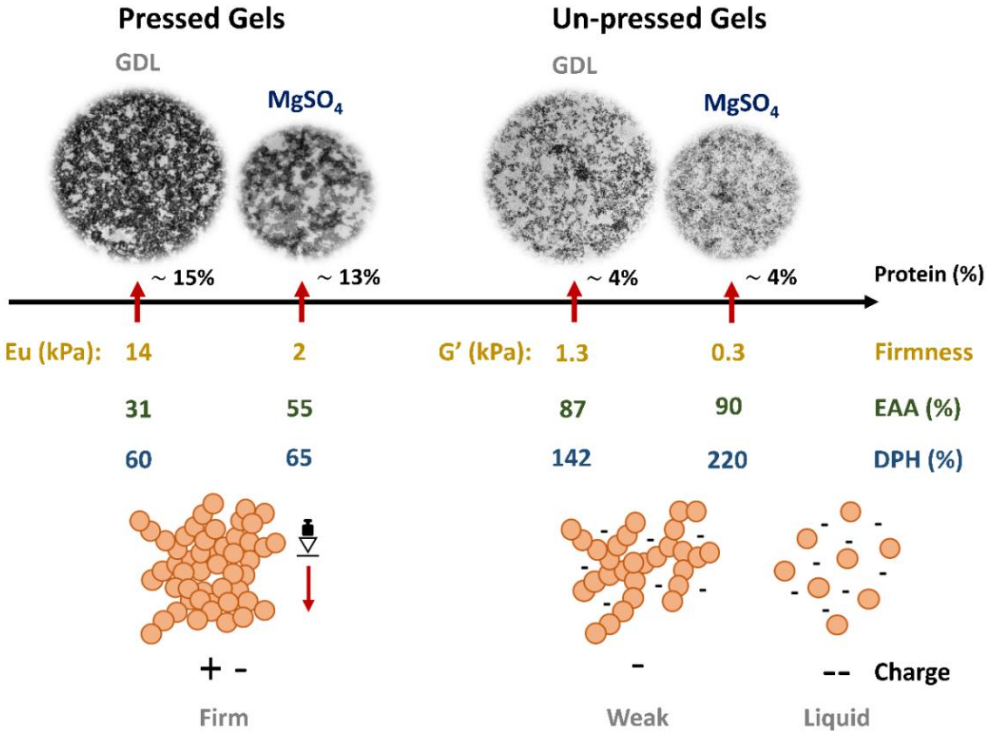


Figure 8.1 Schematic representation of the relationship between texture, microstructure, intestinal bioaccessibility (%) of essential amino acids (EAA) and degree of protein hydrolysis (DPH %).

It seems that the less dense the gel structure, the softer the texture and the more prone to proteolysis. Caution is needed, however, when the different types of gels are compared (unpressed vs pressed), since their protein concentrations differ, which alters the enzyme-to-substrate ratio during *in vitro* digestion (**section 7.3.3)**.

According to Fang et al. (2016), elastic cheeses were less prone to proteolysis compared to ripened and soft cheeses with high-fat content. In addition, other studies have suggested that an increase in the microstructure density of dairy matrices can reduce the penetration of pepsin and thus reduce the rate of proteolysis (Thévenot et al., 2017, Luo et al., 2017). On the other hand, Lorieau et al. (2018) compared different whey protein-based products with different textures/structures and similar composition and did not find any relationship with the proteolysis kinetics.

Another main objective of this research was to compare the bioaccessibility profile of phenolics from the different types of soy protein gels and identify which parameters are important for modulating their release. Comparison of **Chapter 5** and **Chapter 6** showed that both pressed and unpressed gels were good delivery systems since they delivered or released more than 80 % of the bioactive concentration added, within the intestine (**Figure 8.2**). However, the amount eventually released from pressed gels was between 120 to 170 % of the concentration initially added in the mixture. The latter was an effect of the pressing step, which despite the losses in the water/whey serum, increased the concentration of the bioactives available in the gel. The most important characteristics that determined the final concentration of bioactives in the gel after pressing were the production yield of the gel and the retention coefficient of the bioactive. The retention coefficient was dependant on the gelation mechanism (acid or salt) and the chemical structure of the bioactive (**Figure S 1, Figure S 2**). Similarly, Helal et al. (2015) found that the retention of different polyphenols in the cheese curd after pressing varied depending on the structure. Furthermore, the release of bioactives in the oral phase was significantly high (almost complete) in unpressed gels, which is not desirable because the chances for losses (oxidation or interaction with enzymes or other compounds) in later stages are increased.

As it was found in **Chapter 5** the differences seen with the acidified, and salt-induced pressed gels were not because of the texture or microstructure of the gels. On a percentage basis the differences in the release profile of the two pressed gels (GDL versus $MgSO_4$) were non-significant (**Figure 5.8**). Thus, it was concluded that the pressed gels were superior over the unpressed because the final concentration of added bioactives and the textures of the final product can be easily controlled. Also, the added phenolics did not alter the textural characteristics of the gels (in contrast to unpressed

gels), and most importantly the main site of release was the intestine for both bioactives, which is the target site for phenolics bioaccessibility.

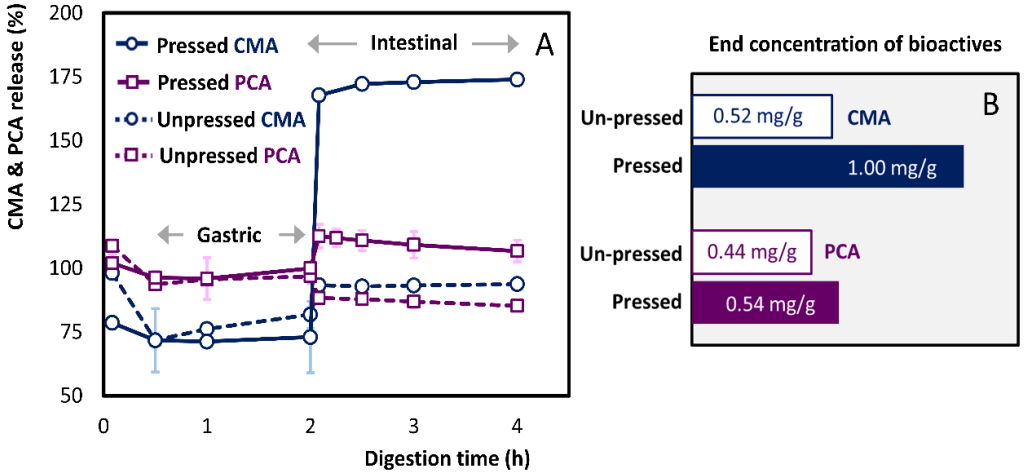


Figure 8.2 Release profile (normalised based on the amount of bioactive initially added in the mixture) (A) and end bioaccessible concentration present in digesta (mg/g of gel) (B) of PCA and CMA embedded in pressed and unpressed gels induced by GDL.

It is worth mentioning that the release of the phenolic acids was independent of the proteolysis rates of the gels, which was confirmed with blank digestion of gels with added phenolics and this agrees with the findings of Bermúdez-Soto et al. (2007). Tagliazucchi et al. (2010) suggested that an *in vitro* digestion model acts as a polyphenol extractor. From both **Chapter 5** and **Chapter 6** became clear that pH during *in vitro* digestion is responsible for the release profile of the PA, regardless of the presence of the digestive enzymes. According to Friedman and Jürgens (2000), the pH affects the stability of various phenolic compounds. Also, Hassan et al. (2013) found that phenolic compounds (including CFA and GLA) interacted strongly with milk proteins at pH 3, while the interaction potential was reduced at pH 7, which agrees with our results. A clear trend between gels' pH and release of PCA in the oral phase was found in **section 7.3.1 (Figure 7.2 A)**. The lower the pH of the gels, the lower the PA release on the aqueous phase of the digesta and thus the lower their bioaccessibility. This trend can be explained by the protonation state of the carboxyl groups of both AA residues and PA. At pH close to pI of proteins the interactions between phenolics and proteins or AA residues in favoured.

Another important finding is that the release profile of PCA from pressed gels had an ascending trend in the intestinal phase, while in the unpressed gels there was a reduction at the same time points (**Figure 8.2** after 2 h), which might be an indication of degradation/ oxidation. Therefore, pressed gels not only deliver higher amounts of bioactives in the intestinal phase but also seem to have a protective effect for some sensitive phenolics.

Some disadvantages of the increased bioactive concentration in pressed gels are the potential for toxicity and adverse effects on the sensory properties. The organoleptic characteristics of the gels were not tested in this study but an increase in bitterness would be expected. The toxicity of phenolics varies depending on the structure; the ones tested in this study are present in fruits and vegetables in a range of 15 to 295 mg/kg for PCA and 7 to 350 mg/ kg for CMA (Herrmann and Nagel, 1989). According to our calculations, a person with a bodyweight of 70 kg would need to consume 58 Kg of tofu to reach the toxicity levels of PCA and CMA (Kakkar and Bais, 2014, Pei et al., 2015) (**Appendix 1**). Therefore, the concentrated phenolic acid amounts detected in the intestinal phase are safe for human consumption.

Finally, a homologous series of structurally related phenolic acids was used to investigate the structure-bioaccessibility profile relationship of phenolic acids embedded in unpressed soy protein gels (**section 6.3.4**). It was found that the phenolic acid bioaccessibility is structure-dependent. A strong positive relationship between bioaccessibility (intestinal phase) and lipophilicity of phenolic acids was found ($r = 0.73$, $p < 0.05$). This suggested that the strength of noncovalent chemical interactions with the gel matrix, i.e. binding strength, can limit release. In addition, the number of -OH groups attached to the phenolic ring appears to have a higher impact on bioaccessibility (**Figure 8.3**). For instance, the lowest bioaccessibility was observed for PCA (~85%), CFA (65%) and GLA (~45%) that have 2 and 3 -OH groups. The latter phenomenon might be an effect of phenolic oxidation at the intestinal phase as it has been suggested by other studies (Friedman and Jürgens, 2000, Bermúdez-Soto et al., 2007), and verified for GLA in **Chapter 6** (**section 6.3.4.3.3**) using N_2-flushed digestion.

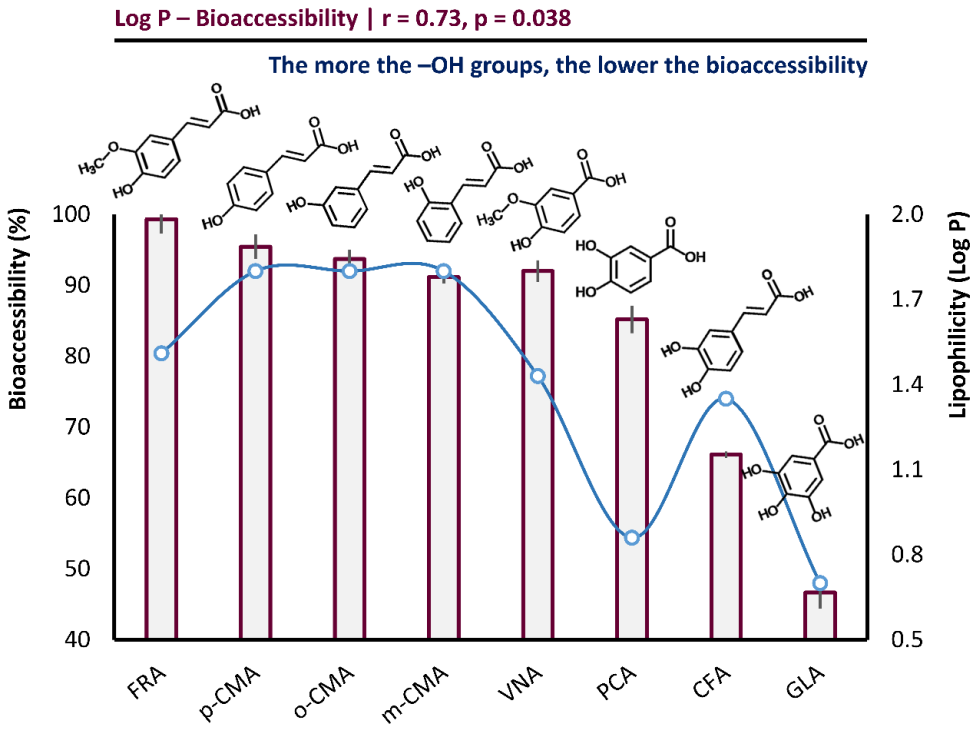


Figure 8.3 Relationship between phenolic acid structure, lipophilicity (open circles) and bioaccessibility (%) (end of intestinal phase) (bars) from unpressed GDL-induced gels.

8.2 Conclusions and future recommendations

Future soy biofunctional products should seek to look at ways to produce products with a dense gel structure and high protein content, similar to pressed tofu, if they want to deliver phenolics in the intestinal phase. An important finding was that acidified pressed gels retained higher bioactive concentration and therefore, they delivered significantly higher phenolic concentrations compared to salt-induced pressed gels. Interestingly, the chemical structure of phenolics affected their retention in the gel after pressing and their release pattern during *in vitro* digestion. Phenolics with 2 or 3 -OH groups attached to the benzene ring were less bioaccessible at the intestinal phase. Future studies should focus on investigating the structure-bioaccessibility relationship of various phenolic structures (increasing complexity) and develop strategies to protect the sensitive structural features of phenolics. Another suggestion is to further research the phenolic to soy protein interactions at conditions more realistic to food systems for instance, acidic and neutral pH and temperatures below 70 °C. Most studies researching the interactions of proteins with phenolics use excessive amounts of phenolics and they focus on alkaline pH conditions (~ 8).

This work gives some insights into the effect of soy protein matrix on proteolysis rates. Gel structures similar to silken tofu exhibited fast rates of protein hydrolysis and high bioaccessibility rates of amino acids. The latter could be useful information for consumers seeking plant protein formulations that promote a faster amino acid release. However, further human digestion studies are needed to confirm the findings of this research.

Finally, we would recommend evaluating proteolysis and phenolics bioaccessibility using soybean tofu. Soybeans contain around 20 % of fat and a small fraction of antinutritional factors that might affect both the release of the bioactives and the protein hydrolysis. In addition, scaling-up of our formulation would test if our results are transferable to tofu/soy gels from industrial production processes.

8.3 Limitations of this research

Proteases have the same proteinaceous nature as their substrate and thus they can self-digest (autolyse). Blank digestion trials showed that the porcine pancreatin extract used was susceptible to autolysis. According to the literature, trypsin of porcine origin (Buck et al., 1962) is less prone to autolysis than other sources. Similarly, in the presence of a small concentration of divalent ions, such as calcium chloride (0.01 M), trypsin is stabilised (Rice et al., 1977). However, the amount of pancreatin used in the INFOGEST protocol is considerably higher than other *in vitro* models, which might intensify the phenomenon. FAA analysis of blank digestions (water as substrate) was equal or higher (**Table S 1, Table S 2**) compared to the ones found in gel samples. The latter indicated that if there was no protein available in the samples, the proteases would start the autolysis faster. Hence, it was not possible to correct the values of the total FAA by subtracting autolysis-derived FAA and identify the fraction of amino acids that originated from soy protein. We hypothesised that the concentration of substrate can affect the autolysis phenomenon, and for this reason, the pressed gels showed lower rates of proteolysis and free amino acids. According to (Rice et al., 1977), trypsin's autolysis susceptibility can be prevented by reductive methylation of lysine residues into e-N,N-dimethyllysine residues, which is used for proteomic quantification (Promega, 2018). Yet, this procedure raises the cost of the enzymes.

Mastication and oral processing are complex procedures that need to be investigated further for the development of delivery systems. In our research, it was found that the bioactives added to the unpressed gels were fully released during the simulated oral processing. However, it is unclear whether a complete PA release in the oral stage occurs under physiological conditions. In the oral phase, the food is transformed into a bolus and the mouth content, including saliva, is directed to the stomach. Also, saliva is a viscous solution that cannot be completely mimicked by the simulated salivary fluid used in the *in vitro* protocols (mixture of aqueous salt solutions). The high viscosity of the saliva could decrease the solubility of the gel structures and thus, the diffusivity of the PA to the water phase of the digesta. In addition, saliva contains proline-rich proteins (PRP) that can interact with phenolic compounds and form complexes. Thus, even if a full release of the phenolics was possible in the oral phase, they could interact with the PRP to some extent and then re-associate at later stages of digestion. An *in vivo* oral processing such as the one from

Guo et al. (2014) could be used to overcome some of the aforementioned limitations and provide additional information such as the particle size distribution of the gel boluses that could potentially influence the release of the PA in the oral phase.

Finally, as it was demonstrated in our study there is a pH dependence of the PA bioaccessibility during *in vitro* digestion which might be due to protein-PA interactions. A static model might not be the right tool to study a pH-dependent bioaccessibility since, the real pH of the GI digestion varies considerably during the digestion, while in static models is adjusted and remains stable.

References

ADACHI, A., HORIKAWA, T., SHIMIZU, H., SARAYAMA, Y., OGAWA, T., SJOLANDER, S., TANAKA, A. & MORIYAMA, T. 2009. Soybean β-conglycinin as the main allergen in a patient with food-dependent exercise-induced anaphylaxis by tofu: Food processing alters pepsin resistance. *Clinical and Experimental Allergy,* 39**,** 167-173. Available: 10.1111/j.1365-2222.2008.03148.x

ADACHI, M., KANAMORI, J., MASUDA, T., YAGASAKI, K., KITAMURA, K., MIKAMI, B. & UTSUMI, S. 2003. Crystal structure of soybean 11S globulin: Glycinin A3B4 homohexamer. *Proceedings of the National Academy of Sciences,* 100**,** 7395-7400. Available: https://doi.org/10.1073/pnas.0832158100

AMBRIZ-PÉREZ, D. L., LEYVA-LÓPEZ, N., GUTIERREZ-GRIJALVA, E. P. & HEREDIA, J. B. 2016. Phenolic compounds: Natural alternative in inflammation treatment. A Review. *Cogent Food & Agriculture,* 2**,** 1131412. Available: https://doi.org/10.1080/23311932.2015.1131412

ARTS, M. J., HAENEN, G. R., WILMS, L. C., BEETSTRA, S. A., HEIJNEN, C. G., VOSS, H. P. & BAST, A. 2002. Interactions between flavonoids and proteins: effect on the total antioxidant capacity. *J Agric Food Chem,* 50**,** 1184-7.

BADLEY, R. A., ATKINSON, D., HAUSER, H., OLDANI, D., GREEN, J. P. & STUBB, J. M. 1975. The structure, physical and chemical properties of the soy bean protein glycinin. *Biochim Biophys Acta,* 412**,** 214-28. Available: https://doi.org/10.1016/0005-2795(75)90036-7

BELLIK, Y., BOUKRAÂ, L., ALZAHRANI, H. A., BAKHOTMAH, B. A., ABDELLAH, F., HAMMOUDI, S. M. & IGUER-OUADA, M. 2013. Molecular Mechanism Underlying Anti-Inflammatory and Anti-Allergic Activities of Phytochemicals: An Update. *Molecules,* 18**,** 322-353. Available: https://doi.org/10.3390/molecules18010322

BENTO-SILVA, A., KOISTINEN, V. M., MENA, P., BRONZE, M. R., HANHINEVA, K., SAHLSTRØM, S., KITRYTĖ, V., MOCO, S. & AURA, A.-M. 2019. Factors affecting intake, metabolism and health benefits of phenolic acids: do we understand individual variability? *European Journal of Nutrition*. Available: https://doi.org/10.1007/s00394-019-01987-6

BERMÚDEZ-SOTO, M. J., TOMÁS-BARBERÁN, F. A. & GARCÍA-CONESA, M. T. 2007. Stability of polyphenols in chokeberry (Aronia melanocarpa) subjected to in vitro gastric and pancreatic digestion. *Food Chemistry,* 102**,** 865-874. Available: https://doi.org/10.1016/j.foodchem.2006.06.025

BIKBOW, T. M., GRINBERG, V. Y., ANTONOV, Y. A., TOLSTOGUZOV, V. B. & SCHMANDKE, H. 1979. On the concentration dependence of the elasticity modulus of soybean globulin gels. *Polymer Bulletin,* 1**,** 865-869. Available: https://doi.org/10.1007/BF00283220

BLAKE, J. S., MUNOZ, K. D. & VOLPE, S. 2019. *Nutrition: From Science to You,* Pearson.

BOHN, T. 2014a. Dietary factors affecting polyphenol bioavailability. *Nutrition Reviews,* 72**,** 429-452. Available: https://doi.org/10.1111/nure.12114

BOHN, T. 2014b. Dietary factors affecting polyphenol bioavailability. *Nutr Rev,* 72**,** 429-52. Available: https://doi.org/10.1111/nure.12114

BOISEN, S. & EGGUM, B. O. 1991. Critical Evaluation of in Vitro Methods for Estimating Digestibility in Simple-Stomach Animals. *Nutrition Research Reviews,* 4**,** 141-162. Available: 10.1079/NRR19910012
BOLAND, M., GOLDING, M. & SINGH, H. 2014. *Food Structures, Digestion and Health*, Elsevier Science.
BOURNE, L. C. & RICE-EVANS, C. 1998. Bioavailability of Ferulic Acid. *Biochemical and Biophysical Research Communications,* 253**,** 222-227. Available: https://doi.org/10.1006/bbrc.1998.9681
BRODKORB, A., EGGER, L., ALMINGER, M., ALVITO, P., ASSUNÇÃO, R., BALLANCE, S., BOHN, T., BOURLIEU-LACANAL, C., BOUTROU, R., CARRIÈRE, F., CLEMENTE, A., CORREDIG, M., DUPONT, D., DUFOUR, C., EDWARDS, C., GOLDING, M., KARAKAYA, S., KIRKHUS, B., LE FEUNTEUN, S., LESMES, U., MACIERZANKA, A., MACKIE, A. R., MARTINS, C., MARZE, S., MCCLEMENTS, D. J., MÉNARD, O., MINEKUS, M., PORTMANN, R., SANTOS, C. N., SOUCHON, I., SINGH, R. P., VEGARUD, G. E., WICKHAM, M. S. J., WEITSCHIES, W. & RECIO, I. 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols,* 14**,** 991-1014. Available: 10.1038/s41596-018-0119-1
BUCK, F. F., VITHAYATHIL, A. J., BIER, M. & NORD, F. F. 1962. On the mechanism of enzyme action. LXXIII. Studies on trypsins from beef, sheep and pig pancreas. *Archives of Biochemistry and Biophysics,* 97**,** 417-424. Available: https://doi.org/10.1016/0003-9861(62)90099-1
BUITIMEA-CANTÚA, N. E., GUTIÉRREZ-URIBE, J. A. & SERNA-SALDÍVAR, S. O. 2018. Phenolic–Protein Interactions: Effects on Food Properties and Health Benefits. *Journal of Medicinal Food,* 21**,** 188-198. Available: https://doi.org/10.1089/jmf.2017.0057
CAI, T. & CHANG, K. C. 1999. Processing effect on soybean storage proteins and their relationship with tofu quality. *J Agric Food Chem,* 47**,** 720-7. Available: https://doi.org/10.1021/jf980571z
CALDWELL, J., GARDNER, I. & SWALES, N. 1995. An Introduction to Drug Disposition: The Basic Principles of Absorption, Distribution, Metabolism, and Excretion. *Toxicologic Pathology,* 23**,** 102-114. Available: https://doi.org/10.1177/019262339502300202
CĂLINOIU, L. F. & VODNAR, D. C. 2018. Whole Grains and Phenolic Acids: A Review on Bioactivity, Functionality, Health Benefits and Bioavailability. *Nutrients,* 10**,** 1615. Available: https://doi.org/10.3390/nu10111615
ÇAM, M., İÇYER, N. C. & ERDOĞAN, F. 2014. Pomegranate peel phenolics: Microencapsulation, storage stability and potential ingredient for functional food development. *LWT - Food Science and Technology,* 55**,** 117-123. Available: https://doi.org/10.1016/j.lwt.2013.09.011
CAO, H., JING, X., WU, D. & SHI, Y. 2013. Methylation of genistein and kaempferol improves their affinities for proteins. *International Journal of Food Sciences and Nutrition,* 64**,** 437-443. Available: 10.3109/09637486.2012.759186
CAO, N., FU, Y. & HE, J. 2007. Mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid. *Food Hydrocolloids,* 21**,** 575-584. Available: https://doi.org/10.1016/j.foodhyd.2006.07.001

CARBONARO, M., CAPPELLONI, M., NICOLI, S., LUCARINI, M. & CARNOVALE, E. 1997. Solubility−Digestibility Relationship of Legume Proteins. *Journal of Agricultural and Food Chemistry,* 45**,** 3387-3394. Available: https://doi.org/10.1021/jf970070y
CARBONARO, M., MASELLI, P. & NUCARA, A. 2015. Structural aspects of legume proteins and nutraceutical properties. *Food Research International,* 76**,** 19-30. Available: https://doi.org/10.1016/j.foodres.2014.11.007
CATSIMPOOLAS, N. & MEYER, E. 1970. Gelation phenomena of soybean globulins. I. Protein-protein interactions. *Cereal Chemistry,* 47**,** 559-570.
CHABANON, G., CHEVALOT, I., FRAMBOISIER, X., CHENU, S. & MARC, I. 2007. Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates. *Process Biochemistry,* 42**,** 1419-1428. Available: https://doi.org/10.1016/j.procbio.2007.07.009
CHANG, S. K. C. 2006. Soymilk and Tofu Manufacturing. *Handbook of Food Products Manufacturing.* Available: https://doi.org/10.1002/9780470113554.ch95
CHELH, I., GATELLIER, P. & SANTÉ-LHOUTELLIER, V. 2006. Technical note: A simplified procedure for myofibril hydrophobicity determination. *Meat science,* 74**,** 681-683. Available: https://doi.org/10.1016/j.meatsci.2006.05.019
CHEYNIER, V., COMTE, G., DAVIES, K. M., LATTANZIO, V. & MARTENS, S. 2013. Plant phenolics: Recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiology and Biochemistry,* 72**,** 1-20. Available: https://doi.org/10.1016/j.plaphy.2013.05.009
CHISARI, E., SHIVAPPA, N. & VYAS, S. 2019. Polyphenol-Rich Foods and Osteoporosis. *Curr Pharm Des,* 25**,** 2459-2466. Available: https://doi.org/10.2174/1381612825666190722093959
CHOUCHOULI, V., KALOGEROPOULOS, N., KONTELES, S. J., KARVELA, E., MAKRIS, D. P. & KARATHANOS, V. T. 2013. Fortification of yoghurts with grape (Vitis vinifera) seed extracts. *LWT - Food Science and Technology,* 53**,** 522-529. Available: https://doi.org/10.1016/j.lwt.2013.03.008
CHRONAKIS, I. S. 1996. Network formation and viscoelastic properties of commercial soy protein dispersions: effect of heat treatment, pH and calcium ions. *Food Research International,* 29**,** 123-134. Available: https://doi.org/10.1016/0963-9969(96)00018-X
CHURCH, F. C., PORTER, D. H., CATIGNANI, G. L. & SWAISGOOD, H. E. 1985. An o-phthalaldehyde spectrophotometric assay for proteinases. *Analytical Biochemistry,* 146**,** 343-348. Available: https://doi.org/10.1016/0003-2697(85)90549-4
CILLIERS, J. J. L. & SINGLETON, V. L. 1989. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *Journal of Agricultural and Food Chemistry,* 37**,** 890-896. Available: https://doi.org/10.1021/jf00088a013
CLIFFORD, M. N. 1999. Chlorogenic acids and other cinnamates – nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science of Food and Agriculture,* 79**,** 362-372. Available: https://doi.org/10.1002/(sici)1097-0010(19990301)79:3<362::Aid-jsfa256>3.0.Co;2-d
CLIFFORD, M. N. 2000. Chlorogenic acids and other cinnamates – nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science*

of Food and Agriculture, 80**,** 1033-1043. Available: https://doi.org/10.1002/(sici)1097-0010(20000515)80:7<1033::Aid-jsfa595>3.0.Co;2-t
COOK, M. T., TZORTZIS, G., CHARALAMPOPOULOS, D. & KHUTORYANSKIY, V. V. 2012. Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release,* 162**,** 56-67. Available: https://doi.org/10.1016/j.jconrel.2012.06.003
CORRING, T. 1980. The adaptation of digestive enzymes to the diet: its physiological significance. *Reprod Nutr Dev,* 20**,** 1217-35. Available: 10.1051/rnd:19800713
CORRING, T., JUSTE, C. & LHOSTE, E. F. 1989. Nutritional Regulation of Pancreatic and Biliary Secretions. *Nutrition Research Reviews,* 2**,** 161-180. Available: 10.1079/NRR19890013
COUNCIL, N. R. 1989. *Recommended Dietary Allowances: 10th Edition,* Washington, DC, The National Academies Press. Available: https://doi.org/10.17226/1349
CUEVA, C., MORENO-ARRIBAS, M. V., MARTÍN-ÁLVAREZ, P. J., BILLS, G., VICENTE, M. F., BASILIO, A., RIVAS, C. L., REQUENA, T., RODRÍGUEZ, J. M. & BARTOLOMÉ, B. 2010. Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in Microbiology,* 161**,** 372-382. Available: https://doi.org/10.1016/j.resmic.2010.04.006
DAMODARAN, S., PARKIN, K. L. & FENNEMA, O. R. 2007. *Fennema's Food Chemistry, Fourth Edition,* Taylor & Francis.
DAY, L. 2013. Proteins from land plants – Potential resources for human nutrition and food security. *Trends in Food Science & Technology,* 32**,** 25-42. Available: https://doi.org/10.1016/j.tifs.2013.05.005
DE FREITAS, V. & MATEUS, N. 2012. Protein/polyphenol interactions: past and present contributions. Mechanisms of astringency perception. *Current Organic Chemistry,* 16**,** 724-746.
DE LUMEN, B. O., GALVEZ, A. F., REVILLEZA, M. J. & KRENZ, D. C. 1999. Molecular Strategies to Improve The Nutritional Quality of Legume Proteins. *In:* SHAHIDI, F., KOLODZIEJCZYK, P., WHITAKER, J. R., MUNGUIA, A. L. & FULLER, G. (eds.) *Chemicals via Higher Plant Bioengineering.* Boston, MA: Springer US. Available: https://10.1007/978-1-4615-4729-7_10
DE QUEIRÓS, L. D., DE ÁVILA, A. R. A., BOTARO, A. V., CHIROTTO, D. B. L., MACEDO, J. A. & MACEDO, G. A. 2020. Combined isoflavones biotransformation increases the bioactive and antioxidant capacity of soymilk. *Applied Microbiology and Biotechnology,* 104**,** 10019-10031. Available: 10.1007/s00253-020-10986-1
DE QUEIRÓS, L. D., DIAS, F. F. G., DE ÁVILA, A. R. A., MACEDO, J. A., MACEDO, G. A. & LEITE NOBREGA DE MOURA BELL, J. M. 2021. Effects of enzyme-assisted extraction on the profile and bioaccessibility of isoflavones from soybean flour. *Food Research International,* 147. Available: 10.1016/j.foodres.2021.110474
DEL BO, C., CIAPPELLANO, S., KLIMIS-ZACAS, D., MARTINI, D., GARDANA, C., RISO, P. & PORRINI, M. 2010. Anthocyanin absorption, metabolism, and distribution from a wild blueberry-enriched diet (Vaccinium

angustifolium) is affected by diet duration in the Sprague-Dawley rat. *J Agric Food Chem,* 58**,** 2491-7. Available: https://doi.org/10.1021/jf903472x
DEMAN, J. M. 1986. Texture and microstructure of soybean curd (tofu) as affected by different coagulants. *Food Microstr.*, 5**,** 83-89.
DÉPREZ, S. P., BREZILLON, C., RABOT, S., PHILIPPE, C., MILA, I., LAPIERRE, C. & SCALBERT, A. 2000. Polymeric Proanthocyanidins Are Catabolized by Human Colonic Microflora into Low-Molecular-Weight Phenolic Acids. *The Journal of Nutrition,* 130**,** 2733-2738. Available: https://doi.org/10.1093/jn/130.11.2733
DERJAGUIN, B. & LANDAU, L. 1941. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta Physicochimica U.R.S.S.,* 14**,** 633-662. Available: https://doi.org/10.1016/0079-6816(93)90013-L
DIAS, K., MYERS, D. J., BIAN, Y., LIHONO, M. A., WU, S. & MURPHY, P. A. 2003. Functional properties of the acidic and basic subunits of the glycinin (11S) soy protein fraction. *Journal of the American Oil Chemists' Society,* 80**,** 551-555. Available: https://doi.org/10.1007/s11746-003-0736-7
DRUGBANK. 2020. *Drugbank. 3,4-Dihydroxybenzoic acid* [Online]. Available: https://go.drugbank.com/drugs/DB03946 [Accessed 20/11/2020].
DUPONT, D., ALRIC, M., BLANQUET-DIOT, S., BORNHORST, G., CUEVA, C., DEGLAIRE, A., DENIS, S., FERRUA, M., HAVENAAR, R., LELIEVELD, J., MACKIE, A. R., MARZORATI, M., MENARD, O., MINEKUS, M., MIRALLES, B., RECIO, I. & VAN DEN ABBEELE, P. 2019. Can dynamic in vitro digestion systems mimic the physiological reality? *Critical Reviews in Food Science and Nutrition,* 59**,** 1546-1562. Available: 10.1080/10408398.2017.1421900
EGGER, L., SCHLEGEL, P., BAUMANN, C., STOFFERS, H., GUGGISBERG, D., BRÜGGER, C., DÜRR, D., STOLL, P., VERGÈRES, G. & PORTMANN, R. 2017. Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Research International,* 102**,** 567-574. Available: https://doi.org/10.1016/j.foodres.2017.09.047
EL-SEEDI, H. R., EL-SAID, A. M. A., KHALIFA, S. A. M., GÖRANSSON, U., BOHLIN, L., BORG-KARLSON, A.-K. & VERPOORTE, R. 2012. Biosynthesis, Natural Sources, Dietary Intake, Pharmacokinetic Properties, and Biological Activities of Hydroxycinnamic Acids. *Journal of Agricultural and Food Chemistry,* 60**,** 10877-10895. Available: https://doi.org/10.1021/jf301807g
FAIRWEATHER-TAIT, S. J. & SOUTHON, S. 2003. Bioavailability of nutrients. *In:* CABALLERO, B. (ed.) *Encyclopedia of Food Sciences and Nutrition (Second Edition).* Oxford: Academic Press. Available: https://doi.org/10.1016/B0-12-227055-X/00096-1
FANG, X., RIOUX, L.-E., LABRIE, S. & TURGEON, S. L. 2016. Commercial cheeses with different texture have different disintegration and protein/peptide release rates during simulated in vitro digestion. *International Dairy Journal,* 56**,** 169-178. Available: https://doi.org/10.1016/j.idairyj.2016.01.023
FAO/WHO 1991. Protein Quality Evaluation: Report of the Joint FAO/WHO. Expert Consulation FAO Food and Nutrition Paper S1

FAO/WHO 2013. Dietary protein quality evaluation in human nutrition. 2013/01/01 ed.: Report of an FAO Expert Consultation.
FELICIANO, R. P., BOERES, A., MASSACESSI, L., ISTAS, G., VENTURA, M. R., NUNES DOS SANTOS, C., HEISS, C. & RODRIGUEZ-MATEOS, A. 2016. Identification and quantification of novel cranberry-derived plasma and urinary (poly)phenols. *Archives of Biochemistry and Biophysics,* 599**,** 31-41. Available: https://doi.org/10.1016/j.abb.2016.01.014
FERREIRA, I. C. F. R., MARTINS, N. & BARROS, L. 2017. Chapter One - Phenolic Compounds and Its Bioavailability: In Vitro Bioactive Compounds or Health Promoters? *In:* TOLDRÁ, F. (ed.) *Advances in Food and Nutrition Research.* Academic Press. Available: https://doi.org/10.1016/bs.afnr.2016.12.004
FERRER-GALLEGO, R., HERNÁNDEZ-HIERRO, J. M., BRÁS, N. F., VALE, N., GOMES, P., MATEUS, N., DE FREITAS, V., HEREDIA, F. J. & ESCRIBANO-BAILÓN, M. T. 2017. Interaction between Wine Phenolic Acids and Salivary Proteins by Saturation-Transfer Difference Nuclear Magnetic Resonance Spectroscopy (STD-NMR) and Molecular Dynamics Simulations. *Journal of Agricultural and Food Chemistry,* 65**,** 6434-6441. Available: 10.1021/acs.jafc.6b05414
FERRUZZI, M. G., BORDENAVE, N. & HAMAKER, B. R. 2012. Does flavor impact function? Potential consequences of polyphenol–protein interactions in delivery and bioactivity of flavan-3-ols from foods. *Physiology & Behavior,* 107**,** 591-597. Available: https://doi.org/10.1016/j.physbeh.2012.02.020
FOODB. 2020. *Foodb. trans-o-Coumaric acid* [Online]. Available: https://foodb.ca/compounds/FDB002589 [Accessed 20/11/2020].
FRIEDMAN, M. 1978. Inhibition of Lysinoalanine Synthesis by Protein Acylation. *In:* FRIEDMAN, M. (ed.) *Nutritional Improvement of Food and Feed Proteins.* Boston, MA: Springer US. Available: https://doi.org/10.1007/978-1-4684-3366-1_30
FRIEDMAN, M. & BRANDON, D. L. 2001. Nutritional and Health Benefits of Soy Proteins. *Journal of Agricultural and Food Chemistry,* 49**,** 1069-1086. Available: https://doi.org/10.1021/jf0009246
FRIEDMAN, M. & JÜRGENS, H. S. 2000. Effect of pH on the Stability of Plant Phenolic Compounds. *Journal of Agricultural and Food Chemistry,* 48**,** 2101-2110. Available: https://doi.org/10.1021/jf990489j
FRIEDMAN, M., LEVIN, C. E. & NOMA, A. T. 1984. Factors Governing Lysinoalanine Formation in Soy Proteins. *Journal of Food Science,* 49**,** 1282-1288. Available: https://10.1111/j.1365-2621.1984.tb14970.x
FRIEDMAN, M. & LIARDON, R. 1985. Racemization kinetics of amino acid residues in alkali-treated soybean protein. *Journal of Agricultural and Food Chemistry,* 33**,** 666-672. Available: https://doi.org/10.1021/jf00064a025
FULCRAND, H., CHEMINAT, A., BROUILLARD, R. & CHEYNIER, V. 1994. Characterization of compounds obtained by chemical oxidation of caffeic acid in acidic conditions. *Phytochemistry,* 35**,** 499-505. Available: https://doi.org/10.1016/S0031-9422(00)94790-3
GAN, J., CHEN, H., LIU, J., WANG, Y., NIRASAWA, S. & CHENG, Y. 2016a. Interactions of beta-Conglycinin (7S) with Different Phenolic Acids-Impact on Structural Characteristics and Proteolytic Degradation of Proteins. *Int J Mol Sci,* 17. Available: 10.3390/ijms17101671

GAN, J., CHEN, H., LIU, J., WANG, Y., NIRASAWA, S. & CHENG, Y. 2016b. Interactions of β-Conglycinin (7S) with Different Phenolic Acids-Impact on Structural Characteristics and Proteolytic Degradation of Proteins. *International journal of molecular sciences,* 17**,** 1671. Available: https://doi.org/10.3390/ijms17101671
GAYOSO, L., CLAERBOUT, A.-S., CALVO, M. I., CAVERO, R. Y., ASTIASARÁN, I. & ANSORENA, D. 2016. Bioaccessibility of rutin, caffeic acid and rosmarinic acid: Influence of the in vitro gastrointestinal digestion models. *Journal of Functional Foods,* 26**,** 428-438. Available: https://doi.org/10.1016/j.jff.2016.08.003
GEORGAKOULI, K., MPESIOS, A., KOURETAS, D., PETROTOS, K., MITSAGGA, C., GIAVASIS, I. & JAMURTAS, A. Z. 2016. The Effects of an Olive Fruit Polyphenol-Enriched Yogurt on Body Composition, Blood Redox Status, Physiological and Metabolic Parameters and Yogurt Microflora. *Nutrients,* 8**,** 344. Available: https://doi.org/10.3390/nu8060344
GIROUX, H. J., DE GRANDPRÉ, G., FUSTIER, P., CHAMPAGNE, C. P., ST-GELAIS, D., LACROIX, M. & BRITTEN, M. 2013. Production and characterization of Cheddar-type cheese enriched with green tea extract. *Dairy Science & Technology,* 93**,** 241-254. Available: https://doi.org/10.1007/s13594-013-0119-4
GOLDING, M. 2019. Exploring and Exploiting the Role of Food Structure in Digestion. *In:* GOUSETI, O., BORNHORST, G. M., BAKALIS, S. & MACKIE, A. (eds.) *Interdisciplinary Approaches to Food Digestion.* Cham: Springer International Publishing. Available: https://doi.org/10.1007/978-3-030-03901-1_5
GONZÀLEZ-TELLO, P., CAMACHO, F., JURADO, E., PÁEZ, M. P. & GUADIX, E. M. 1994. Enzymatic hydrolysis of whey proteins: I. Kinetic models. *Biotechnol Bioeng,* 44**,** 523-8. Available: https://doi.org/10.1002/bit.260440415
GOODMAN, B. E. 2010. Insights into digestion and absorption of major nutrients in humans. *Advances in Physiology Education,* 34**,** 44-53. Available: https://doi.org/10.1152/advan.00094.2009
GOSAL, W. S. & ROSS-MURPHY, S. B. 2000. Globular protein gelation. *Current Opinion in Colloid & Interface Science,* 5**,** 188-194. Available: https://doi.org/10.1016/S1359-0294(00)00057-1
GRAEFE, E. U. & VEIT, M. 1999. Urinary metabolites of flavonoids and hydroxycinnamic acids in humans after application of a crude extract from Equisetum arvense. *Phytomedicine,* 6**,** 239-246. Available: https://doi.org/10.1016/S0944-7113(99)80015-4
GRAY, G. M. & COOPER, H. L. 1971. Protein Digestion and Absorption. *Gastroenterology,* 61**,** 535-544. Available: https://doi.org/10.1016/S0016-5085(19)33506-1
GUNASEKARAN, S. & AK, M. M. 2002. *Cheese Rheology and Texture,* CRC Press.
GUO, J., YANG, X.-Q., HE, X.-T., WU, N.-N., WANG, J.-M., GU, W. & ZHANG, Y.-Y. 2012. Limited Aggregation Behavior of β-Conglycinin and Its Terminating Effect on Glycinin Aggregation during Heating at pH 7.0. *Journal of Agricultural and Food Chemistry,* 60**,** 3782-3791. Available: https://doi.org/10.1021/jf300409y

GUO, Q., YE, A., LAD, M., DALGLEISH, D. & SINGH, H. 2014. Behaviour of whey protein emulsion gel during oral and gastric digestion: effect of droplet size. *Soft Matter,* 10**,** 4173-4183. Available: 10.1039/C4SM00598H
HAGERMAN, A. E. 1992. Tannin—Protein Interactions. *Phenolic Compounds in Food and Their Effects on Health I.* American Chemical Society. Available: doi:10.1021/bk-1992-0506.ch019
10.1021/bk-1992-0506.ch019
HAGERMAN, A. E. & BUTLER, L. G. 1978. Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry,* 26**,** 809-812. Available: https://doi.org/10.1021/jf60218a027
HAN, J., BRITTEN, M., ST-GELAIS, D., CHAMPAGNE, C. P., FUSTIER, P., SALMIERI, S. & LACROIX, M. 2011. Effect of polyphenolic ingredients on physical characteristics of cheese. *Food Research International,* 44**,** 494-497. Available: https://doi.org/10.1016/j.foodres.2010.10.026
HAPIOT, P., NEUDECK, A., PINSON, J., FULCRAND, H., NETA, P. & ROLANDO, C. 1996. Oxidation of caffeic acid and related hydroxycinnamic acids. *Journal of Electroanalytical Chemistry,* 405**,** 169-176. Available: https://doi.org/10.1016/0022-0728(95)04412-4
HASLAM, E., LILLEY, T. H. & BUTLER, L. G. 1988. Natural astringency in foodstuffs — A molecular interpretation. *Critical Reviews in Food Science and Nutrition,* 27**,** 1-40. Available: 10.1080/10408398809527476
HASSAN, Z., DIN, H. M. F., ALI, A., MEHANNA, N. & EL-MESSERY, T. 2013. Interaction of some low molecular weight phenolics with milk proteins. *World Applied Sciences Journal,* 23**,** 182-187. Available: 10.5829/idosi.wasj.2013.23.02.13057
HAVEA, P., WATKINSON, P. & KUHN-SHERLOCK, B. 2009. Heat-Induced Whey Protein Gels: Protein−Protein Interactions and Functional Properties. *Journal of Agricultural and Food Chemistry,* 57**,** 1506-1512. Available: https://doi.org/10.1021/jf802559z
HE, G., SHANKAR, R. A., CHZHAN, M., SAMOUILOV, A., KUPPUSAMY, P. & ZWEIER, J. L. 1999. Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 4586-4591. Available: https://doi.org/10.1073/pnas.96.8.4586
HELAL, A. & TAGLIAZUCCHI, D. 2018. Impact of in-vitro gastro-pancreatic digestion on polyphenols and cinnamaldehyde bioaccessibility and antioxidant activity in stirred cinnamon-fortified yogurt. *LWT,* 89**,** 164-170. Available: https://doi.org/10.1016/j.lwt.2017.10.047
HELAL, A., TAGLIAZUCCHI, D., VERZELLONI, E. & CONTE, A. 2015. Gastro-pancreatic release of phenolic compounds incorporated in a polyphenols-enriched cheese-curd. *LWT - Food Science and Technology,* 60**,** 957-963. Available: https://doi.org/10.1016/j.lwt.2014.10.037
HELENO, S. A., MARTINS, A., QUEIROZ, M. J. R. P. & FERREIRA, I. C. F. R. 2015. Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food Chemistry,* 173**,** 501-513. Available: https://doi.org/10.1016/j.foodchem.2014.10.057
HELRICH, K. 1990. Official methods of Analysis of the AOAC. Volume 2. Food composition; additives; natural contaminants. 685-1298.

HERMANSSON, A. M. 1978. Physico-chemical aspects of soy proteins structure formation. *Journal of Texture Studies,* 9**,** 33-58. Available: https://doi.org/10.1111/j.1745-4603.1978.tb01293.x
HERMANSSON, A. M. 1986. Soy protein gelation. *Journal of the American Oil Chemists Society,* 63**,** 658-666. Available: https://doi.org/10.1007/BF02638232
HERRMANN, K. & NAGEL, C. W. 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science and Nutrition,* 28**,** 315-347. Available: https://doi.org/10.1080/10408398909527504
HIDER, R. C., LIU, Z. D. & KHODR, H. H. 2001. Metal chelation of polyphenols. *Methods in Enzymology*. Academic Press. Available: https://doi.org/10.1016/S0076-6879(01)35243-6
HIRAMATSU, E. Y., DE ÁVILA, A. R. A., GÊNOVA, V. M., DE QUEIRÓS, L. D., MACEDO, G. A., MARTINS, I. M. & MACEDO, J. A. 2020. Biotransformation processes in soymilk isoflavones to enhance anti-inflammatory potential in intestinal cellular model. *Journal of Food Biochemistry,* 44. Available: 10.1111/jfbc.13149
HODGSON, J. M., YEE CHAN, S., PUDDEY, I. B., DEVINE, A., WATTANAPENPAIBOON, N., WAHLQVIST, M. L., LUKITO, W., BURKE, V., WARD, N. C., PRINCE, R. L. & CROFT, K. D. 2004. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. *British Journal of Nutrition,* 91**,** 301-305. Available: https://doi.org/10.1079/BJN20031046
HOLE, A. S., RUD, I., GRIMMER, S., SIGL, S., NARVHUS, J. & SAHLSTRØM, S. 2012. Improved Bioavailability of Dietary Phenolic Acids in Whole Grain Barley and Oat Groat following Fermentation with Probiotic Lactobacillus acidophilus, Lactobacillus johnsonii, and Lactobacillus reuteri. *Journal of Agricultural and Food Chemistry,* 60**,** 6369-6375. Available: https://doi.org/10.1021/jf300410h
HSIA, S.-Y., HSIAO, Y.-H., LI, W.-T. & HSIEH, J.-F. 2016. Aggregation of soy protein-isoflavone complexes and gel formation induced by glucono-δ-lactone in soymilk. *Scientific Reports,* 6**,** 35718. Available: https://doi.org/10.1038/srep35718
ISTFAN, N., MURRAY, E., JANGHORBANI, M. & YOUNG, V. R. 1983. An evaluation of the nutritional value of a soy protein concentrate in young adult men using the short-term N-balance method. *J Nutr,* 113**,** 2516-23. Available: https://doi.org/10.1093/jn/113.12.2516
IWABUCHI, S. & YAMAUCHI, F. 1987. Electrophoretic analysis of whey proteins present in soybean globulin fractions. *Journal of Agricultural and Food Chemistry,* 35**,** 205-209. Available: https://doi.org/10.1021/jf00074a010
JENNER, A. M., RAFTER, J. & HALLIWELL, B. 2005. Human fecal water content of phenolics: The extent of colonic exposure to aromatic compounds. *Free Radical Biology and Medicine,* 38**,** 763-772. Available: https://doi.org/10.1016/j.freeradbiomed.2004.11.020
KAHLE, K., KRAUS, M., SCHEPPACH, W. & RICHLING, E. 2005. Colonic availability of apple polyphenols--a study in ileostomy subjects. *Mol Nutr Food Res,* 49**,** 1143-50. Available: https://doi.org/10.1002/mnfr.200500132

KAKKAR, S. & BAIS, S. 2014. A review on protocatechuic acid and its pharmacological potential. *International Scholarly Research Notices,* 2014. Available: https://doi.org/10.1155/2014/952943
KAMEI, Y., HATAZAWA, Y., UCHITOMI, R., YOSHIMURA, R. & MIURA, S. 2020. Regulation of Skeletal Muscle Function by Amino Acids. *Nutrients,* 12. Available: https://doi.org/10.3390/nu12010261
KARAASLAN, M., OZDEN, M., VARDIN, H. & TURKOGLU, H. 2011. Phenolic fortification of yogurt using grape and callus extracts. *LWT - Food Science and Technology,* 44**,** 1065-1072. Available: https://doi.org/10.1016/j.lwt.2010.12.009
KERN, S. M., BENNETT, R. N., MELLON, F. A., KROON, P. A. & GARCIA-CONESA, M.-T. 2003. Absorption of Hydroxycinnamates in Humans after High-Bran Cereal Consumption. *Journal of Agricultural and Food Chemistry,* 51**,** 6050-6055. Available: https://doi.org/10.1021/jf0302299
KIMBALL, S. R. & JEFFERSON, L. S. 2006. Signaling Pathways and Molecular Mechanisms through which Branched-Chain Amino Acids Mediate Translational Control of Protein Synthesis. *The Journal of Nutrition,* 136**,** 227S-231S. Available: https://doi.org/10.1093/jn/136.1.227S
KOHYAMA, K., SANO, Y. & DOI, E. 1995. Rheological Characteristics and Gelation Mechanism of Tofu (Soybean Curd). *Journal of Agricultural and Food Chemistry,* 43**,** 1808-1812. Available: https://doi.org/10.1021/jf00055a011
KOHYAMA, K., SASAKI, T. & HAYAKAWA, F. 2008. Characterization of Food Physical Properties by the Mastication Parameters Measured by Electromyography of the Jaw-Closing Muscles and Mandibular Kinematics in Young Adults. *Bioscience, Biotechnology, and Biochemistry,* 72**,** 1690-1695. Available: https://doi.org/10.1271/bbb.70769
KONISHI, Y., HITOMI, Y., YOSHIDA, M. & YOSHIOKA, E. 2005. Pharmacokinetic study of caffeic and rosmarinic acids in rats after oral administration. *J Agric Food Chem,* 53**,** 4740-6. Available: https://doi.org/10.1021/jf0478307
KONISHI, Y., KOBAYASHI, S. & SHIMIZU, M. 2003. Transepithelial transport of p-coumaric acid and gallic acid in Caco-2 cell monolayers. *Biosci Biotechnol Biochem,* 67**,** 2317-24. Available: https://doi.org/10.1271/bbb.67.2317
KONISHI, Y. & SHIMIZU, M. 2003. Transepithelial transport of ferulic acid by monocarboxylic acid transporter in Caco-2 cell monolayers. *Biosci Biotechnol Biochem,* 67**,** 856-62. Available: https://doi.org/10.1271/bbb.67.856
KONISHI, Y., ZHAO, Z. & SHIMIZU, M. 2006. Phenolic acids are absorbed from the rat stomach with different absorption rates. *J Agric Food Chem,* 54**,** 7539-43. Available: https://doi.org/10.1021/jf061554+
KOZU, H., NAKATA, Y., NAKAJIMA, M., NEVES, M. A., UEMURA, K., SATO, S., KOBAYASHI, I. & ICHIKAWA, S. 2014. Development of a Human Gastric Digestion Simulator Equipped with Peristalsis Function for the Direct Observation and Analysis of the Food Digestion Process. *Food Science and Technology Research,* 20**,** 225-233. Available: https://doi.org/10.3136/fstr.20.225

KRISHNAN, H. B. & COE, E. H. 2001. Seed Storage Proteins. *In:* BRENNER, S. & MILLER, J. H. (eds.) *Encyclopedia of Genetics.* New York: Academic Press. Available: https://doi.org/10.1006/rwgn.2001.1714
KROLL, J., RAWEL, H. M., ROHN, S. & CZAJKA, D. 2001. Interactions of glycinin with plant phenols--influence on chemical properties and proteolytic degradation of the proteins. *Nahrung,* 45**,** 388-9. Available: https://doi.org/10.1002/1521-3803(20011001)45:6<388::Aid-food388>3.0.Co;2-8
KROLL, J., UUML, RGEN, RAWEL, H. M. & ROHN, S. 2003. Reactions of Plant Phenolics with Food Proteins and Enzymes under Special Consideration of Covalent Bonds. *Food Science and Technology Research,* 9**,** 205-218. Available: 10.3136/fstr.9.205
KUCUK, O. 2017. Soy foods, isoflavones, and breast cancer. *Cancer,* 123**,** 1901-1903. Available: 10.1002/cncr.30614
KUMAR, N. & GOEL, N. 2019. Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnology Reports,* 24**,** e00370. Available: https://doi.org/10.1016/j.btre.2019.e00370
LADDOMADA, B., CARETTO, S. & MITA, G. 2015. Wheat Bran Phenolic Acids: Bioavailability and Stability in Whole Wheat-Based Foods. *Molecules,* 20**,** 15666-85. Available: https://doi.org/10.3390/molecules200915666
LAFAY, S. & GIL-IZQUIERDO, A. 2007. Bioavailability of phenolic acids. *Phytochemistry Reviews,* 7**,** 301. Available: https://doi.org/10.1007/s11101-007-9077-x
LAMOTHE, S., AZIMY, N., BAZINET, L., COUILLARD, C. & BRITTEN, M. 2014. Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment. *Food & Function,* 5**,** 2621-2631. Available: https://doi.org/10.1039/C4FO00203B
LAMOTHE, S., LANGLOIS, A., BAZINET, L., COUILLARD, C. & BRITTEN, M. 2016. Antioxidant activity and nutrient release from polyphenol-enriched cheese in a simulated gastrointestinal environment. *Food & Function,* 7**,** 1634-1644. Available: https://doi.org/10.1039/C5FO01287B
LE BOURVELLEC, C. & RENARD, C. M. 2012. Interactions between polyphenols and macromolecules: quantification methods and mechanisms. *Crit Rev Food Sci Nutr,* 52**,** 213-48. Available: 10.1080/10408398.2010.499808
LE, T., MAKI, H., OKAZAKI, E., OSAKO, K. & TAKAHASHI, K. 2018. Influence of Various Phenolic Compounds on Properties of Gelatin Film Prepared from Horse Mackerel Trachurus japonicus Scales. *J Food Sci,* 83**,** 1888-1895. Available: https://doi.org/10.1111/1750-3841.14193
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. Available: https://doi.org/10.1007/s11746-003-0656-6
LEOPOLDINI, M., MARINO, T., RUSSO, N. & TOSCANO, M. 2004. Antioxidant Properties of Phenolic Compounds: H-Atom versus Electron Transfer Mechanism. *The Journal of Physical Chemistry A,* 108**,** 4916-4922. Available: https://doi.org/10.1021/jp037247d
LI, C., YU, W., WU, P. & CHEN, X. D. 2020a. Current in vitro digestion systems for understanding food digestion in human upper gastrointestinal tract. *Trends in Food Science & Technology,* 96**,** 114-126. Available: https://doi.org/10.1016/j.tifs.2019.12.015

LI, N., WU, X., ZHUANG, W., XIA, L., CHEN, Y., ZHAO, R., YI, M., WAN, Q., DU, L. & ZHOU, Y. 2020b. Soy and Isoflavone Consumption and Multiple Health Outcomes: Umbrella Review of Systematic Reviews and Meta-Analyses of Observational Studies and Randomized Trials in Humans. *Molecular Nutrition & Food Research,* 64**,** 1900751. Available: https://doi.org/10.1002/mnfr.201900751
LI TAY, S., YAO TAN, H. & PERERA, C. 2006. The Coagulating Effects of Cations and Anions on Soy Protein. *International Journal of Food Properties,* 9**,** 317-323. Available: https://doi.org/10.1080/10942910600596340
LI, X., LI, Y., CHEN, C., ZHAO, D., WANG, X., ZHAO, L., SHI, H., MA, G. & SU, Z. 2015. Pore size analysis from low field NMR spin–spin relaxation measurements of porous microspheres. *Journal of Porous Materials,* 22**,** 11-20. Available: 10.1007/s10934-014-9864-x
LIENER, I. E. 1994. Implications of antinutritional components in soybean foods. *Crit Rev Food Sci Nutr,* 34**,** 31-67. Available: https://doi.org/10.1080/10408399409527649
LINDSLEY, C. W. 2010. Lipophilicity. *In:* STOLERMAN, I. P. & PRICE, L. H. (eds.) *Encyclopedia of Psychopharmacology.* Berlin, Heidelberg: Springer Berlin Heidelberg. Available: https://doi.org/10.1007/978-3-642-27772-6_7015-1
LIPINSKI, C. A., LOMBARDO, F., DOMINY, B. W. & FEENEY, P. J. 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev,* 46**,** 3-26. Available: https://doi.org/10.1016/s0169-409x(00)00129-0
LIU, F. & TANG, C.-H. 2016. Soy glycinin as food-grade Pickering stabilizers: Part. III. Fabrication of gel-like emulsions and their potential as sustained-release delivery systems for β-carotene. *Food Hydrocolloids,* 56**,** 434-444. Available: https://doi.org/10.1016/j.foodhyd.2016.01.002
LIU, H. 2000. Measurement of Blood Plasma Amino Acids in Ultrafiltrates by High-Performance Liquid Chromatography with Automatic Precolumn O-Phthaldialdehyde Derivatization. *In:* COOPER, C., PACKER, N. & WILLIAMS, K. (eds.) *Amino Acid Analysis Protocols.* Totowa, NJ: Humana Press. Available: https://doi.org/10.1385/1-59259-047-0:123
LORIEAU, L., HALABI, A., LIGNEUL, A., HAZART, E., DUPONT, D. & FLOURY, J. 2018. Impact of the dairy product structure and protein nature on the proteolysis and amino acid bioaccessiblity during in vitro digestion. *Food Hydrocolloids,* 82**,** 399-411. Available: https://doi.org/10.1016/j.foodhyd.2018.04.019
LOVEDAY, S. M. 2019. Food Proteins: Technological, Nutritional, and Sustainability Attributes of Traditional and Emerging Proteins. *Annual Review of Food Science and Technology,* 10**,** 311-339. Available: https://doi.org/10.1146/annurev-food-032818-121128
LU, J. Y., CARTER, E. & CHUNG, R. A. 1980. Use of calcium salts for soybean curd preparation. *Journal of Food Science,* 45**,** 32-34. Available: https://doi.org/10.1111/j.1365-2621.1980.tb03864.x
LUO, N., YE, A., WOLBER, F. M. & SINGH, H. 2019. Structure of whey protein emulsion gels containing capsaicinoids: Impact on in-mouth breakdown behaviour and sensory perception. *Food Hydrocolloids,* 92**,** 19-29. Available: https://doi.org/10.1016/j.foodhyd.2019.01.019

LUO, N., YE, A., WOLBER, F. M. & SINGH, H. 2020. In-mouth breakdown behaviour and sensory perception of emulsion gels containing active or inactive filler particles loaded with capsaicinoids. *Food Hydrocolloids*, 106076. Available: https://doi.org/10.1016/j.foodhyd.2020.106076
LUO, Q., BORST, J. W., WESTPHAL, A. H., BOOM, R. M. & JANSSEN, A. E. M. 2017. Pepsin diffusivity in whey protein gels and its effect on gastric digestion. *Food Hydrocolloids*, 66, 318-325. Available: https://doi.org/10.1016/j.foodhyd.2016.11.046
MALTAIS, A., REMONDETTO, G. E. & SUBIRADE, M. 2009. Soy protein cold-set hydrogels as controlled delivery devices for nutraceutical compounds. *Food Hydrocolloids*, 23, 1647-1653. Available: https://doi.org/10.1016/j.foodhyd.2008.12.006
MALTAIS, A., REMONDETTO, G. E. & SUBIRADE, M. 2010. Tabletted soy protein cold-set hydrogels as carriers of nutraceutical substances. *Food Hydrocolloids*, 24, 518-524. Available: 10.1016/j.foodhyd.2009.11.016
MANACH, C., WILLIAMSON, G., MORAND, C., SCALBERT, A. & RÉMÉSY, C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition*, 81, 230S-242S. Available: https://doi.org/10.1093/ajcn/81.1.230S
MARGALEF, M., PONS, Z., BRAVO, F. I., MUGUERZA, B. & AROLA-ARNAL, A. 2015. Tissue distribution of rat flavanol metabolites at different doses. *J Nutr Biochem*, 26, 987-95. Available: https://doi.org/10.1016/j.jnutbio.2015.04.006
MCCLEMENTS, D. J., DECKER, E. A., PARK, Y. & WEISS, J. 2009. Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Crit Rev Food Sci Nutr*, 49, 577-606. Available: https://doi.org/10.1080/10408390902841529
MCMURRY, J. 2011. *Organic Chemistry with Biological Applications*, Brooks/Cole Cengage Learning.
MEDIC, J., ATKINSON, C. & HURBURGH, C. R. 2014. Current Knowledge in Soybean Composition. *Journal of the American Oil Chemists' Society*, 91, 363-384. Available: https://doi.org/10.1007/s11746-013-2407-9
MENNAH-GOVELA, Y. A., SINGH, R. P. & BORNHORST, G. M. 2019. Buffering capacity of protein-based model food systems in the context of gastric digestion. *Food & Function*, 10, 6074-6087. Available: https://doi.org/10.1039/C9FO01160A
MINEKUS, M., ALMINGER, M., ALVITO, P., BALLANCE, S., BOHN, T., BOURLIEU, C., CARRIERE, F., BOUTROU, R., CORREDIG, M., DUPONT, D., DUFOUR, C., EGGER, L., GOLDING, M., KARAKAYA, S., KIRKHUS, B., LE FEUNTEUN, S., LESMES, U., MACIERZANKA, A., MACKIE, A., MARZE, S., MCCLEMENTS, D. J., MENARD, O., RECIO, I., SANTOS, C. N., SINGH, R. P., VEGARUD, G. E., WICKHAM, M. S., WEITSCHIES, W. & BRODKORB, A. 2014. A standardised static in vitro digestion method suitable for food - an international consensus. *Food Funct*, 5, 1113-24. Available: https://doi.org/10.1039/c3fo60702j
MOLINA ORTIZ, S. E. & WAGNER, J. R. 2002. Hydrolysates of native and modified soy protein isolates: structural characteristics, solubility and foaming properties. *Food Research International*, 35, 511-518. Available: https://doi.org/10.1016/S0963-9969(01)00149-1

MOSELE, J. I., MACIÀ, A., ROMERO, M.-P., MOTILVA, M.-J. & RUBIÓ, L. 2015. Application of in vitro gastrointestinal digestion and colonic fermentation models to pomegranate products (juice, pulp and peel extract) to study the stability and catabolism of phenolic compounds. *Journal of Functional Foods,* 14**,** 529-540. Available: https://doi.org/10.1016/j.jff.2015.02.026
MOSER, S., CHEGENI, M., JONES, O. G., LICEAGA, A. & FERRUZZI, M. G. 2014. The effect of milk proteins on the bioaccessibility of green tea flavan-3-ols. *Food Research International,* 66**,** 297-305. Available: https://doi.org/10.1016/j.foodres.2014.09.018
MUNIALO, C. D., EUSTON, S. R. & DE JONGH, H. H. J. 2018. 19 - Protein gels. *In:* YADA, R. Y. (ed.) *Proteins in Food Processing (Second Edition).* Woodhead Publishing. Available: https://doi.org/10.1016/B978-0-08-100722-8.00020-6
NARDINI, M., FORTE, M., VRHOVSEK, U., MATTIVI, F., VIOLA, R. & SCACCINI, C. 2009. White Wine Phenolics Are Absorbed and Extensively Metabolized in Humans. *Journal of Agricultural and Food Chemistry,* 57**,** 2711-2718. Available: https://doi.org/10.1021/jf8034463
NARDINI, M., NATELLA, F., SCACCINI, C. & GHISELLI, A. 2006. Phenolic acids from beer are absorbed and extensively metabolized in humans. *The Journal of Nutritional Biochemistry,* 17**,** 14-22. Available: https://doi.org/10.1016/j.jnutbio.2005.03.026
NIELSEN, P. M., PETERSEN, D. & DAMBMANN, C. 2001. Improved Method for Determining Food Protein Degree of Hydrolysis. *Journal of Food Science,* 66**,** 642-646. Available: https://doi.org/10.1111/j.1365-2621.2001.tb04614.x
NISHINARI, K., FANG, Y., GUO, S. & PHILLIPS, G. O. 2014. Soy proteins: A review on composition, aggregation and emulsification. *Food Hydrocolloids,* 39**,** 301-318. Available: https://doi.org/10.1016/j.foodhyd.2014.01.013
NISHINARI, K., FANG, Y., NAGANO, T., GUO, S. & WANG, R. 2018. 6 - Soy as a food ingredient. *In:* YADA, R. Y. (ed.) *Proteins in Food Processing (Second Edition).* Woodhead Publishing. Available: https://doi.org/10.1016/B978-0-08-100722-8.00007-3
NORDESTGAARD, B. G. & ROSTGAARD, J. 1985. Critical-point drying versus freeze drying for scanning electron microscopy: a quantitative and qualitative study on isolated hepatocytes. *J Microsc,* 137**,** 189-207. Available: https://doi.org/10.1111/j.1365-2818.1985.tb02577.x
NUTRIRIONDATA. 2021. *Nutritiondata. MORI-NU, Tofu, silken, soft Nutrition facts* [Online]. Available: https://nutritiondata.self.com/facts/legumes-and-legume-products/4410/2 [Accessed 25/05/2021].
OGAWA, K., HIROSE, S., NAGAOKA, S. & YANASE, E. 2016. Interaction between Tea Polyphenols and Bile Acid Inhibits Micellar Cholesterol Solubility. *Journal of Agricultural and Food Chemistry,* 64**,** 204-209. Available: https://doi.org/10.1021/acs.jafc.5b05088
OZDAL, T., CAPANOGLU, E. & ALTAY, F. 2013. A review on protein–phenolic interactions and associated changes. *Food Research International,* 51**,** 954-970. Available: https://doi.org/10.1016/j.foodres.2013.02.009

PEI, K., OU, J., HUANG, J. & OU, S. 2015. p-Coumaric acid and its conjugates: Dietary sources, pharmacokinetic properties and biological activities. *Journal of the science of food and agriculture,* 96. Available: https://doi.org/10.1002/jsfa.7578
PEREIRA, D. M., VALENTÃO, P., PEREIRA, J. A. & ANDRADE, P. B. 2009. Phenolics: From Chemistry to Biology. *Molecules,* 14**,** 2202-2211. Available: https://doi.org/10.3390/molecules14062202
PÉREZ-JIMÉNEZ, J., HUBERT, J., HOOPER, L., CASSIDY, A., MANACH, C., WILLIAMSON, G. & SCALBERT, A. 2010. Urinary metabolites as biomarkers of polyphenol intake in humans: a systematic review. *The American Journal of Clinical Nutrition,* 92**,** 801-809. Available: 10.3945/ajcn.2010.29924
PETROTOS, K., KARKANTA, F., GKOUTSIDIS, P. E., GIAVASIS, I., PAPATHEODOROU, K. & NTONTOS, A. C. 2012. Production of Novel Bioactive Yogurt Enriched with Olive Fruit Polyphenols. *World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering,* 6**,** 170-175.
PETSKO, G. A. & RINGE, D. 2004. From sequence to structure. *Protein structure and function,* 1**,** 49.
PHILLIPS, S. M., TANG, J. E. & MOORE, D. R. 2009. The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons. *J Am Coll Nutr,* 28**,** 343-54. Available: https://doi.org/10.1080/07315724.2009.10718096
PINELO, M., ARNOUS, A. & MEYER, A. S. 2006. Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends in Food Science & Technology,* 17**,** 579-590. Available: https://doi.org/10.1016/j.tifs.2006.05.003
PIZZINO, G., IRRERA, N., CUCINOTTA, M., PALLIO, G., MANNINO, F., ARCORACI, V., SQUADRITO, F., ALTAVILLA, D. & BITTO, A. 2017. Oxidative Stress: Harms and Benefits for Human Health. *Oxidative medicine and cellular longevity,* 2017**,** 8416763-8416763. Available: https://doi.org/10.1155/2017/8416763
POCKER, Y. & GREEN, E. 1973. Hydrolysis of D-glucono-.delta.-lactone. I. General acid-base catalysis, solvent deuterium isotope effects, and transition state characterization. *Journal of the American Chemical Society,* 95**,** 113-119. Available: https://doi.org/10.1021/ja00782a019
PRABHAKARAN, M. P., PERERA, C. O. & VALIYAVEETTIL, S. 2006. Effect of different coagulants on the isoflavone levels and physical properties of prepared firm tofu. *Food Chemistry,* 99**,** 492-499. Available: https://doi.org/10.1016/j.foodchem.2005.08.011
PREEDY, V. R. 2012. *Isoflavones: Chemistry, Analysis, Function and Effects*, Royal Society of Chemistry.
PROMEGA. 2018. *Sequencing Grade Modified Trypsin Certificate of Analysis 9PIV511*.
PUPPO, M. C., LUPANO, C. E. & ANON, M. C. 1995. Gelation of Soybean Protein Isolates in Acidic Conditions. Effect of pH and Protein Concentration. *Journal of Agricultural and Food Chemistry,* 43**,** 2356-2361. Available: https://doi.org/10.1021/jf00057a008

QIAO, Y., GUMPERTZ, M. & VAN KEMPEN, T. 2002. Stability of pepsin (EC 3.4.23.1) during in vitro protein digestibility assay. *Journal of Food Biochemistry,* 26**,** 355-375. Available: https://doi.org/10.1111/j.1745-4514.2002.tb00759.x
QIAO, Y., GUMPERTZ, M. & VAN KEMPEN, T. 2005. Stability of a pancreatic enzyme coctail during in vitro protein digestibility assays. *Journal of Food Biochemistry,* 29**,** 205-220. Available: https://doi.org/10.1111/j.1745-4514.2005.00003.x
RACCACH, M. 1984. The antimicrobial activity of phenolic antioxidants in foods: a review. *Journal of Food Safety,* 6**,** 141-170. Available: https://doi.org/10.1111/j.1745-4565.1984.tb00479.x
RAND, W. M., PELLETT, P. L. & YOUNG, V. R. 2003. Meta-analysis of nitrogen balance studies for estimating protein requirements in healthy adults. *Am J Clin Nutr,* 77**,** 109-27. Available: https://doi.org/10.1093/ajcn/77.1.109
RAWEL, H. M., CZAJKA, D., ROHN, S. & KROLL, J. 2002a. Interactions of different phenolic acids and flavonoids with soy proteins. *Int J Biol Macromol,* 30**,** 137-50.
RAWEL, H. M., CZAJKA, D., ROHN, S. & KROLL, J. 2002b. Interactions of different phenolic acids and flavonoids with soy proteins. *International Journal of Biological Macromolecules,* 30**,** 137-150. Available: https://doi.org/10.1016/S0141-8130(02)00016-8
RAWEL, H. M., MEIDTNER, K. & KROLL, J. 2005. Binding of Selected Phenolic Compounds to Proteins. *Journal of Agricultural and Food Chemistry,* 53**,** 4228-4235. Available: https://doi.org/10.1021/jf0480290
RECORD, I. R. & LANE, J. M. 2001. Simulated intestinal digestion of green and black teas. *Food Chemistry,* 73**,** 481-486. Available: https://doi.org/10.1016/S0308-8146(01)00131-5
REIN, M. J., RENOUF, M., CRUZ-HERNANDEZ, C., ACTIS-GORETTA, L., THAKKAR, S. K. & DA SILVA PINTO, M. 2013. Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. *British journal of clinical pharmacology,* 75**,** 588-602. Available: https://doi.org/10.1111/j.1365-2125.2012.04425.x
RENKEMA, J. M., LAKEMOND, C. M., DE JONGH, H. H., GRUPPEN, H. & VAN VLIET, T. 2000. The effect of pH on heat denaturation and gel forming properties of soy proteins. *J Biotechnol,* 79**,** 223-30. Available: https://doi.org/10.1016/s0168-1656(00)00239-x
RENKEMA, J. M. & VAN VLIET, T. 2002. Heat-induced gel formation by soy proteins at neutral pH. *J Agric Food Chem,* 50**,** 1569-73. Available: https://doi.org/10.1021/jf010763l
RENKEMA, J. M. S., GRUPPEN, H. & VAN VLIET, T. 2002. Influence of pH and Ionic Strength on Heat-Induced Formation and Rheological Properties of Soy Protein Gels in Relation to Denaturation and Their Protein Compositions. *Journal of Agricultural and Food Chemistry,* 50**,** 6064-6071. Available: https://doi.org/10.1021/jf020061b
REYNAUD, Y., LOPEZ, M., RIAUBLANC, A., SOUCHON, I. & DUPONT, D. 2020. Hydrolysis of plant proteins at the molecular and supra-molecular scales during in vitro digestion. *Food Research International,* 134**,** 109204. Available: https://doi.org/10.1016/j.foodres.2020.109204
RICE-EVANS, C. A., MILLER, N. J. & PAGANGA, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free*

Radical Biology and Medicine, 20**,** 933-956. Available: https://doi.org/10.1016/0891-5849(95)02227-9
RICE, R. H., MEANS, G. E. & BROWN, W. D. 1977. Stabilization of bovine trypsin by reductive methylation. *Biochimica et Biophysica Acta (BBA) - Protein Structure,* 492**,** 316-321. Available: https://doi.org/10.1016/0005-2795(77)90082-4
RIZZO, G. & BARONI, L. 2018a. Soy, Soy Foods and Their Role in Vegetarian Diets. *Nutrients,* 10**,** 43. Available: https://doi.org/10.3390/nu10010043
RIZZO, G. & BARONI, L. 2018b. Soy, Soy Foods and Their Role in Vegetarian Diets. *Nutrients,* 10. Available: https://doi.org/10.3390/nu10010043
ROCHA, L., MONTEIRO, M. & TEODORO, A. 2012. Anticancer Properties of Hydroxycinnamic Acids -A Review. *Cancer and Clinical Oncology,* 1. Available: https://doi.org/10.5539/cco.v1n2p109
RODRÍGUEZ-ROQUE, M. J., ROJAS-GRAÜ, M. A., ELEZ-MARTÍNEZ, P. & MARTÍN-BELLOSO, O. 2013. Soymilk phenolic compounds, isoflavones and antioxidant activity as affected by in vitro gastrointestinal digestion. *Food Chemistry,* 136**,** 206-212. Available: 10.1016/j.foodchem.2012.07.115
RUI, X., FU, Y., ZHANG, Q., LI, W., ZARE, F., CHEN, X., JIANG, M. & DONG, M. 2016. A comparison study of bioaccessibility of soy protein gel induced by magnesiumchloride, glucono-δ-lactone and microbial transglutaminase. *LWT - Food Science and Technology,* 71**,** 234-242. Available: https://doi.org/10.1016/j.lwt.2016.03.032
SAIO, K. & WATANABE, T. 1978. Differences in functional properties of 7S and 11S soybean proteins. *Journal of Texture Studies,* 9**,** 135-157. Available: https://doi.org/10.1111/j.1745-4603.1978.tb01298.x
SANCHÓN, J., FERNÁNDEZ-TOMÉ, S., MIRALLES, B., HERNÁNDEZ-LEDESMA, B., TOMÉ, D., GAUDICHON, C. & RECIO, I. 2018. Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chemistry,* 239**,** 486-494. Available: https://doi.org/10.1016/j.foodchem.2017.06.134
SARWAR, G. 1997. The protein digestibility-corrected amino acid score method overestimates quality of proteins containing antinutritional factors and of poorly digestible proteins supplemented with limiting amino acids in rats. *J Nutr,* 127**,** 758-64. Available: https://doi.org/10.1093/jn/127.5.758
SASTRY, M. C. S. & RAO, M. S. N. 1990. Binding of chlorogenic acid by the isolated polyphenol-free 11 S protein of sunflower (Helianthus annuus) seed. *Journal of Agricultural and Food Chemistry,* 38**,** 2103-2110. Available: 10.1021/jf00102a001
SCALBERT, A., MORAND, C., MANACH, C. & REMESY, C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother,* 56**,** 276-82. Available: https://doi.org/10.1016/s0753-3322(02)00205-6
SCHASTEEN, C. S., WU, J., SCHULZ, M. G. & PARSONS, C. M. 2007. Correlation of an immobilized digestive enzyme assay with poultry true amino acid digestibility for soybean meal. *Poult Sci,* 86**,** 343-8. Available: https://doi.org/10.1093/ps/86.2.343

SCHMIDT, R. H. 1981. Gelation and Coagulation. *Protein Functionality in Foods.* American Chemical Society. Available: https://doi.org/10.1021/bk-1981-0147.ch007
SCRIMSHAW, N. S., WAYLER, A. H., MURRAY, E., STEINKE, F. H., RAND, W. M. & YOUNG, V. R. 1983. Nitrogen Balance Response in Young Men Given One of Two Isolated Soy Proteins or Milk Proteins. *The Journal of Nutrition,* 113**,** 2492-2497. Available: https://doi.org/10.1093/jn/113.12.2492
SEVERIN, S. & XIA, W. S. 2006. Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry,* 30**,** 77-97. Available: https://10.1111/j.1745-4514.2005.00048.x
SHAHIDI, F. & YEO, J. D. 2016. Insoluble-Bound Phenolics in Food. *Molecules,* 21. Available: 10.3390/molecules21091216
SHURTLEFF, W. & AOYAGI, A. 1975. *The Book of Tofu: Food for Mankind,* Autumn Press.
SHURTLEFF, W. & AOYAGI, A. 2000. *Tofu & Soymilk Production: A Craft and Technical Manual,* Soyfoods Center.
SI, W., GONG, J., TSAO, R., ZHOU, T., YU, H., POPPE, C., JOHNSON, R. & DU, Z. 2006. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *Journal of Applied Microbiology,* 100**,** 296-305. Available: https://doi.org/10.1111/j.1365-2672.2005.02789.x
SIEBERT, K. J. 1999. Effects of Protein−Polyphenol Interactions on Beverage Haze, Stabilization, and Analysis. *Journal of Agricultural and Food Chemistry,* 47**,** 353-362. Available: 10.1021/jf980703o
SIGMA-ALDRICH. *Bradford reagent. Technical Bulletin*
SILVA, J. V. C., LEGLAND, D., CAUTY, C., KOLOTUEV, I. & FLOURY, J. 2015. Characterization of the microstructure of dairy systems using automated image analysis. *Food Hydrocolloids,* 44**,** 360-371. Available: https://doi.org/10.1016/j.foodhyd.2014.09.028
STALIKAS, C. D. 2010. Phenolic Acids and Flavonoids: Occurrence and Analytical Methods. *In:* UPPU, R. M., MURTHY, S. N., PRYOR, W. A. & PARINANDI, N. L. (eds.) *Free Radicals and Antioxidant Protocols.* Totowa, NJ: Humana Press. Available: https://doi.org/10.1007/978-1-60327-029-8_5
STEFFE, J. F. 1996. *Rheological methods in food process engineering,* Freeman press.
STEWART, R. J. C., MORTON, H., COAD, J. & PEDLEY, K. C. 2019. In vitro digestion for assessing micronutrient bioavailability: the importance of digestion duration. *International Journal of Food Sciences and Nutrition,* 70**,** 71-77. Available: https://doi.org/10.1080/09637486.2018.1481200
STRAUSS, G. & GIBSON, S. M. 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids,* 18**,** 81-89. Available: https://doi.org/10.1016/S0268-005X(03)00045-6
ŚWIECA, M., GAWLIK-DZIKI, U., SĘCZYK, Ł., DZIKI, D. & SIKORA, M. 2018. Interactions of green coffee bean phenolics with wheat bread matrix in a model of simulated in vitro digestion. *Food Chemistry,* 258**,** 301-307. Available: https://doi.org/10.1016/j.foodchem.2018.03.081
SZWAJGIER, D., BARANOWSKA-WOJCIK, E. & BOROWIEC, K. 2018. Phenolic Acids Exert Anticholinesterase and Cognition-Improving Effects.

Curr Alzheimer Res, 15**,** 531-543. Available: 10.2174/1567205014666171128102557
SZWAJGIER, D., BOROWIEC, K. & PUSTELNIAK, K. 2017. The Neuroprotective Effects of Phenolic Acids: Molecular Mechanism of Action. *Nutrients,* 9. Available: https://doi.org/10.3390/nu9050477
TAGLIAZUCCHI, D., VERZELLONI, E., BERTOLINI, D. & CONTE, A. 2010. In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry,* 120**,** 599-606. Available: https://doi.org/10.1016/j.foodchem.2009.10.030
TANG, C.-H. 2017. Emulsifying properties of soy proteins: A critical review with emphasis on the role of conformational flexibility. *Critical Reviews in Food Science and Nutrition,* 57**,** 2636-2679. Available: https://doi.org/10.1080/10408398.2015.1067594
TANG, C.-H. 2019. Nanostructured soy proteins: Fabrication and applications as delivery systems for bioactives (a review). *Food Hydrocolloids,* 91**,** 92-116. Available: https://doi.org/10.1016/j.foodhyd.2019.01.012
TANG, J. E., MOORE, D. R., KUJBIDA, G. W., TARNOPOLSKY, M. A. & PHILLIPS, S. M. 2009. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J Appl Physiol (1985),* 107**,** 987-92. Available: https://doi.org/10.1152/japplphysiol.00076.2009
THÉVENOT, J., CAUTY, C., LEGLAND, D., DUPONT, D. & FLOURY, J. 2017. Pepsin diffusion in dairy gels depends on casein concentration and microstructure. *Food Chemistry,* 223**,** 54-61. Available: https://doi.org/10.1016/j.foodchem.2016.12.014
THRANE, M., PAULSEN, P. V., ORCUTT, M. W. & KRIEGER, T. M. 2017. Chapter 2 - Soy Protein: Impacts, Production, and Applications. *In:* NADATHUR, S. R., WANASUNDARA, J. P. D. & SCANLIN, L. (eds.) *Sustainable Protein Sources.* San Diego: Academic Press. Available: https://doi.org/10.1016/B978-0-12-802778-3.00002-0
TOKEDE, O. A., ONABANJO, T. A., YANSANE, A., GAZIANO, J. M. & DJOUSSÉ, L. 2015. Soya products and serum lipids: a meta-analysis of randomised controlled trials. *British Journal of Nutrition,* 114**,** 831-843. Available: https://doi.org/10.1017/S0007114515002603
TSAO, R. 2010. Chemistry and biochemistry of dietary polyphenols. *Nutrients,* 2**,** 1231-1246. Available: https://doi.org/10.3390/nu2121231
URBONAITE, V., DE JONGH, H. H. J., VAN DER LINDEN, E. & POUVREAU, L. 2014. Origin of Water Loss from Soy Protein Gels. *Journal of Agricultural and Food Chemistry,* 62**,** 7550-7558. Available: https://doi.org/10.1021/jf501728t
VALLEJO, F., GIL-IZQUIERDO, A., PÉREZ-VICENTE, A. & GARCÍA-VIGUERA, C. 2004. In Vitro Gastrointestinal Digestion Study of Broccoli Inflorescence Phenolic Compounds, Glucosinolates, and Vitamin C. *Journal of Agricultural and Food Chemistry,* 52**,** 135-138. Available: https://doi.org/10.1021/jf0305128
VAN DIJK, E., HOOGEVEEN, A. & ABELN, S. 2015. The Hydrophobic Temperature Dependence of Amino Acids Directly Calculated from Protein Structures. *PLOS Computational Biology,* 11**,** e1004277. Available: https://doi.org/10.1371/journal.pcbi.1004277

VAN KLEEF, F. S. M. 1986. Thermally induced protein gelation: Gelation and rheological characterization of highly concentrated ovalbumin and soybean protein gels. *Biopolymers,* 25**,** 31-59. Available: https://doi.org/10.1002/bip.360250105
VENABLE , J. H. & COGGESHALL , R. 1965. A simplified lead citrate stain for use in electron microscopy. *Journal of Cell Biology,* 25**,** 407-408. Available: https://doi.org/10.1083/jcb.25.2.407
VERWEY, E. J. W. & OVERBEEK, J. T. G. 1947. Theory of the Stability of Lyophobic Colloids. *The Journal of Physical and Colloid Chemistry,* 51**,** 631-636. Available: https://doi.org/10.1021/j150453a001
VINAYAGAM, R., JAYACHANDRAN, M. & XU, B. 2016. Antidiabetic Effects of Simple Phenolic Acids: A Comprehensive Review. *Phytotherapy Research,* 30**,** 184-199. Available: https://doi.org/10.1002/ptr.5528
VIRGILI, F., PAGANA, G., BOURNE, L., RIMBACH, G., NATELLA, F., RICE-EVANS, C. & PACKER, L. 2000. Ferulic acid excretion as a marker of consumption of a French maritime pine (Pinus maritima) bark extract. *Free Radical Biology and Medicine,* 28**,** 1249-1256. Available: https://doi.org/10.1016/S0891-5849(00)00244-6
WAHLE, K. W., BROWN, I., ROTONDO, D. & HEYS, S. D. 2010. Plant phenolics in the prevention and treatment of cancer. *Adv Exp Med Biol,* 698**,** 36-51. Available: https://doi.org/10.1007/978-1-4419-7347-4_4
WALSTRA, P. 2002. *Physical Chemistry of Foods,* Taylor & Francis.
WANG, J.-M., XIA, N., YANG, X.-Q., YIN, S.-W., QI, J.-R., HE, X.-T., YUAN, D.-B. & WANG, L.-J. 2012. Adsorption and Dilatational Rheology of Heat-Treated Soy Protein at the Oil–Water Interface: Relationship to Structural Properties. *Journal of Agricultural and Food Chemistry,* 60**,** 3302-3310. Available: https://doi.org/10.1021/jf205128v
WEN, D., LI, C., DI, H., LIAO, Y. & LIU, H. 2005. A universal HPLC method for the determination of phenolic acids in compound herbal medicines. *J Agric Food Chem,* 53**,** 6624-9. Available: https://doi.org/10.1021/jf0511291
WENG, C. J. & YEN, G. C. 2012. Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: phenolic acids, monophenol, polyphenol, and their derivatives. *Cancer Treat Rev,* 38**,** 76-87. Available: https://doi.org/10.1016/j.ctrv.2011.03.001
WINTER, M. & HERRMANN, K. 1986. Esters and glucosides of hydroxycinnamic acids in vegetables. *Journal of Agricultural and Food Chemistry,* 34**,** 616-620. Available: https://doi.org/10.1021/jf00070a007
WRIGHT, J. S., JOHNSON, E. R. & DILABIO, G. A. 2001. Predicting the Activity of Phenolic Antioxidants: Theoretical Method, Analysis of Substituent Effects, and Application to Major Families of Antioxidants. *Journal of the American Chemical Society,* 123**,** 1173-1183. Available: https://doi.org/10.1021/ja002455u
WU, C., MA, W. & HUA, Y. 2019. The relationship between breaking force and hydrophobic interactions or disulfide bonds involved in heat-induced soy protein gels as affected by heating time and temperature. *International Journal of Food Science & Technology,* 54**,** 231-239. Available: https://doi.org/10.1111/ijfs.13931
WU, J., CHIU, S.-C., PEARCE, E. M. & KWEI, T. K. 2001. Effects of phenolic compounds on gelation behavior of gelatin gels. *Journal of Polymer Science Part A: Polymer Chemistry,* 39**,** 224-231. Available:

https://doi.org/10.1002/1099-0518(20010101)39:1<224::Aid-pola250>3.0.Co;2-x
WU, S., ZHANG, Y., REN, F., QIN, Y., LIU, J., LIU, J., WANG, Q. & ZHANG, H. 2018. Structure–affinity relationship of the interaction between phenolic acids and their derivatives and β-lactoglobulin and effect on antioxidant activity. *Food Chemistry,* 245**,** 613-619. Available: https://doi.org/10.1016/j.foodchem.2017.10.122
YANG, I., JAYAPRAKASHA, G. K. & PATIL, B. 2018. In vitro digestion with bile acids enhances the bioaccessibility of kale polyphenols. *Food & Function,* 9**,** 1235-1244. Available: https://doi.org/10.1039/C7FO01749A
YANG, Y., CHURCHWARD-VENNE, T. A., BURD, N. A., BREEN, L., TARNOPOLSKY, M. A. & PHILLIPS, S. M. 2012. Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. *Nutrition & metabolism,* 9**,** 57-57. Available: https://doi.org/10.1186/1743-7075-9-57
YOUNG, V. R., PUIG, M., QUEIROZ, E., SCRIMSHAW, N. S. & RAND, W. M. 1984. Evaluation of the protein quality of an isolated soy protein in young men: relative nitrogen requirements and effect of methionine supplementation. *Am J Clin Nutr,* 39**,** 16-24. Available: https://doi.org/10.1093/ajcn/39.1.16
YUAN, S., CHANG, S. K. C., LIU, Z. & XU, B. 2008. Elimination of Trypsin Inhibitor Activity and Beany Flavor in Soy Milk by Consecutive Blanching and Ultrahigh-Temperature (UHT) Processing. *Journal of Agricultural and Food Chemistry,* 56**,** 7957-7963. Available: https://doi.org/10.1021/jf801039h
ZHANG, Q., CHENG, Z., WANG, Y. & FU, L. 2020. Dietary protein-phenolic interactions: characterization, biochemical-physiological consequences, and potential food applications. *Critical Reviews in Food Science and Nutrition***,** 1-27. Available: 10.1080/10408398.2020.1803199
ZHANG, S. & VARDHANABHUTI, B. 2014. Effect of initial protein concentration and pH on in vitro gastric digestion of heated whey proteins. *Food Chemistry,* 145**,** 473-480. Available: https://doi.org/10.1016/j.foodchem.2013.08.076
ZHANG, X., DO, M. D., CASEY, P., SULISTIO, A., QIAO, G. G., LUNDIN, L., LILLFORD, P. & KOSARAJU, S. 2010. Chemical Modification of Gelatin by a Natural Phenolic Cross-linker, Tannic Acid. *Journal of Agricultural and Food Chemistry,* 58**,** 6809-6815. Available: https://doi.org/10.1021/jf1004226
ZHANG, Y. J., WU, L., ZHANG, Q. L., LI, J., YIN, F. X. & YUAN, Y. 2011. Pharmacokinetics of phenolic compounds of Danshen extract in rat blood and brain by microdialysis sampling. *J Ethnopharmacol,* 136**,** 129-36. Available: https://doi.org/10.1016/j.jep.2011.04.023
ZHAO, Y.-Y., CAO, F.-H., LI, X.-J., MU, D.-D., ZHONG, X.-Y., JIANG, S.-T., ZHENG, Z. & LUO, S.-Z. 2020. Effects of different salts on the gelation behaviour and mechanical properties of citric acid-induced tofu. *International Journal of Food Science & Technology,* 55**,** 785-794. Available: https://doi.org/10.1111/ijfs.14348

Appendix 1.

Supplementary Materials & Calculations

Chapter 5

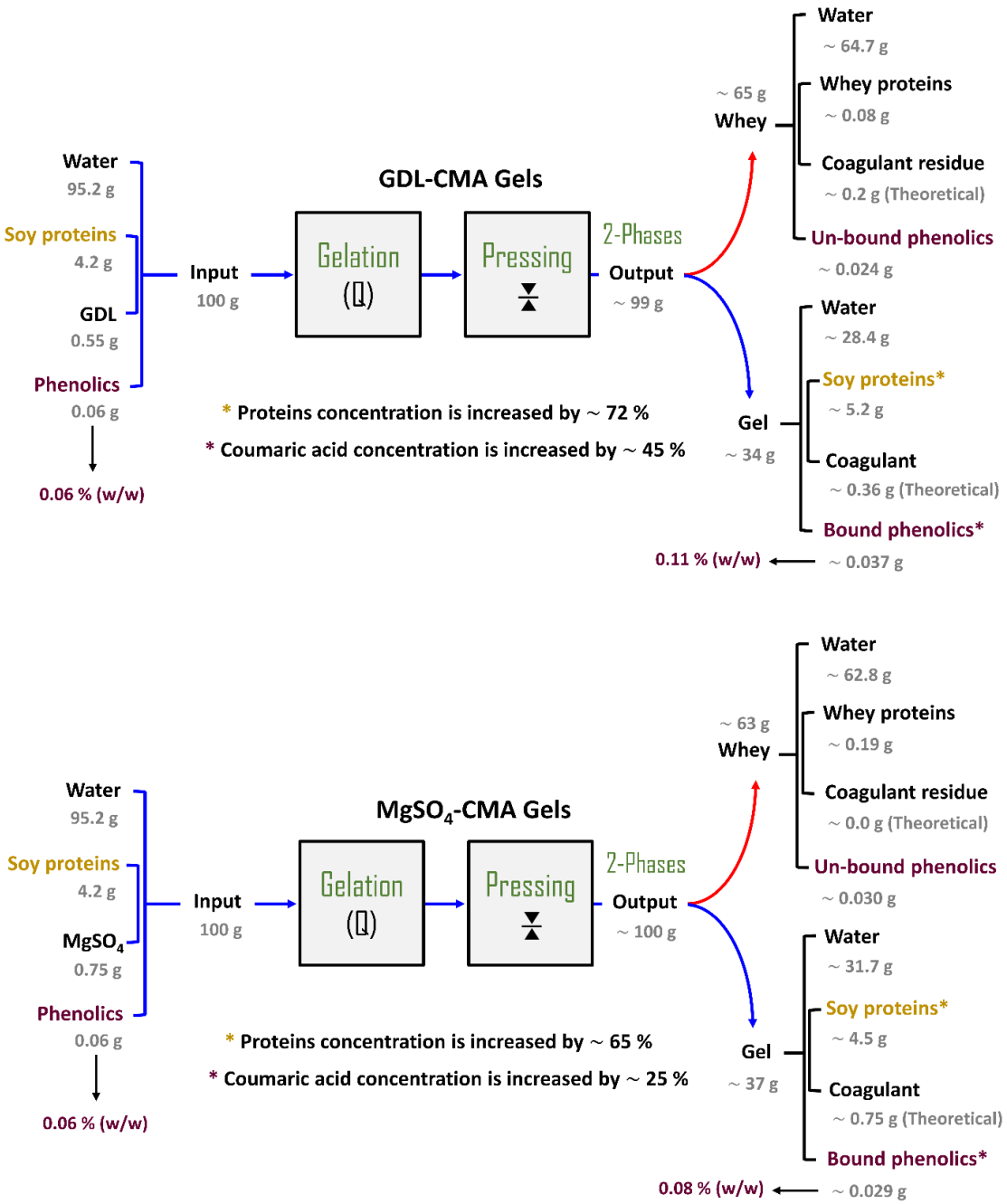


Figure S 1. Mass balance diagram of gelation procedure of GDL-CMA and $MgSO_4$-CMA pressed gels. Protein and bounded phenolic acid concentration after pressing depends on coagulant and used.

Table S 1. Free amino acid concentration (µmol/ g of digesta at the intestinal phase) of *in vitro* blank digestions (without substrate) and large-scale pressed control gels induced by GDL and $MgSO_4$.

AA	Concentration (µmol/ g of Digesta)		
	Blank	$MgSO_4$ gels	GDL gels
Aspartic acid	1.1 ± 0.2 [a]	0.8 ± 0.1 [a]	0.5 ± 0.1 [b]
Glutamic acid	2.6 ± 0.6 [a]	2.5 ± 0.4 [a]	1.3 ± 0.2 [b]
Serine	2.9 ± 1.0 [a]	2.9 ± 0.3 [a]	1.6 ± 0.3 [a]
Histidine	0.8 ± 0.1 [b]	1.4 ± 0.2 [a]	0.7 ± 0.3 [b]
Glycine	4.1 ± 1.9 [a]	3.0 ± 0.3 [a]	1.8 ± 0.6 [a]
Threonine	1.9 ± 0.5 [ab]	2.1 ± 0.2 [a]	1.1 ± 0.2 [b]
Arginine	3.4 ± 1.0 [b]	6.5 ± 0.7 [a]	4.0 ± 1.2 [b]
Alanine	3.3 ± 1.2 [a]	3.6 ± 0.4 [a]	2.2 ± 0.3 [a]
Tyrosine	2.4 ± 0.6 [a]	3.3 ± 0.5 [a]	2.3 ± 0.3 [a]
Valine	1.9 ± 0.2 [b]	2.6 ± 0.3 [a]	1.3 ± 0.2 [b]
Methionine	0.6 ± 0.0 [b]	0.9 ± 0.1 [a]	0.6 ± 0.1 [b]
Phenylalanine	1.4 ± 0.3 [a]	5.0 ± 0.7 [c]	3.2 ± 0.6 [b]
Isoleucine	1.4 ± 0.3 [b]	2.9 ± 0.3 [a]	1.7 ± 0.2 [b]
Leucine	3.4 ± 1.5 [b]	8.1 ± 1.0 [a]	4.8 ± 1.2 [b]
Lysine	8.0 ± 7.2 [a]	6.5 ± 0.5 [a]	3.9 ± 1.3 [a]

[1] Values were represented as means ± standard deviations (n≥3). [2] The letters a to c indicate that the values in the same row are significantly different (p≤0.05).

Chapter 6

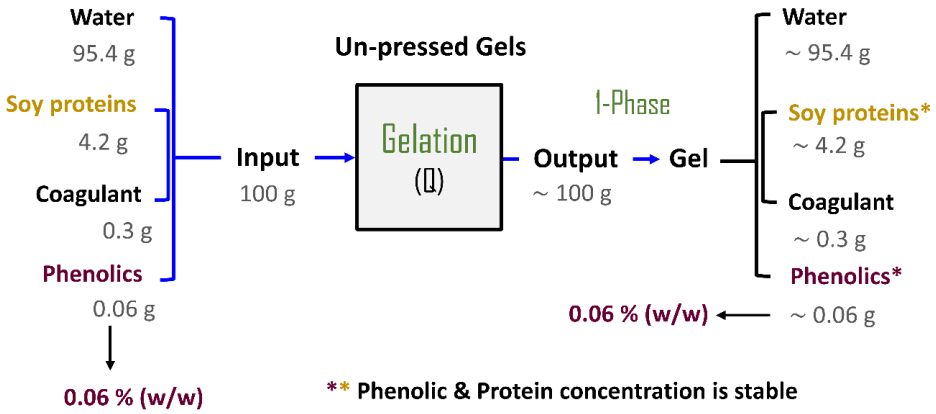


Figure S 2. Mass balance diagram of gelation procedure of GDL-CMA unpressed gels. Protein and bounded phenolic acid concentration remain stable.

Table S 2. Free amino acid concentration (µmol/ g of digesta at the intestinal phase) of *in vitro* blank digestions (without substrate) and unpressed control samples induced by GDL and $MgSO_4$.

AA	Concentration (µmol/ g of Digesta)		
	Blank	$MgSO_4$ gels	GDL gels
Aspartic acid	1.1 ± 0.2 [a]	0.8 ± 0.1 [ab]	0.5 ± 0.2 [b]
Glutamic acid	2.6 ± 0.6 [a]	2.0 ± 0.3 [ab]	1.3 ± 0.5 [b]
Serine	2.9 ± 1.0 [a]	2.5 ± 0.3 [a]	1.7 ± 0.8 [a]
Histidine	0.8 ± 0.1 [a]	1.0 ± 0.1 [a]	0.6 ± 0.3 [a]
Glycine	4.1 ± 1.9 [a]	2.9 ± 0.3 [a]	2.0 ± 0.8 [a]
Threonine	1.9 ± 0.5 [a]	1.6 ± 0.2 [a]	1.1 ± 0.5 [a]
Arginine	3.4 ± 1.0 [a]	3.5 ± 0.4 [a]	2.5 ± 0.9 [a]
Alanine	3.3 ± 1.2 [a]	2.9 ± 0.3 [a]	2.0 ± 0.8 [a]
Tyrosine	2.4 ± 0.6 [a]	1.6 ± 0.4 [ab]	1.2 ± 0.3 [b]
Valine	1.9 ± 0.2 [a]	1.7 ± 0.2 [ab]	1.2 ± 0.4 [b]
Methionine	0.6 ± 0.0 [a]	0.6 ± 0.1 [a]	0.4 ± 0.2 [a]
Phenylalanine	1.4 ± 0.3 [a]	2.0 ± 0.2 [a]	1.5 ± 0.5 [a]
Isoleucine	1.4 ± 0.3 [a]	1.6 ± 0.2 [a]	1.0 ± 0.4 [a]
Leucine	3.4 ± 1.5 [a]	3.7 ± 0.4 [a]	2.9 ± 0.9 [a]
Lysine	8.0 ± 7.2 [a]	4.2 ± 0.5 [a]	3.3 ± 1.3 [a]

[1] Values were represented as means ± standard deviations (n=3-6). [2] The letters a to b indicate that the values in the same row are significantly different ($p \leq 0.05$).

Chapter 7

Table S 3. Enzyme activities and the corresponding concentrations (% w/w).

Enzyme	Activity (U/mg)	Activity during digestion (U/mL)	Concentration % (w/w)
Pepsin	479	~ 2000	0.42
Pancreatin	6.5	~ 100	1.57

Table S 4. The substrate of pressed soy protein GDL-induced gel was expressed as intact protein concentration during the *in vitro* digestion phases. The F corresponds to the dilution factor tested in section 7.3.3.

	Protein content (theoretical) % (w/w)		
	F=1	F=2	F=3
Undigested pressed gel	13.9	6.7	4.6
***Oral phase**	6.9	3.4	2.3
***Gastric phase**	3.6	1.8	1.2
***Intestinal phase**	1.9	0.9	0.6
E_G: S	$\frac{0.42}{3.6} \sim 0.12$	$\frac{0.42}{1.8} \sim 0.24$	$\frac{0.42}{1.2} \sim 0.36$
E_I: S	$\frac{1.57}{1.9} \sim 0.8$	$\frac{1.57}{0.9} \sim 1.7$	$\frac{1.57}{0.6} \sim 2.5$

Table S 5. The substrate of unpressed soy protein GDL-induced gel was expressed as intact protein concentration during the *in vitro* digestion phases.

Protein content (theoretical) % (w/w)	
Undigested unpressed gel	4.3
***Oral phase**	2.1
***Gastric phase**	1.2
***Intestinal phase**	0.6
E_G: S	$\frac{0.42}{1.2} \sim 0.35$
E_I: S	$\frac{1.57}{0.6} \sim 2.6$

***The protein content during the *in vitro* digestion phases was calculated according to the following equation:

$$\frac{M_S \times C_P}{M_{TD}}$$

Where M_S is the mass (g) of the sample (gel) subjected to digestion, C_P is the protein concentration (% w/w) of the un-digested gel, measured using Kjedhal method (Helrich, 1990) and M_{TD} is the total mass (g) of digesta in each phase after the addition of the simulated fluids, enzymes, water, acids or base.

Chapter 8

Fruits and vegetables contain PCA and CMA concentrations in a range of 15 to 295 mg/kg and 7 to 350 mg/ kg respectively (Herrmann and Nagel, 1989). The amounts of PCA and CMA detected in the intestinal phase after digestion of pressed gels were equivalent to 601.8 ± 25.5 mg/Kg and 1005.3 ± 13.1 mg/Kg of tofu. According to Kakkar and Bais (2014), protocatechuic aldehyde, which is a derivative of PCA, was toxic in mice after oral administration of 500 mg/Kg of bodyweight. Thus, a person with a body weight of 70 kg would need to consume 58 Kg of tofu to reach the above toxicity levels. Similar results were found for coumaric acid (Pei et al., 2015).

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