PROCESSING AND CHARACTERIZATION OF SOY PROTEIN-BASED MATERIALS

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A Koro

Considero más valiente al que conquista sus deseos que al que conquista a sus enemigos, ya que la victoria más dura es la victoria sobre uno mismo.

Aristóteles

Acknowledgements

Al finalizar un trabajo tan arduo y lleno de dificultades como el desarrollo de una tesis doctoral se siente satisfacción del trabajo realizado. Sin embargo, realizando un análisis objetivo se debe concluir que la magnitud de los logros obtenidos hubiesen sido imposibles sin la participación de personas e instituciones. Por ello, es para mí un verdadero placer utilizar este espacio para ser justo y consecuente con ellas, expresándoles mis agradecimientos.

Debo agradecer de manera especial y sincera a la Dr. Koro de la Caba Ciriza por aceptarme para realizar esta tesis doctoral bajo su dirección. Su apoyo y confianza así como el grado de libertad otorgado en el desarrollo del presente trabajo han sido un aporte incalculable. Las ideas propias, siempre enmarcadas en su orientación y rigurosidad, han sido la clave del buen trabajo que hemos realizado juntos. Le agradezco también el haberme facilitado siempre los medios suficientes para llevar a cabo todas las actividades propuestas durante el desarrollo de esta tesis.

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Finalmente, debo agradecer a los servicios generales de investigación (SGIker) de la EHU/UPV por la disposición de los servicios técnicos en la realización de la tesis doctoral. También debo agradecer a la Diputación Foral de Gipuzkoa, al Ministerio de Ciencia e Innovación MICINN (Project MAT2009-07735) y al Plan E por la financiación del trabajo realizado.

En tres tiempos se divide la vida: en presente, pasado y futuro. De éstos, el presente es brevísimo; el futuro, dudoso y el pasado, cierto.

Séneca

Summary

Plastics are one of the most important materials employed in daily life. However, environmental pollution from their consumption has become a serious issue, particularly when they are disposed after one-time use, like packaging materials. To overcome this problem, biopolymers, which are natural and biodegradable polymers, are regarded as an attractive alternative. Among biopolymers, soy protein could be a potential replacement for petroleum-based products since it is abundant, inexpensive, renewable and biodegradable, and therefore environmentally friendly. Soy protein is extracted from soybeans used to obtain soy oil. During this process, soy flour is obtained as a secondary product and it can be purified to obtain soy protein concentrate (SPC) and soy protein isolate (SPI), adding value to agricultural by-products. Although optimization in processing methods is required, this kind of proteins could offer significant opportunities to develop packaging materials in the future. These soy-based plastics could be employed as short-term use or one-time use plastic products in place of the non-biodegradable materials currently used. Furthermore, soy protein can be used for food packaging purposes since it meets food grade standards.

The general objective of this work was to process and characterize soy protein-based materials for packaging applications. The work consists of nine chapters, which present the research carried out in this area. Chapter 1 is an overview of biopolymers, especially proteins, and gives a general introduction to the interest of biobased materials, focusing in chemical, environmental and economical aspects. The materials and methods employed to prepare those biobased materials are described in the second chapter.

Although soy protein plastics without any additive have a brittle behaviour, which makes processing difficult, addition of plasticizers is an

effective way to obtain flexible SPI-based films. In the third chapter, final properties of glycerol-plasticized soy protein films prepared by casting and compression are analyzed; the effects of glycerol content and processing method are studied. The differences in the protein-glycerol interactions, as a function of the pH on the unfolding of the protein, are studied in chapter 4.

Taking into account the previous results, chapters 5 and 6 investigate the effect of the addition of natural substances on the functional properties of SPI-based films prepared by compression. The influence of gelatin type and content on mechanical properties and water uptake are related to SPI-gelatin interactions in chapter 5, while chapter 6 shows the effect of different contents of lactic acid, epoxydized soybean oil and olive oil on optical, barrier, and mechanical properties of SPI-based films modified with gelatin.

Based on the results obtained in the previous chapters and the motivation of the potential industrial applications, the purpose of chapter 7 is to study the effect of moisture, gelatin and sugars on system parameters and product properties, thus providing good characterization of the extrusion process in order to make it highly energy efficient and cost effective.

Finally, the general conclusions of this work are summarized in chapter 8 and references cited along the text are listed in chapter 9.

Objectives

It is well-known that the use of long-lasting polymers for short-term applications, such as packaging is not sustainable. Proteins are biopolymers with potential properties for applications in the field of food packaging because they are able to form films with good barrier properties in dry conditions. Also, they are biodegradable/compostable and come from renewable resources, so they offer important advantages from the environmental and economic point of view. Nevertheless, films based on biopolymers are brittle and have low resistance to moisture, thus some modifications are needed to turn them into useful materials in service conditions.

The aim of this thesis was to reduce the moisture absorption and, simultaneously, to improve mechanical properties of the materials prepared with soy protein. For this purpose, it was necessary to add substances that could interact with the polar groups of the protein, reducing their hydrophilic character, and simultaneously could act like plasticizers, reducing the brittleness of the final product. Moreover, processing conditions also influence final properties of protein-based materials, so the optimization of the processing was also an objective of this work.

To be able to improve material properties and, in particular, those related to food packaging, mechanical properties and barrier properties, this work focused on these three areas:

1. Plasticization by the addition of glycerol.
2. Blending with natural substances, such as gelatins, acids, oils, and sugars.
3. Processing by wet and dry methods under different conditions.

Index

INTRODUCTION

1. Introduction

This chapter is intended to provide a brief outline of work in the field of biodegradable polymers' research and development, and the areas in which this research is being applied, with emphasis in packaging.

1.1. Background

The rising oil prices helped to grow interest in natural polymers back in the 1970s (Chandra and Rustgi, 1998), and concerns over the dwindling availability of landfill sites are reviving interests in biodegradable materials today (Gandini and Belgacem, 2008; Luckachan and Pillai, 2011). The durability of traditional petroleum-derived plastics, which makes them ideal for applications such as packaging, can also lead to waste-disposal problems, as these materials are not biodegradable. Biodegradable polymers offer scientists a possible solution to these waste-disposal problems (Bastioli, 2005; Fritz et al., 2001; Vroman and Tighzert, 2009, Yu et al., 2006).

Apart from the disposal, the fabrication of industrial products should also take into account raw materials from renewable resources in order to preserve fossil resources. Currently, two approaches are explored to minimise the impact of the use of non-biodegradable petroleum-derived polymers: first, the design of polymeric materials for long duration, such as biocomposites based on plant oil resin or wood fiber, for construction materials (Wuambua et al., 2003; Sudin and Swamy, 2006; Ashori, 2008); second, technological innovations designed for the production of polymers for short duration, such as agricultural mulches, horticultural pots or disposable packages (Guilbert and Gontard, 2005; Platt, 2006; Rudnik, 2008), which is within the aim of this work. Combining these two properties, durability and biodegradability, suppose the design of materials to be resistant during their use and biodegradable at the end of their useful lives.

In the last decades, there has been an increasing interest in biodegradable polymers for packaging to replace non-biodegradable synthetic polymers at a low cost, thereby producing a positive effect both environmentally and economically (Gross and Kalra, 2002). Therefore, biobased packaging materials are interesting from a sustainable point of view. However, designing biobased packaging materials involves many considerations (**Figure 1.1**) to successfully manufacture the package with the properties required for the desired application.

Figure 1.1. Aspects to take into account for the design of biobased packaging materials.

As a result of the vast amount of packaging materials used and the waste associated with it, governments have implemented legislation to reduce the amount of municipal waste packaging being sent to landfill, so biodegradability/compostability is one of the main focuses for choosing biopolymers as packaging materials. This property provides the opportunity to degrade material after useful life and enables to close the idealised life cycle for bioplastics, taking into account the “cradle to cradle” consideration (**Figure 1.2**). Life Cycle Assessment (LCA) is a practical key tool to measure products’ environmental impact from the extraction of resources to ultimate disposal (Bier et al., 2011; Hospido et al., 2010; Panicelli et al., 2009; Roy et al., 2009; Vercalsteren et al., 2010; Vidal et al., 2007).

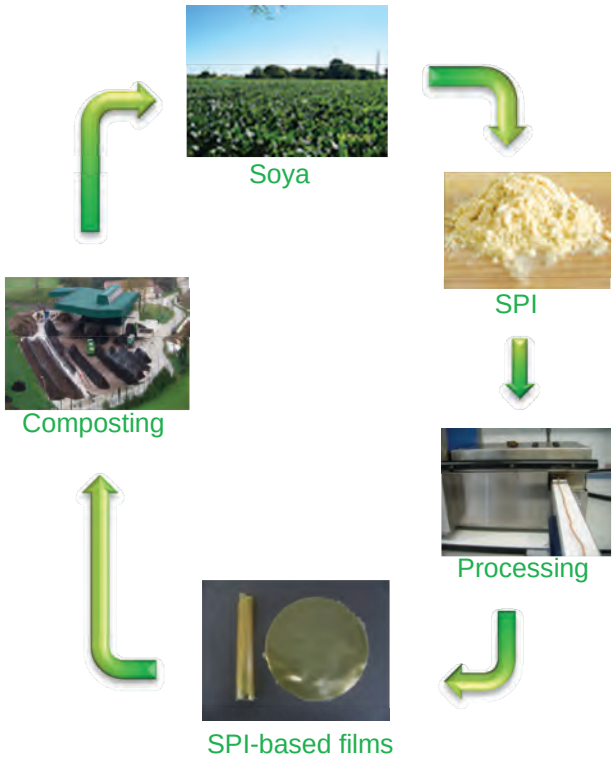


Figure 1.2. Idealised closed loop life cycle for bioplastics.

On the other hand, consumers are more likely to purchase products that are ecofriendly packaged. Moreover, the rise in the average age of the population and in the number of single person households in developed markets is influencing new products. Consumers are looking for products that are easy to use, healthy, and have good price/quality ratio. Although these trends are global, they impact on the packaging market, which is an essential component of modern living (Butler, 2009). The global market for packaging in 2010 was valued at just over US$ 395 billion and long term growth is expected to be on average around 3% a year to 2015 (Chipchase, 2011). Food packaging is the largest segment, accounting for 51% of the total market value **(Figure 1.3)**, and must be designed with some specific requirements in order to

improve food product quality and safety and extend shelf life product stability, which are important market demands. Non-food market sectors such as pharmaceuticals, beverages, health and beauty, and households products also offer significant packaging opportunities.

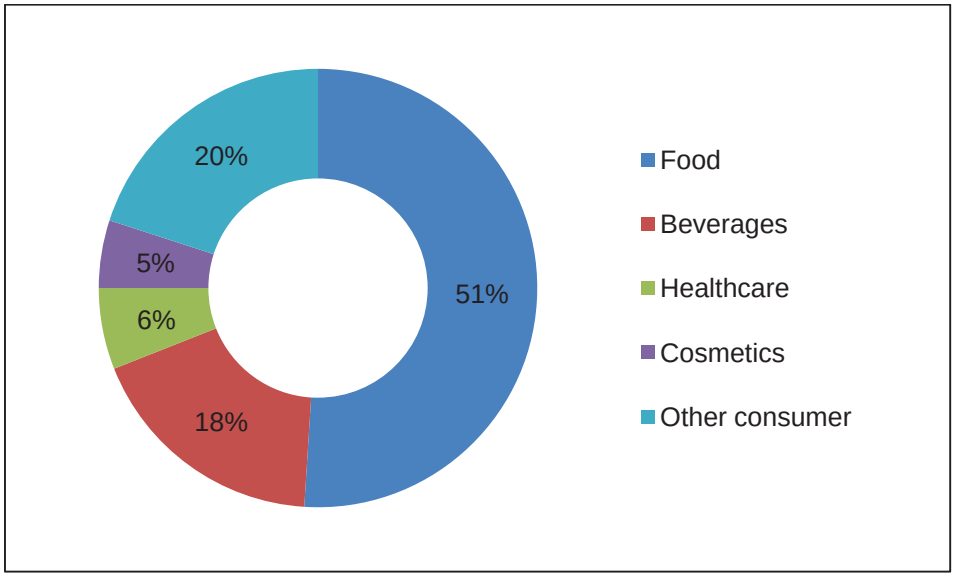


Figure 1.3. Packaging global market in 2010.

Plastic (rigid and flexible) accounted for 37% of all global packaging sales in 2010, the largest share of the market (**Figure 1.4**). It is expected to be the fastest growing packaging material during the period 2010 to 2015 with a predicted average annual growth rate of around 4% (**Figure 1.5**). The commercial success of plastics as packaging products is due to a combination of flexibility from film to rigid applications, strength and lightness. While over 50% of all European goods are packaged in plastics, these plastics account only for 17% of all packaging weight. Moreover, plastic food packaging does not affect the taste and quality of the food. In fact, plastics ensure that food keeps its natural taste while protecting it from external contamination. Plastics versatility can be seen in the variety of applications like films for fresh meat,

bottles for beverages or yoghurt cups (Chipchase, 2011).

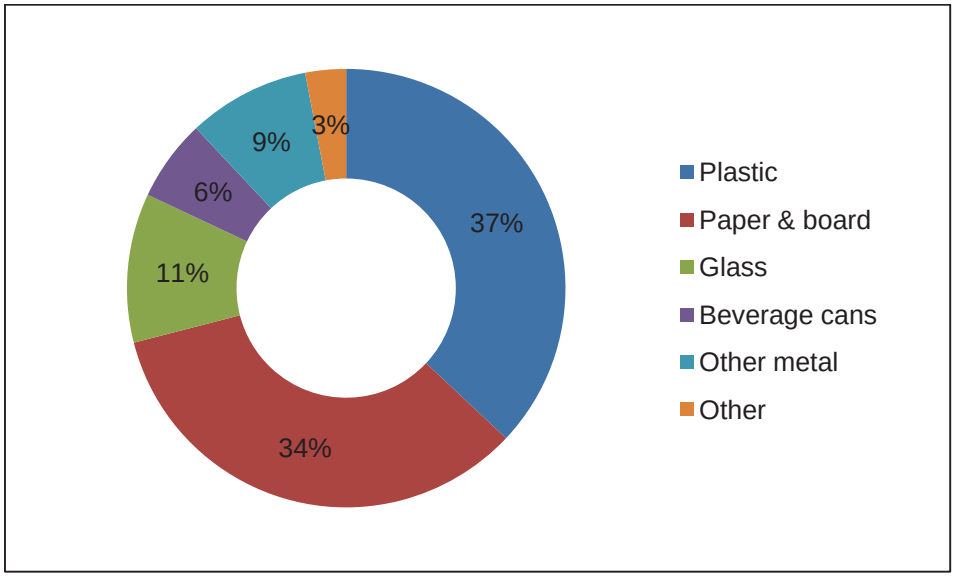


Figure 1.4. Packaging materials in 2010.

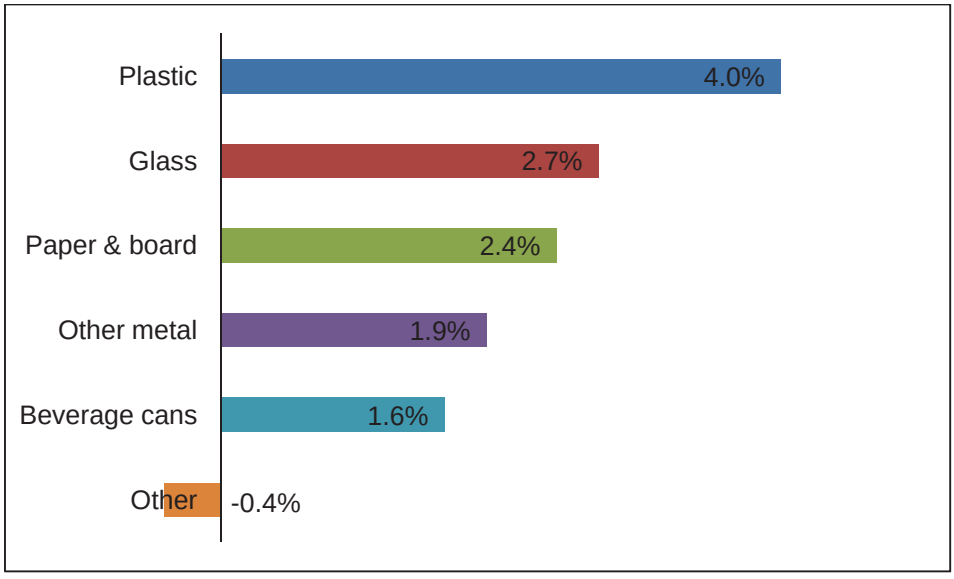


Figure 1.5. Predicted annual growth rate for packaging materials in 2010-2015.

Within plastics, the commodity polymers, which were provided to develop durability and resistance to degradation, have been the most widely used polymers. To date, packaging materials have been, to a large extent, based on non-renewable materials. However, the field of biopolymers, while still in its early stages, is growing in the packaging market, mainly due to environmental concerns. Although the most widely used renewable packaging materials are paper and board, which are based on cellulose, major efforts are under way to find alternative biopolymers.

In the specific context of food packaging materials, food packages must serve some important functions, including containment and protection of food. In addition to sensory and safety aspects relating to foods, the development and selection of biobased materials also involve other issues such as logistics, marketing, legislation and environmental, and financial contrasts, as cited above. Although this work does not go into details on these issues, these aspects require consideration. As foods are dynamic systems with limited shelf life, food packaging materials must serve some specific requirements. These relate to barrier properties (oxygen and water vapour permeability), optical properties (colour and transparency), and mechanical properties (strength and flexibility), as well as disposal requirements and price-competitiveness. However, matching the durability of the material with the product shelf life is one of the most important requirements. In the case of biobased materials, they must remain stable, maintaining functional properties such as mechanical and barrier properties, during storage of the food. In addition, the material should biodegrade efficiently on disposal, thus, biodegradation must be avoided during storage, whereas optimal conditions for biodegradation/composting must exist after discarding (Haugaard et al, 2001). Research and development in the area of food biopackaging have been intensified over the last years. However, the low presence of biobased packages on the market is evident, which indicates that further studies of these

materials must be carried out.

1.2. Biopolymers

Biopolymers are biodegradable polymers generated from natural sources. According to the American Society for Testing and Materials (ASTM), biodegradable polymers are defined as degradable polymers in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae (ASTM, 1999). Biodegradable polymers are those that undergo significant deterioration in their properties under the influence of microorganisms in specific conditions aided by chemical reactions like photodegradation, oxidation, and hydrolysis (Chandra and Rustgi, 1998; Wang et al., 2003). In fact, biodegradation is considered to take place through three stages: biodeterioration, biofragmentation and assimilation, so it is a natural complex phenomenon. The term “biodegradation” indicates the predominance of biological activity, however, outdoor conditions associated to weather or burying can undergo transformations (mechanical, thermal, and chemical) that change the ability of the material to be biodegraded (Kyrikou and Briassoulis, 2007). In most cases, these parameters initiate the biodegradation process (Lucas et al., 2008). Biodegradability depends not only on the environmental conditions, but also on the polymer origin and its chemical structure (Briassoulis, 2006; Scarascia-Mugnozza et al., 2006; Rudnik and Briassoulis, 2011).

Biopolymers may be divided into three main categories based on their origin and production (Krochta and De Mulder-Johnston, 1997; Chandra and Rustgi, 1998; Mohanty et al., 2000): polymers produced by microorganisms, polymers produced by classical chemical synthesis using biomonomers, and polymers directly extracted from biomass (**Figure 1.6**). Most commonly available polymers from biomass are extracted from plants or animals (Vroman and Tighzert, 2009). Plants naturally produce a number of structural and carbon-reserve polymers (Mooney, 2009).

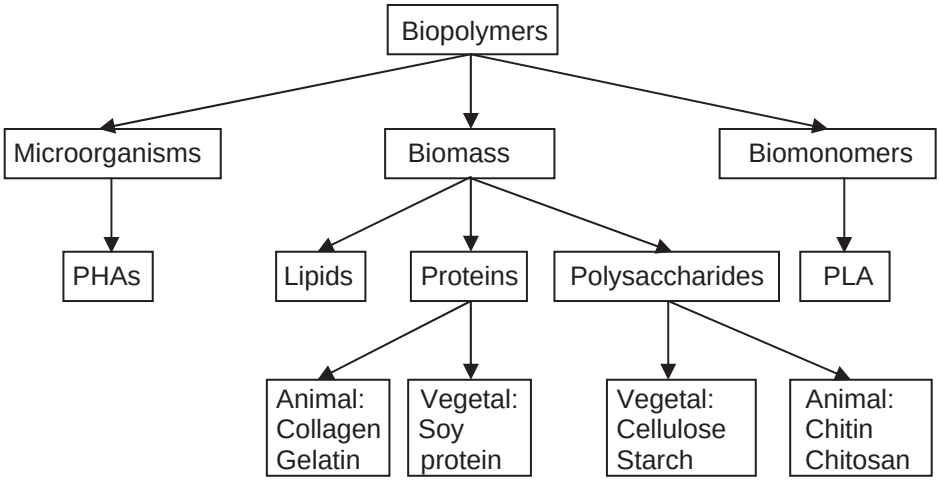


Figure 1.6. Schematic presentation of biopolymers based on their origin.

Biopolymers are a growing research issue and represent an interesting alternative to synthetic polymers for a short-life range of applications like packaging. Nowadays, chemical companies worldwide produce biopolymers, which are commercially available under different names (Martín-Closas and Pelacho, 2011), as it can be seen in **Table 1.1.**

Biopolymer	Commercial name	Manufacturer
PHAs	Goodfellow PHB	Goodfellow (UK)
	Biomer®	Biomer (Germany)
PLA	Ingeo™	NatureWorks LLC (USA)
	Bio-Flex®	FKuR Plastics (Germany)
Starch	Mater-Bi®	Novamont (Italy)
	Bioplast®	Biosphere (France)
Cellulose	Naturflex™	Innovia Films (UK)
	Biograde®	FKuR Plastics (Germany)
Soy protein	PRO-FAM®	ADM (Netherlands)

Table 1.1. Some commercial biopolymers.

To date, polymers produced by microorganisms consist mainly of poly(hydroxyalkonoates) (PHAs), but developments with bacterial cellulose are in progress (Retegi et al., 2010, 2012). Some organisms accumulate PHA from

30 to 80% of their cellular dry weight, in the presence of an abundant source of carbon and under limited nitrogen (Lunego et al., 2003, Albuquerque et al., 2011). PHAs are polyesters of various chain lengths. According to the size of the alkyl substituent, mechanical properties of PHAs differ; brittle plastics or flexible plastics can be obtained (Dias et al., 2006; Mooney, 2009). The PHA with a methyl substituent group, poly(hydroxibutyrate) (PHB), is the most common. As the pure homopolymer is a brittle material, its copolymer with hydroxyvalerate (PHBV) is synthesized and mechanical properties of the copolymer can be modified by changing the hydroxyvalerate unit content (Juzwa, 2006). Actually, PHAs are produced by microorganisms, cultured under different nutrient and environmental conditions; nevertheless, increasing attention is given to PHAs produced by plants (Tilbrook et al., 2011).

Another biodegradable polyester is poly(lactic acid) (PLA), which is the best known polymer produced by classical chemical synthesis using renewable biobased monomers (Courgneau et al., 2011). PLA is polymerised from lactic acid monomer, which may be produced via fermentation of carbohydrate feedstock (Kricheldorf, 2001). PLA is considered the most versatile material among biodegradable polymers because of its biocompatibility and high mechanical strength (Martino et al., 2011a), comparable with that of polyethylene (PE) and polystyrene (PS), and the easy availability from renewable agricultural sources. These attributes make them a leading candidate in biomedical applications such as orthopaedics, drug delivery, sutures, and scaffolds (Albersson and Varma, 2003; Zhou et al., 2004). It is also used as an environment friendly plastic, although its market is still limited due to its low degradation rate as compared to the waste accumulation rate (Tuominen et al., 2002; Luckachan and Pillai, 2011). Copolymerization of lactide with other monomers or polymers is used to modify the properties of PLA and to control its degradation behaviour suitable for specific applications.

As cited above, PLA may be produced by fermentation of carbohydrate

feedstock, which may be agricultural products like wheat or corn, which can also be the source of other biopolymers, such as those directly extracted from biomass. Examples of this kind of biopolymers are proteins like gluten and polysaccharides such as starch. Nowadays, starch is one of the principal polysaccharides of interest for material production (Avérous and Halley, 2009). In fact, starch is the most commonly used agricultural raw material for biodegradable materials. It is the main storage supply in botanical resources (cereals, legumes and tubers), thus a widely available raw material. Starch granules can be easily isolated from plants sources, mainly from corn, wheat, potato, rice, and pea. Starch is a polysaccharide consisting of α-1, 4 glucose units. It is composed of two different macromolecules, linear amylose and highly branched amylopectin. Starch composition and structure are dependent on its origin. Indeed, some species present amylose-rich starch and some amylopectin-rich starch. Except for applications as fillers to produce reinforced plastics, native starch is chemically or/and physically modified (Thirathumthavorn and Charoenrein, 2007; Lopéz et al., 2011). Starch is gelatinized with heat combined with high water content, which causes the disruption of the highly granular organization, forming a viscous paste with destruction of most of intermolecular hydrogen links. At a high destructuring level plasticized starch, so-called “thermoplastic starch” (TPS), is obtained. The first patents on TPS were published at the end of the 1980s. In practice, TPS combines starch with a plasticizer and then it is transformed under thermomechanical treatment as a thermoplastic, using extrusion or injection. Depending on the plasticizer level and the starch source, a wide range of properties may be obtained (Godbillot et al., 2006). However, TPS shows high moisture sensitivity and weak mechanical properties compared to conventional synthetic polymers, properties which are important for the most widespread polymer applications (Avérous, 2004).

Another polysaccharide of great interest is cellulose. It is the most

abundantly occurring natural polymer, consisting of β-1, 4 glucose units. In the packaging context, paper and board are the most familiar forms. Cellulose is a cheap raw material, but difficult to use because of its hydrophilic nature, insolubility, and crystalline structure. However, a number of cellulose derivates are produced commercially (Cunha and Gandini, 2010a), most commonly methyl cellulose, carboxymethyl cellulose, ethyl cellulose, hydroxymethyl cellulose, and cellulose acetate, but only cellulose acetate is widely used in food packaging (Edgar et al., 2001; Heinze and Liebert, 2001). However, the most innovative aspect related to cellulose is associated with its nanofibers (Habibi et al., 2010; Siró and Plackett, 2010), which deal with their preparation, characterization, surface treatment, and potential applications. Unlike cellulose, hemicelluloses are non-crystalline polysaccharides. Since they represent 20-30% of the biomass of plants, they have become an important natural source of biopolymers in the last few decades (Cunha and Gandini, 2010b). Their wide diversity has been exploited in several areas, including food, biomedical and cosmetic applications (Hansen and Plackett, 2008).

The second most abundant polysaccharide in nature is chitin, which can be regarded as cellulose with hydroxyl at position C-2 replaced by an acetoamide group (Ravi Kumar, 2000; Rinaudo, 2006). Chitin appears mostly in the exoskeleton of crustaceans and insects, but also in certain species of fungi (Pillai et al., 2009; Rinaudo, 2008). Chitin is a structural biopolymer, which has a role analogous to that of cellulose in plants. Plants produce cellulose in their cell walls and insects and crustaceans produce chitin in their shells, thus cellulose and chitin are two important and related polysaccharides that provide structural integrity to plants and animals, respectively (Raabe et al., 2007). Chitin has strong hydrogen bonds between OH and NH groups and, as a consequence, it is insoluble in most common solvents. By extensive (higher than 50%) deacetylation of chitin, chitosan is obtained. While chitin is insoluble, chitosan is soluble in dilute acidic solutions below pH 6.0. Although on a lesser

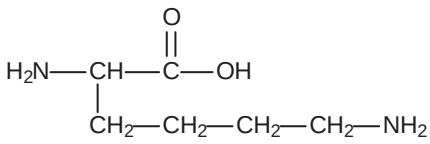
scale than other polysaccharides, chitosan has been exploited. The characteristics of chitosan that may be varied as required for a particular application are the degree of deacetylation and the molecular weight (Baspar and Sampath Kumar, 2009). Chitosan forms films (Kerch and Korkhov, 2011) and it has been used for the production of edible coatings (Epure et al., 2011; Martino et al., 2011b).

Among biopolymers, polysaccharides are constituted of a few or even one monomer, while proteins are based on several amino acids. Therefore, proteins are known for their structural complexity and functional diversity.

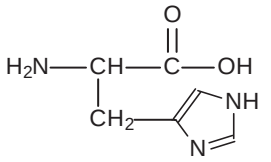
1.3. Proteins

Proteins are polymers formed from α-amino acids, in which amino group is attached to the carbon atom immediately adjacent to the carboxylic group. There are 20 standard amino acids that differ from each other in the structure of the side chains, which can be non-ioinized or ionized polar (basic and acidic amino acids) or non-polar, as shown in **Table 1.2.** Ten of those amino acids are essential amino acids, since they are not synthesized by human metabolic processes and are diet components.

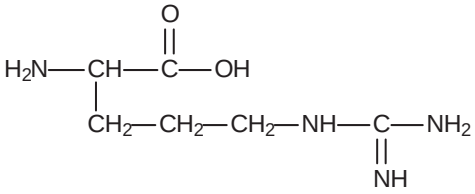
Basic amino acids



Lysine* (Lys)

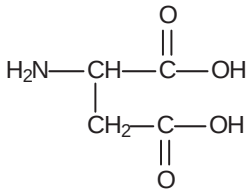


Histidine* (His)

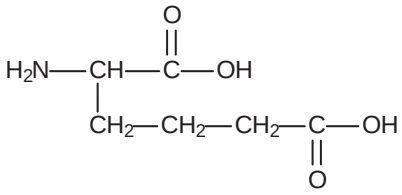


Arginine* (Arg)

Acidic amino acids

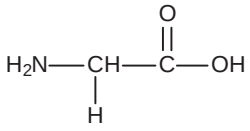


Aspartic acid (Asp)

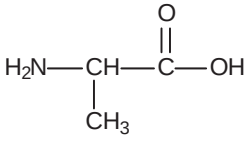


Glutamic acid (Glu)

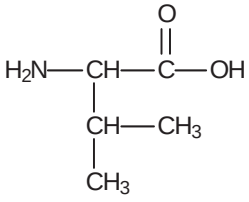
Non-polar amino acids



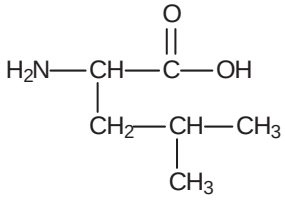
Glycine (Gly)



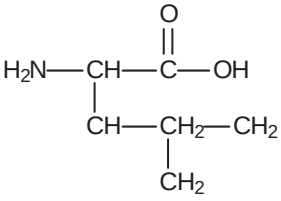
Alanine (Ala)



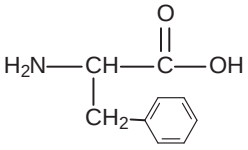
Valine* (Val)



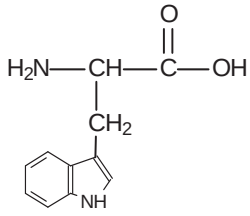
Leucine* (Leu)



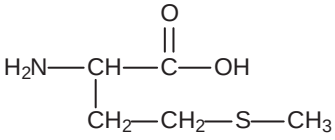
Isoleucine* (Ile)



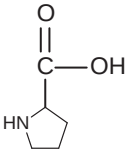
Phenylalanine* (Phe)



Tryptophan* (Trp)



Methionine* (Met)



Proline (Pro)

Polar amino acids

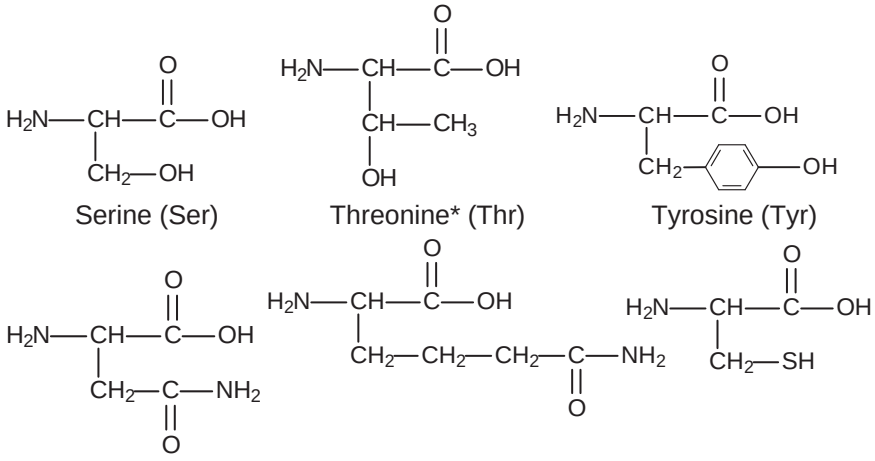


Table 1.2. Standard and essential (*) amino acids.

If amine and carboxylic acid functional groups in amino acids join together to form amide (peptide) bonds, polypeptides are formed. Some proteins consist of only single polypeptides, but most involve two or more aggregated polypeptides. The amino acids' composition is different from one protein to another, and therefore the properties of the materials manufactured from them. Most proteins contain 100-500 amino acid residues. Peptide bonds are the main links in proteins, but disulfide bonds are possible when cysteine appears in the composition (**Figure 1.7**). These many possibilities of bonding and structures derive in complex polymers with varied properties.

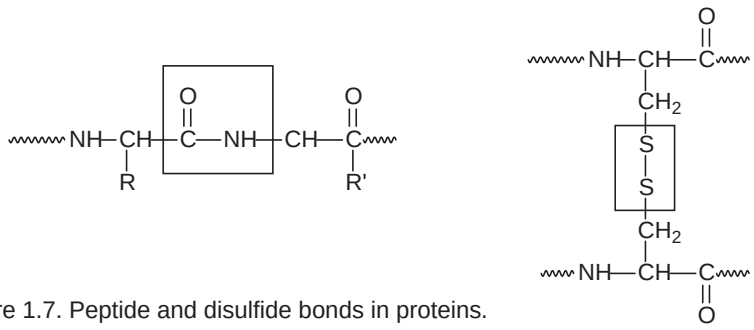


Figure 1.7. Peptide and disulfide bonds in proteins.

Due to the rigid nature of the peptide bond, peptide links are relatively planar and resistant to conformational changes. This aspect of proteins' structure is an important factor influencing the conformations adopted by proteins. Fibrous proteins have fiber-like structures, and serve as the structural material in tissues. Corresponding to this structural function, they are relatively insoluble in water and unaffected by moderate changes in temperature and pH. Fibrous proteins include collagens, the proteins of connective tissues, and keratins, proteins that are major components of skin and hair. On the other hand, globular proteins serve maintenance roles in living organisms and either dissolve or disperse in water. Such proteins are generally more sensitive to temperature and pH change than fibrous proteins.

Since proteins incorporate both acidic and basic functional groups, the proton transfer from the acidic carboxyl function to the basic amino group results in the ammonium carboxylate structure, commonly referred to as a zwitterions. However, the net charge in an aqueous solution will depend on the pH of the solution. At an acidic pH, both the carboxylate and amine functions are protonated, so the protein has a net positive charge. At a basic pH, the amine exists as a neutral base and the carboxyl as its conjugate base, so the protein has a net negative charge. At intermediate pHs, the positively charged groups are exactly balanced by the negatively charged groups and the protein, on average, has no net charge. This characteristic pH is called the isoelectric point. Many globular proteins precipitate if the pH of the solution is set at the isoelectric point (pI) of the protein.

The different amino acids that make up a protein and the order in which they are joined together by peptide bonds are referred as the primary structure. The properties of proteins depend not only on their component amino acids and their bonding sequence in protein chains, but also on the way in which the peptide chains are folded in space (McMurry, 2009).

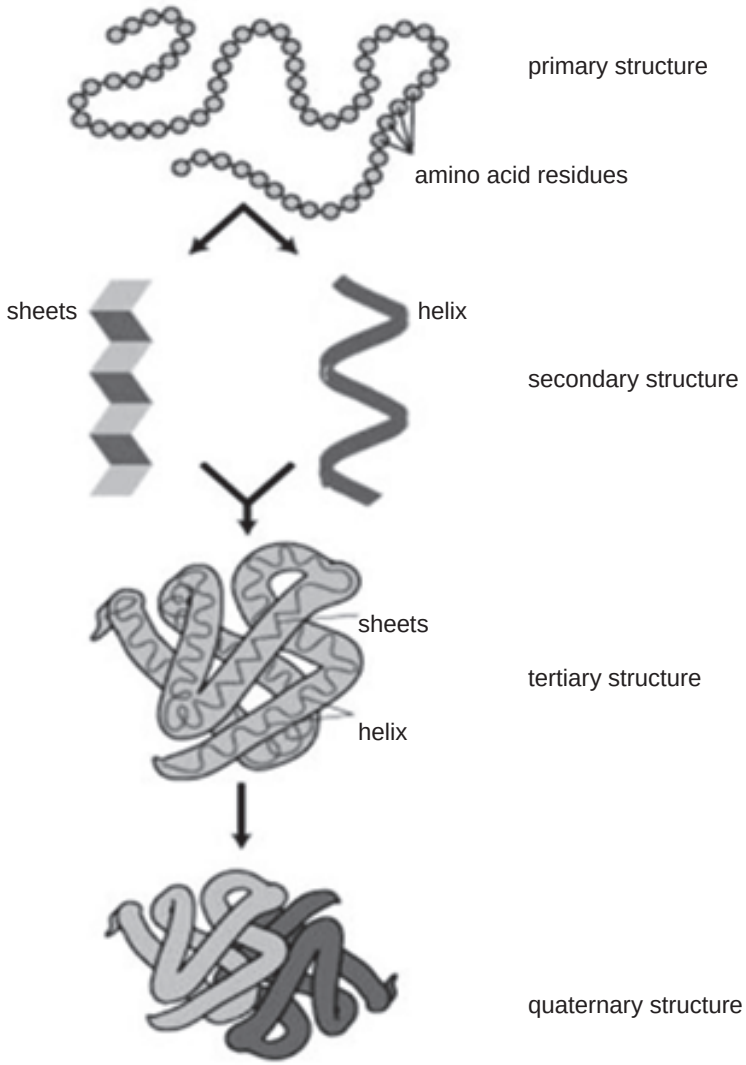


Figure 1.8. Protein structures.

Because of their size, the orientation options of proteins could be enormous, but several factors narrow the structural options, and it is possible to identify some common structures that appear repeatedly, such as helix or sheets, which are referred as secondary structures. Most proteins do not adopt

completely uniform conformations, and full descriptions of their preferred three dimensional arrangements are defined as tertiary structures. Some factors that influence the conformational equilibria of protein chains are the planarity of peptide bonds, hydrogen bonding of amide carbonyl groups to NH groups, steric crowding of neighbouring groups, repulsion and attraction of charged groups, and the hydrophilic and hydrophobic character of side groups. In addition to the tertiary structures that will be displayed, attention must also be given to the way in which peptide structures may aggregate, which is related to the quaternary structure (**Figure 1.8**).

The natural or native structures of proteins may be altered by treatment that does not disrupt the primary structure. The secondary, tertiary and quaternary structure of proteins can be modified by various physical and chemical agents, including heat, pressure, acids and alkalis. This structural unfolding (denaturation) is often done deliberately in the course of modifying proteins. Following denaturation, some proteins will return to their native structures under proper conditions; but extreme conditions, such as strong heating, usually cause irreversible change.

The proteins used for the manufacture of materials are those found in greatest quantities (Samarasinghe and Easteal, 2008): proteins of animal tissue structure (collagen, keratin), cereal co-products (gluten, zein), or reserve proteins of grains (sunflower, soybean).

Collagen is the primary protein component of animal connective tissues. It is composed of different polypeptides, which contain mostly glycine, proline, hydroxyproline and lysine. It has been extensively investigated for biomedical applications (Gelse et al., 2003; Huang and Fu, 2010). By denaturation of collagen, a high molecular weight polypepetide is produced, called gelatin, which consists of 19 amino acids, is water soluble, and has good film forming abilities (Cao et al., 2009; Gómez-Guillén et al., 2009). Gelatin is a

protein derived from the chemical denaturation of collagen and it is a mixture of α-chains (one polymer chain), β-chains (two α-chains covalently crosslinked), and γ-chains (three covalently crosslinked α-chains) (Papon et al., 2007). Depending on the method in which the collagen is treated, two different types of gelatin can be produced. Type A gelatin (pI 6–9) is produced from acid-treated collagen, and type B gelatin (pI 5) is produced from alkali-treated collagen (Eysturskaro et al., 2009; Stainsby, 1987). The amino acid composition of gelatin is very close to that of its parent collagen. It is mostly composed of glycine (Gly, 34%), proline and hydroxyproline (Pro + Hyp, 16%) imino acids, and alanine residues (Ala, 10%) (Gómez-Guillén et al., 2002; Karim and Bhat, 2009).

Wheat gluten is a protein by-product of the starch fabrication. It is available in high quantity and at low cost. Wheat gluten contains two main groups of proteins, gliadin and glutenin. Gliadins are proteins with disulphide bonds and low molecular weight, while glutenins have at least ten times higher molecular weight. Wheat gluten is excellent film forming but, without plasticizer, films are brittle (Chen et al., 2012; Lagrain et al., 2010).

Forming packaging materials from proteins requires three steps: first, the rupture of low-energy intermolecular bonds that stabilize polymers in the native state; second, the arrangement and orientation of polymer chains; and third, the formation of a three dimensional network stabilized by new interactions and bonds. Two technological processes are used to make materials based on proteins: a wet process based on dispersion or solubilisation of proteins, and a dry process based on the thermoplastic properties of proteins (Cuq et al., 1998).

The solvents used to prepare protein film-forming solutions or dispersions are generally based on water and ethanol. However, dissolving or dispersing proteins in solvents may require pH adjustment by addition of acids

or bases. Film formation by the wet process is based on separation of the protein from the solvent due to changes in solvent conditions (pH), thermal treatments (heating), or solvent removal (drying), therefore, film formation from a protein solution or dispersion under controlled laboratory conditions requires the knowledge of physicochemical properties of proteins in aqueous solvents systems (Chou and Morr, 1979). Sensitivity of proteins to pH is usually associated with a high content of ionized polar amino acids. For instance, zein and keratin films form over a wide pH range because they have low ionized amino acids contents, 10.0 and 10.7%, respectively, and thus are not sensitive to pH changes (Cuq et al., 1998). On the other hand, high contents of ionized polar amino acids in soy proteins (25.4%) limit film formation at low pH (Mauri and Añón, 2006). The functional properties of packaging materials obtained by the wet process depend on protein concentration, pH, additives, drying rate and temperature (Krochta, 2002). Most protein-based packaging materials have been fabricated by this process.

Regarding to the dry process, protein-based materials can be shaped by traditionally called thermoplastic processing technologies, such as extrusion or thermomechanical moulding, depending on type and density of interactions in proteins, which determine the behaviour of proteins to be able to change in a reversible way from a rigid state to a soft state through a temperature increase or plasticizer addition. Plasticization by water or polyhydroxy compounds is critical for the interactions of protein to form a continuous network from powdered raw materials. However, complexity and heterogeneity of intermolecular interactions within proteins are responsible for the lack of flow region and fabrication of materials by these technologies could be expected only in the presence of agents that break intermolecular bonds that stabilize native proteins (Hernández-Izquierdo and Krochta, 2008). Therefore, although thermoplastic processing is adapted to synthetic materials for which fabrication parameters are optimized, process parameters such as temperature, plasticizer

concentration, and residence time must be optimized for proteins.

1.4. Soy protein

It is widely believed that soybean was originated in China, and introduced in Europe in the early 1700s. However, soybean production has been limited in Europe due to poor climate and soil conditions. Soybean was first introduced in North America in the 18th century, but large-scale production did not occur until the early 1900s, when the first soybean processing plant was opened by 1920. Before the 1980s, USA produced approximately 77% of the world's soybeans, while China was the second leading producer (15%). At the moment, USA is the world leader in soybean production, but Brazil and Argentina have high soybean production, which makes South America the main producer. In 2011, USA represented 32% of the world soybean market, Brazil 29%, Argentina 20%, and China 5% (USDA-FAS, 2012).

Soy proteins are composed of a mixture of albumins and globulins, 90% of which are storage proteins with globular structure. Soy protein consists of four major fractions: 2S, 7S, 11S and 15S, where S stands for Svedburg units, based on the rate of sedimentation. 7S (β-conglycinin) and 11S (glycinin) globulin fractions make up 70% of the total proteins in soybeans (Kinsella, 1979). The ratio 11S/7S may vary from 0.5 to 3 (Ning and Villota, 1994). These globulins are protein fractions in which the subunits are associated via hydrophobic and hydrogen bonding (Thanh and Shibasaki, 1976). The polypeptides are compactly folded, though considerable unstructured regions exist internally (Wolf, 1972). In both 7S and 11S globular structures, disulfide bonds also bind the polypeptide subunits together (Catsimpoolas et al., 1971; Koshiyama and Fukushima, 1976; Utsumi and Kinsella, 1985).

Soy protein is obtained as a by-product from soybeans through an extraction process to obtain soy oil. The most common technique used in the production of soy oil consists of hexane extraction, due to its high oil extraction

efficiency (Russin et al., 2011). However, the solvent has some considerable economic, environmental, and safety drawbacks. Economically, one of the main concerns is the stability of both hexane supply and price due to fluctuation in the fossil fuel market (Gandhi et al., 2003). Coupled with economic concerns, growing awareness of the effects of organic solvents on environmental and human health has increased public scrutiny of environmental emissions from oil processing plants. In addition, hexane is highly flammable and safety precautions need to be taken into account during extraction. Based on economic, environmental and safety reasons, alternative soy oil extraction techniques have been investigated. Aqueous extraction processing (AEP) is an emerging technology that may be a viable alternative to conventional hexane extraction to obtain oil from soybeans (Rosenthal et al., 1996, 1998, 2001). In addition to oil, soy is also highly value for its protein, which is obtained in the same extraction process. It is therefore important to preserve the quality of soy protein during defatting and so, the influence of the conditions used must be evaluated in order to identify suitable extraction techniques and conditions which are economically viable and respectful of the environment (Friedman and Brandon, 2001; Singh et al., 2008). In AEP, water is used as an extracting medium to remove oil as an emulsion. Typical steps in AEP are mechanical disruption of soybean cells by grinding; extraction of oil and protein with or without enzymes; centrifugal separation of an oil-rich emulsion, insoluble solids, and a liquid containing water solubles; and demulsification of the oil-rich cream fraction to recover free oil. AEP results in three distinct fractions: an insoluble fraction (residual fraction) rich in cellulose, insoluble proteins and other insoluble material; a liquid fraction (skim) of soluble proteins, minerals, and carbohydrates; and an oil-in-water emulsion stabilized by proteins and phospholipids (cream). Each of these three fractions presents a challenge to the economic viability of AEP of soybeans. Economic viability, therefore, depends upon employing better separation technologies to recover more of the oil in the cream and/or creating value-added products. However, AEP oil yield

is still lower than yield by hexane extraction (Campbell et al., 2011).

On the global scale, soybean represents the second largest source of vegetable oil. Soy oil shares 28% of the global trade in vegetable oil next to palm oil (32%). A good understanding of microstructural characteristics of soybeans is important for understanding extraction principles. Typical soybean composition is 20% oil, 40% protein, 35% carbohydrate (16% soluble and 19% insoluble), and 5% ash on dry basis. About 80% of the total protein in soybeans is stored in the so-called protein bodies, which occupy most of the cell volume. Protein bodies range in size from 10 to 50 μm in diameter. In aqueous media, large protein bodies are disrupted more easily than smaller protein bodies. On the other hand, soybean oil bodies are much smaller than protein bodies, ranging from 0.2 to 0.5 μm in diameter. They fill the space between protein bodies (Wolf, 1970).

The vast majority of soybeans are processed by cracking, producing about 79.0% meal (edible deffated flakes), 18.5% oil, and 2.5% waste. Typically, soybeans are cracked into approximately 4-6 fragments per bean, conditioned at 60 ºC, and then passed through smooth-surfaced roller mills, resulting in flakes approximately 1 cm across by 250 μm thick. The traditional process of producing edible defatted flakes or meal with a protein content of 40 to 50% is shown in **Figure 1.9**. It is necessary to further process soy meal and flakes to remove some low molecular weight components in order to have higher protein content. Soy protein concentrate (SPC), which contains at least 70% protein on a dry-weight basis, and soy protein isolate (SPI), which contains 90-95% protein, are obtained by removing soluble carbohydrate, ash, and other minor constituents (Kasai et al., 2003).

The traditional procedure for SPI production is by using aqueous or mild alkali extraction (pH 7-10) of the protein and soluble carbohydrates. The extract is then centrifuged, where suspension is used in the isoelectric

precipitation procedure (pH 4.5). The precipitated protein is then washed, neutralized (pH 6.8) and spray dried. The produced SPI is therefore almost pure protein, making it to be practically free of odour, flavour, and colour.

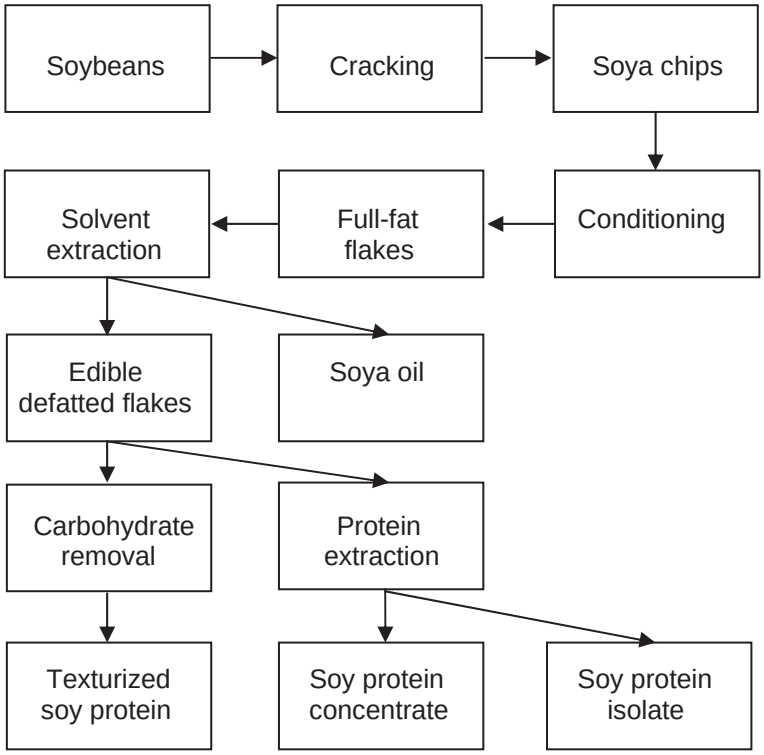


Figure 1.9. Process to obtain defatted soy proteins.

Soy proteins are the first biopolymers from agriculture which were used for the manufacture of moulded materials. Indeed, in the 1930s some parts for Ford cars were manufactured with a phenolformaldehyde/soybean flour mixture (Rouilly and Rigal., 2002), but this was stopped due to the emergence of synthetic plastics.

As co-products of processing cereal crops for food and fuel, plant proteins such as soy proteins are available in abundance, inexpensive and are

derived from renewable resources (Hernández-Izquierdo and Krochta, 2008). However, there are limited industrial applications and most of the proteins currently have low value applications such as animal feed. Poor thermoplasticity, water resistance and brittleness are some of the main reasons for its limited use (Lagrain et al., 2010). Plasticizers are commonly used to develop thermoplastic products and also to improve flexibility. However, adding plasticizers, which are usually hydrophilic substances, makes soy protein more vulnerable to water. Alternatively, chemical modifications can be considered to develop products with good mechanical properties and water resistance. Similarly, blending of soy protein with other biopolymers or reinforcing with natural fibers could be another approach to develop soy protein-based products. Taking the above into consideration, the aim of this work consists of processing soy protein-based materials with improved mechanical and barrier properties.

MATERIALS AND METHODS

2. Materials and methods

2.1. Materials

Soy protein isolate (PROFAM 974) with 90% protein on a dry basis was supplied by ADM Protein Specialties Division (Rozenburg, Netherlands). SPI has 5% moisture, 4% fat and 5% ash. It has acid character and the isoelectric point is 4.6 due to the high content of glutamic acid (Glu, 19.2%) and aspartic acid (Asp, 11.5%), as it can be seen in **Table 2.1**.

Amino acids	SPI	Bovine gelatin	Fish gelatin
Hyp	0.00	8.20	7.69
Asp	11.47	4.55	4.29
Thr	3.69	3.26	2.41
Ser	5.48	3.85	3.71
Glu	19.14	7.31	6.88
Pro	5.18	12.55	11.82
Gly	4.09	33.79	34.91
Ala	4.29	11.17	12.59
Cys	1.20	0.00	0.00
Val	4.79	1.88	1.67
Met	1.40	0.40	1.02
Ile	4.79	1.09	0.77
Leu	7.98	2.37	2.05
Tyr	3.79	0.40	0.28
Phe	5.18	1.19	1.22
His	2.69	0.40	0.47
Lys	6.28	2.47	2.28
Arg	7.48	4.64	5.12
Trp	1.10	0.00	0.00
Hyl	0.00	0.49	0.82

Table 2.1. Amino acid composition of SPI, bovine gelatin and fish gelatin, expressed as number of residues per 100 residues.

The commercial bovine gelatins type A (100/120 and 200/220 bloom values) were obtained from Sancho de Borja S.L. (Zaragoza, Spain). Two commercial cod fish gelatins type A were employed in this study. The fish

gelatin with 140/160 bloom value was obtained from Junca S.L. (Girona, Spain) and the fish gelatin with 200 bloom value was supplied by Weishardt International (Liptovsky Mikulas, Slovakia). All gelatins met the quality standard for edible gelatins (1999/724/CE). The amino acid composition was supplied by the manufacturer and it is shown in **Table 2.1**. Bovine and fish gelatins exhibited typical type I collagen glycine content, representing approximately 1/3 of the total amino acids. The proline plus hydroxyproline content was higher in bovine gelatin than in fish gelatin. It is known that 50-60% of α-chains consist of tripeptides having the general formula glycine-proline-hydroxyproline.

Glycerol used in this study was food grade reagent obtained from Panreac, as well as L-lactic acid (LA). Epoxydized soybean oil (ESO), rich in unsaturated fatty acids (84.5%), was supplied by Hebron S.A. (Barcelona, Spain), and virgin extra olive oil (OO) variety picual, with oleic acid (78.1%) as the major component, by Cooperative La Carrera (Jaén, Spain). Sucrose (SUC) and lactose monohydrate (LAC) sugars used in this study were food grade reagents obtained from Panreac.

2.2. Processing

There are two technologies to prepare materials based on proteins: wet and dry processes. Wet process, also called solution casting, is based on the dispersion or solubilisation of proteins in a solvent medium. Dry process often includes hot-pressing or compression moulding as well as extrusion technique.

In order to evaluate the relationship between processing and final properties of the materials, four processing methods were employed in this study: casting, compression, freeze-drying followed by compression and extrusion, as it is illustrated in **Figure 2.1**. In all the cases, the protein was dried for 24 h in an air-circulating circulating oven at 105 ºC before processing.

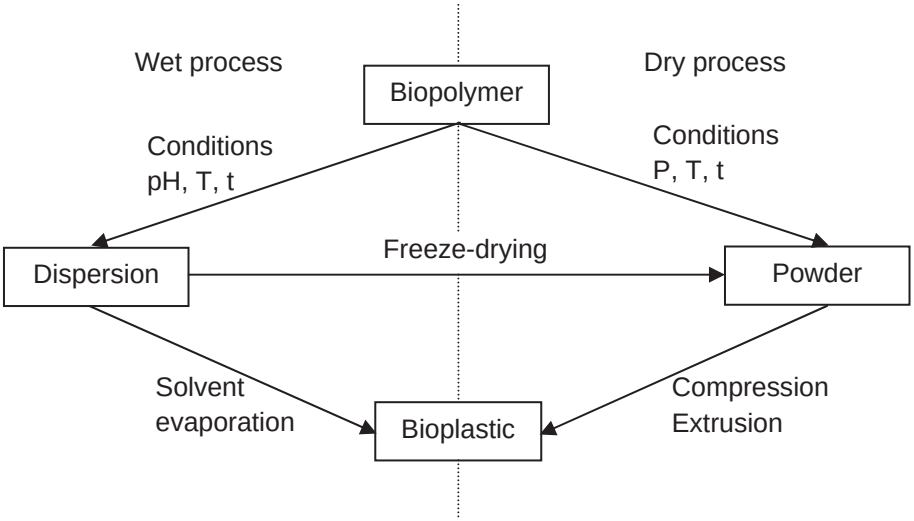


Figure 2.1**.** Processing methods.

All samples were conditioned in a controlled bio-chamber (ACS Sunrise 700V) at 25 ºC and 50% relative humidity for 48 h before testing.

2.3. Physico-chemical characterization

2.3.1. Moisture Content (MC) and Total Soluble Matter (TSM)

TSM was expressed as the percentage of film dry matter solubilised after 24 h immersion in distilled water. Two methods of determination were used and compared. One of them was previously used in some studies of films from proteins (Cuq et al., 1996; Guerrero et al., 2011; Kunte et al., 1997). Using the first method (method 1), three specimens of each sample were weighed (m_w) and subsequently dried in an air-circulating oven at 105 ºC for 24 h. After this time, samples were reweighed (m_0) to determine MC values. Afterwards, samples were immersed in 30 mL of distilled water in the presence of sodium azide (0.02%) in order to prevent the microbial growth. The beakers were stored in an environmental chamber at 25 ºC for 24 h with occasional gentle

stirring. After this time, specimens were dried in an air-circulating oven at 105 ºC for 24 h and weighed (m_f). Using the second method (method 2), dry matter and soluble matter were not determined on the same sample in an effort to avoid heating sample prior to immersion in water. Instead, three samples were directly immersed in water and beakers were stored in environmental chamber at 25 ºC for 24 h with occasional gentle stirring to determine soluble dry matter. Initial dry matter values needed for TSM calculation were the ones obtained from MC measurements for the same sample.

MC and TSM values were calculated as:

$$MC(\%)=\frac{m_w-m_0}{m_w}\times 100 \qquad TSM\ (\%)=\frac{m_0-m_f}{m_0}\times 100$$

2.3.2. Fourier Transformed Infrared (FTIR) spectroscopy

FTIR spectra of pure components, films and extrudated samples were carried out on a Nicolet Nexus FTIR spectrometer using ATR Golden Gate (Specac). A total of 32 scans were performed at 4 cm^{-1} resolution. The measurements were recorded between 4000-400 cm^{-1}.

2.3.3. X-Ray Diffraction (XRD)

XRD studies of powders were performed with a diffraction unit (PANalytical Xpert PRO) operating at 40 kV and 40 mA. The radiation was generated from a Cu-Kα (λ = 1,54060 Á) source. The diffraction data were collected from 2θ values from 2.5º to 50º, where θ is the angle of incidence of the X-ray beam on the sample.

2.4. Optical and morphological properties

2.4.1. Light absorption

The light-barrier properties of films were determined by measuring their light absorption at wavelengths ranging from 200 nm to 800 nm, using a UV-

Jasco spectrophotometer (Model V-630). The transparency of the films was calculated by the equation A_{600}/T, where A_{600} is the absorbance at 600 nm and T is the film thickness (mm). Three specimens were tested for each composition.

2.4.2. Colour measurements

Colour values of the films were measured using a portable colorimeter (CR-400 Minolta Croma Meter). Film specimens were placed on a white plate, and the CIELAB colour scale was used to measure colour: L* = 0 (black) to L*= 100 (white), -a* (greenness) to +a* (redness), and -b* (blueness) to +b* (yellowness). Standard values for the white calibration plate were L*= 97.39, a*= 0.03, and b*= 1.77. Considering standard light source D65 and standard observer 2 degrees, colour parameters L*, a*, b* were measured. Chrome (C^*_{ab}) and hue angle (h^*_{ab}) were calculated from:

$$C^*=\sqrt{(a^*)^2+(b^*)^2}$$

$$h^*=\text{arctg}\frac{b^*}{a^*}$$

The whiteness index (WI) and total colour differences (ΔE^*) with respect to the control film were also calculated as:

$$WI=100-\sqrt{(100-L^*)^2+(a^*)^2+(b^*)^2}$$

$$\Delta E^*=\sqrt{(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2}$$

Values were expressed as the means of ten measurements on different areas of each film.

2.4.3. Scanning electron microscopy (SEM)

The morphology of the fracture surface of the extruded pellets was visualized using a field emission scanning electron microscope (Hitachi S-4800) at an acceleration voltage of 15 kV. Samples were fractured under liquid nitrogen prior to morphology visualization. The fracture surfaces were mounted on a metal stub with double-side adhesive tape and coated under vacuum with gold (JFC-1100) in an argon atmosphere prior to observation.

2.5. Thermal properties

2.5.1. Differential Scanning Calorimetry (DSC)

DSC experiments were performed using a Mettler Toledo DSC 822. Sample weights were in the range of 3 mg, and all runs were carried out at a heating rate of 10 ºC/min from -50 ºC up to 250 ºC. Experiments were carried out under nitrogen atmosphere, and the nitrogen gas flow employed was 10 mL/min. Sealed aluminium pans were used to prevent mass loss during the experiment.

2.5.2. Thermogravimetric Analysis (TGA)

Thermal stability of films was analysed by thermogravimetric analysis (TGA). Non-isothermal degradation measurements were performed in a Mettler Toledo TGA SDTA 851. Tests were running from room temperature up to 600 ºC at a heating rate of 10 ºC/min under nitrogen atmosphere (10 mL/min) to avoid thermo-oxidative reactions.

2.6. Barrier properties

2.6.1. Contact angle determination

A contact angle meter (model Oca20, dataphysics instruments) was used to perform contact angle measurements on the surface of films. A film sample (20 mm x 80 mm) was put on a movable sample stage and levelled

horizontally; then a drop of about 3 µL of distilled water was placed on the surface of the film using a microsyringe. The contact angle was measured in a conditioned room by recording contact angle values. Image analyses were carried out using SCA20 software. Ten replicates were made per formulation.

2.6.2. Water Vapour Permeability (WVP)

WVP of the films was determined according to ASTM E96-00 (ASTM, 2000). The film was cut into a circle of 7.40 cm diameter and the test area was 33 cm^2. The setup was subjected to a temperature and relative humidity of 38 ºC and 90%, respectively. Water vapour transmission rate (WVTR) was calculated as:

$$WVTR\left(\frac{g}{s \cdot cm^2}\right) = \frac{G}{t \cdot A}$$

where, G is the change in weight (g), t is the time (s), and A is the test area (cm^2). WVP was calculated as:

$$WVP\left(\frac{g}{cm \cdot s \cdot Pa}\right) = \frac{WVTR \cdot T}{\Delta P}$$

where T is the thickness of the test specimen (cm) and ΔP is the partial pressure difference of the water vapour across the film (Pa). WVP for three specimens of each sample was calculated and reported.

2.6.3. Oxygen Permeability (OP)

Oxygen permeability was measured under controlled conditions (50% relative humidity, 25 °C) according to the method of Papkovsky (2000). Film was mounted between the upper lid and rubber ring with silicon lubricant and fixed to the lower cup by screws with an oxygen sensor housed inside. Nitrogen gas was blown into the chamber through one pipe, while leaving the other pipe open to evacuate the chamber until the nitrogen reading became

stable. Both pipes were then shut. The sensor measured the increase in oxygen content over time during the testing period. Data was graphed and oxygen permeability was calculated using the following equation:

$$OP = [S/(60 \cdot \beta) \cdot (V/20.5) \cdot (273/298) \cdot (T \cdot 1000)/A]/101.625 \cdot 10^{-9}/24$$

where OP = oxygen permeability ($cm^3 \cdot \mu m/m^2 \cdot d \cdot kPa$), S = slope indicating the transmission rate of oxygen, β = permeability coefficient (4.776), a constant value in this study as all treatments were conducted at the same conditions (50 ± 3% relative humidity, 23 ± 2 °C), A = surface area of the film (m^2), T = thickness of the test film samples (μm), V = volume of the chamber (mL).

2.7. Mechanical properties

2.7.1. Tensile testing

An electromechanical testing system (MTS Insight 10) was used to determine mechanical properties. Tensile strength and elongation at break were determined according to ASTM D1708-93 (ASTM, 1993). Bone-shaped specimens (4.75 mm wide and 22.25 mm long) were cut. Five replicates were tested for each composition.

2.7.2. Puncture testing

Puncture tests were performed to determine puncture strength and deformation. Films were fixed in a 2.6 cm diameter cell and were perforated to breaking point using a Mecmesing Imperial 2500 testing instrument with a round-ended stainless steel plunger of 3 mm diameter and with a cross-head speed of 60 mm/min. Puncture strength and deformation were determined using Emperor software. Five replicates were tested for each composition.

2.8. Statistical analysis

The data were subjected to one-way analysis of variant (ANOVA) by means of an SPSS computer program (SPSS Statistic 17.0). Post hoc multiple

comparisons were determined by the Duncan and Tukey´s test with the level of significance set at $P < 0.05$.

EFFECT OF PROCESSING METHOD

Published as: Guerrero P., Retegi, A., Gabilondo, N., de la Caba, K., 2010. Mechanical and thermal properties of soy protein films processed by casting and compression. *Journal of Food Engineering* 100 (1), 145-151.

3. Effect of processing method

3.1. Summary

Soy protein plastics without any additive have a brittle behaviour, which makes processing difficult (Lian et al., 1999). Addition of plasticizers is an effective way to obtain flexible SPI-based films. It is known that plasticizers with characteristics such as small size, high polarity, more than one polar group per molecule, generally impart great plasticizing effect on polymeric systems. Currently, biopolymeric films are usually plasticized by hydroxyl compounds (Cao et al., 2009). Glycerol has a high boiling point and good stability and is regarded as one of the most efficient plasticizers for soy protein plastics.

Three theories have been proposed to explain the mechanism of the plasticizer effect (Sears and Darby, 1982). According to the lubricity theory, a plasticizer is considered as a lubricant to facilitate the movements of the macromolecules over each other; related to the gel theory, a plasticizer disrupts the polymer-polymer interactions including hydrogen bonds and van der Waals and ionic forces; and in accordance with the free volume theory, a plasticizer may depress the glass transition temperature by increasing polymer free volume. The fundamental concept underlying these theories is that a plasticizer can interpose itself between the polymer chains and decrease the forces holding the chains together (Entwistle and Rowe, 1979).

In this chapter, we analysed the final properties of glycerol-plasticized soy protein films prepared by casting and compression. Also, freeze-drying followed by compression has been employed in order to analyze the effect of previous solution in water on the unfolding of protein, and thus on the mechanical properties. The effect of glycerol content was also studied. Results were related to data obtained by infrared analysis (FTIR), differential scanning calorimetry (DSC), and thermo-gravimetric analysis (TGA).

3.2. Film preparation

In the case of compression, SPI/glycerol mixtures with 70/30, 60/40 and 50/50 (w/w) compositions (designed as SPI30, SPI40, and SPI50) were manually mixed. Mixtures were intensively blended in a beaker for a period of about 5 min, and then thermally compacted using a caver laboratory press (Atlas^{TM}). About 1.3 g of SPI/glycerol mixture was placed between 2 sheets of aluminium (0.2 mm thick and 100 mm diameter). These sheets were placed between the platens of the press, which had been previously heated to 150 ºC. A pressure of 12 MPa was applied for 2 min. The plates were allowed to cool for 3 min before removing film samples. Then, samples were cut to the required gage dimensions for further testing.

In the case of processing by casting, film-forming solutions were prepared by mixing 125 mL of distilled water and 7.5 g of SPI and heated at 80 ºC at 150 rpm on a magnetic stirrer for 30 min. After that, glycerol was added to the solutions to obtain SPI30, SPI40, and SPI50 mixtures. The solutions so obtained were maintained at 80 ºC for other 30 min under stirring at 150 rpm. Subsequently, solution was separated in two equal fractions. One of them was poured into polystyrene Petri (140 mm diameter) which was kept at a conditioned room for 48 h to evaporate water. The other fraction was freeze-dried using and Alpha 1-4 LD freeze-dryer (Martin Chirst) and the powder so obtained was hot-pressed as described above.

3.3. Results and discussion

3.3.1. FTIR analysis

The FTIR spectrum of pure SPI is shown in **Figure 3.1.** The main absorption bands of peptide linkage are related to C=O stretching at 1630 cm^{-1} (amide I), N-H bending at 1530 cm^{-1} (amide II) and C-H deformation at 1430 cm^{-1}. The absorption band at 1230 cm^{-1} is attributable to the C-N stretching and N–H bending (amide III) (Schmidt et al., 2005). The band at 1100 cm^{-1} is

apparently formed by a contribution of different groups such as out of plane C-H bending (from aromatic structures).

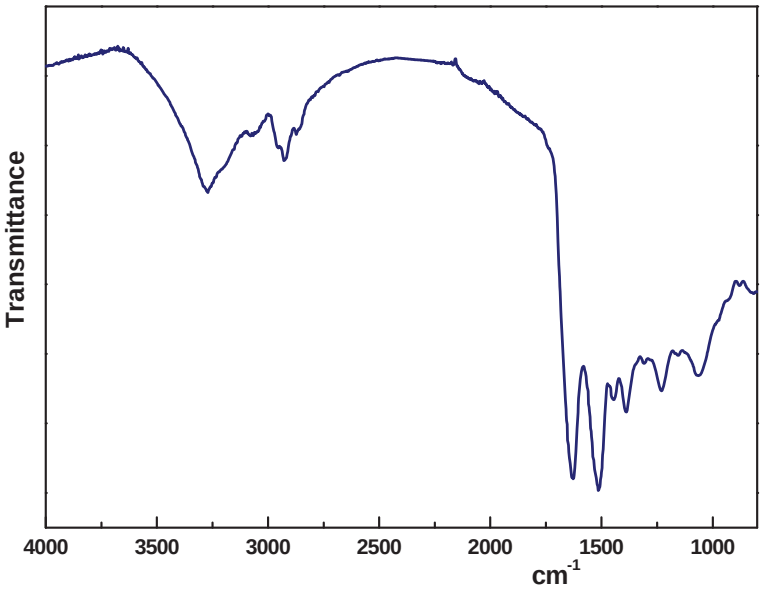


Figure 3.1. FTIR spectrum of SPI used in this study.

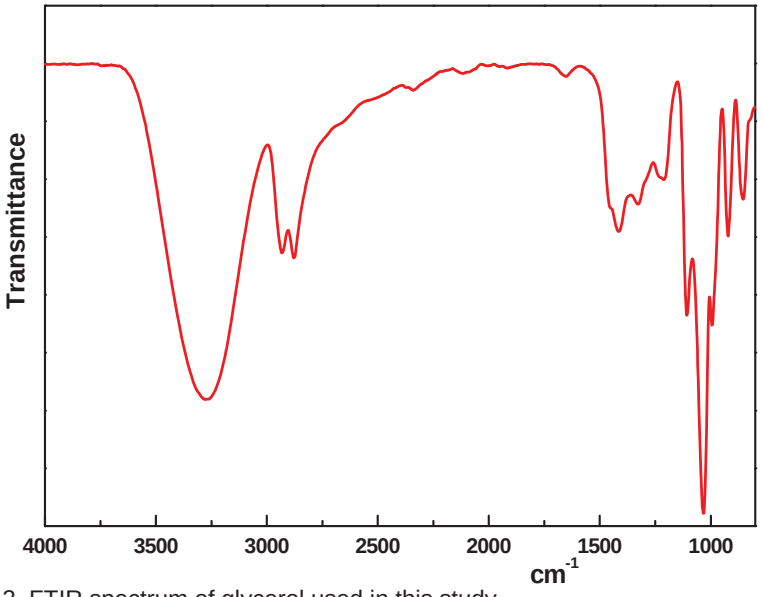


Figure 3.2**.** FTIR spectrum of glycerol used in this study.

The broad band observed in the 3500-3000 cm^{-1} range is attributable to free and bound O-H and N-H groups, which are able to form hydrogen bonding with the carbonyl group of the peptide linkage in the protein. Absorption peak at 2929 cm^{-1} is attributable to CH_2 asymmetrical stretching (Karnnet et al., 2005).

The FTIR spectral data of pure glycerol are show in **Figure 3.2**. Typical absorption bands of glycerol are located in the region from 800 cm^{-1} up to 1150 cm^{-1}, where five peaks corresponding to the vibrations of C-C and C-O linkages appear. The peaks at 850, 925, and 995 cm^{-1} are assigned to the vibration of the skeleton C-C, the band at 1045 cm^{-1} is associated to the stretching of the C-O linkage in C1 and C3, and the one at 1117 cm^{-1} is related to the stretching of C-O in C2.

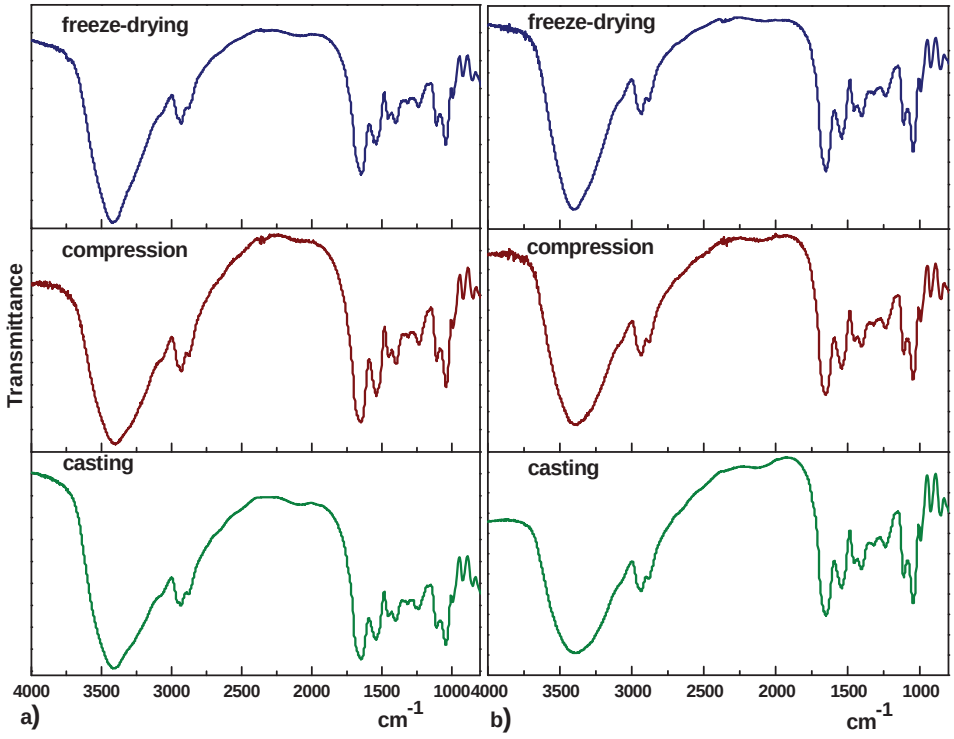


Figure 3.3. FTIR spectra of a) SPI30 and b) SPI40 films processed by casting, compression, and freeze-dried followed by compression.

Spectral data for SPI plasticized with 30 and 40% by weight of glycerol are shown in **Figure 3.3**. Comparing these spectra with the previous ones corresponding to pure SPI and glycerol, it can be seen that no change takes place in the characteristic peaks of both protein and glycerol. In particular, there is no change in the 1200-800 cm^{-1} region; the bands at 850, 925, 995, 1045 and 1117 cm^{-1} appear for SPI/glycerol system in the same position as in **Figure 3.2**. This fact indicates that glycerol does not react with the protein through covalent linkages. In the case of the films obtained by casting, it can be seen a broader peak corresponding to the hydroxyl group, which indicates a major moisture content in the films processed in solution.

3.3.2. Thermal properties

The major components of soy proteins are globular proteins, 7S (about 35%) and 11S (about 52%) (Kinsella, 1979; Kumar et al., 2008; Mori et al., 1981). Temperature, pressure and time are the main parameters in soy protein processing in order to denature the protein, unfold globular structure, and permit interaction and entanglement between protein chains to modify material properties (Denavi et al., 2009b; Ghanbarzadeh and Oromiehi, 2009; Hermansson, 1978; Thanh and Shibasaki, 1976). Similar unfolding could be obtained with high temperature and short time or low temperature and long time, although time-temperature relation must be optimized in order to avoid colour change from light to dark yellow (Mo et al., 1999). Therefore, inter- and intramolecular interactions will be significantly influenced by the processing temperature, pressure and time employed. **Figure 3.4** shows typical DSC thermograms for soy proteins, in which the two characteristic denaturation temperatures for 7S and 11S globulins are shown. The first peak at around 75 ºC corresponds to the denaturation of the lower molecular weight fraction (7S) and the second one at around 225 ºC is related to the high molecular weight fraction (11S).

These results are consistent with similar DSC curves that have been

obtained by other researchers (Hermansson, 1979; Kitabatake and Doi, 1990; Mo and Sun, 2002; Morales and Kokini, 1997; Ning and Villota, 1994; Tang et al., 2007). As it was also shown by these authors, denaturation temperatures of 7S and 11S globulins are strongly dependant on moisture content, shifting to higher values at low moisture contents. It seems that the different processing method employed did not affect the unfolding of the protein. Only a shift of the 7S peak to higher temperatures was observed when the dry method was used to process the films due to the low water content of the film.

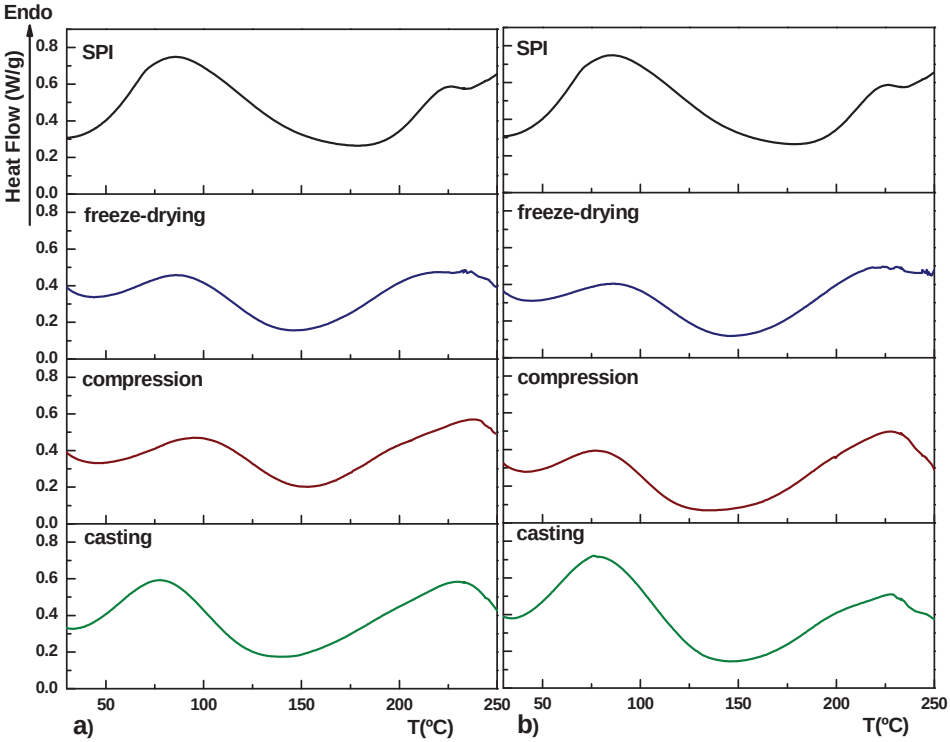


Figure 3.4. DSC thermograms of a) SPI30 and b) SPI40 films processed by casting, compression, and freeze-dried followed by compression.

The effect of plasticizer content on the thermal degradation of soy protein films was investigated by thermogravimetry. **Figure 3.5** shows the

weight loss as a function of temperature for pure SPI powder and for the protein films containing 30 and 40% by weight of glycerol.

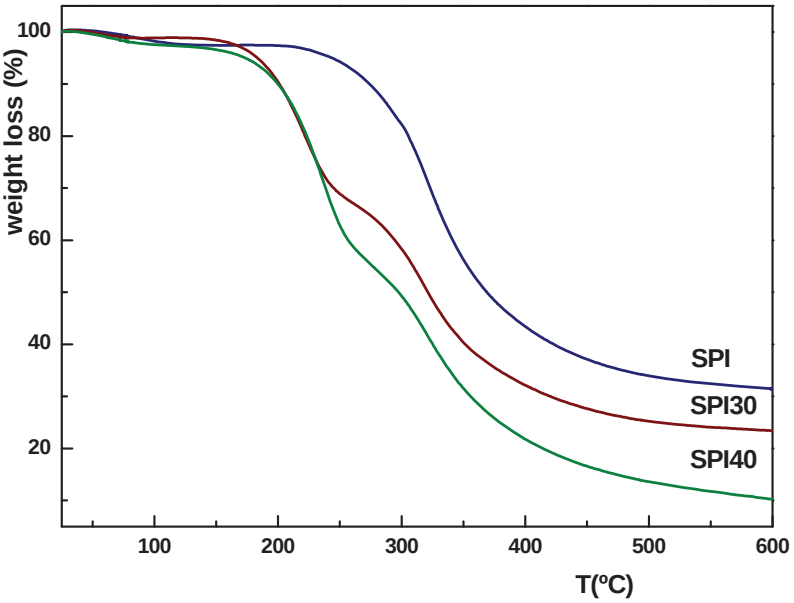


Figure 3.5. Thermogravimetric analysis of SPI films with 0, 30, and 40% by weight of glycerol.

For pure SPI films, there is a small weight loss at temperatures below 100 ºC, which is due to the loss of moisture. Above 100 ºC the rate of weight loss is small until 200 ºC, but starts to become significant above 225 ºC. These values are in good agreement with the ones obtained in others works (Ogale et al., 2000; Swain et al., 2005; Wang et al., 2007). For the plasticized films, the weight loss starts to become significant above 200 ºC. The higher weight loss of plasticized samples can be explained by the high vapour pressure of glycerol. It must be noted that the values of weight loss from TGA can not be used as absolute values in processing under pressure since weight loss during film formation at elevated pressures can be different from that at ambient or low pressures. Nevertheless, thermogravimetric analysis indicates that soy protein films exhibit substantial thermal degradation at temperatures above 180 ºC, so

that the processing temperature chosen has been 150 ºC, which is consistent with the temperature found to be the optimum processing temperature in other studies (Ogale et al., 2000).

3.3.3. Mechanical properties

The colour of the soy protein films changed from powderlike white yellow to transparent yellow when the glycerol content was increased, as it is shown in **Figure 3.6**. The films with glycerol content lower than 30% by weight were brittle, and it was no possible to cut samples for mechanical analysis. On the other hand, the films obtained with 50% by weight of glycerol were very sticky. However, films obtained with 30 and 40% by weight of glycerol are flexible and present good mechanical properties, as it is shown in **Table 3.1**. In these cases, glycerol acts as a plasticizer without forming any covalent linkages with SPI, as it was shown by FTIR analysis. However, glycerol can interact by hydrogen bonds with protein at amine, amide, carboxyl and hydroxyl sites, increasing the free volume of the system.

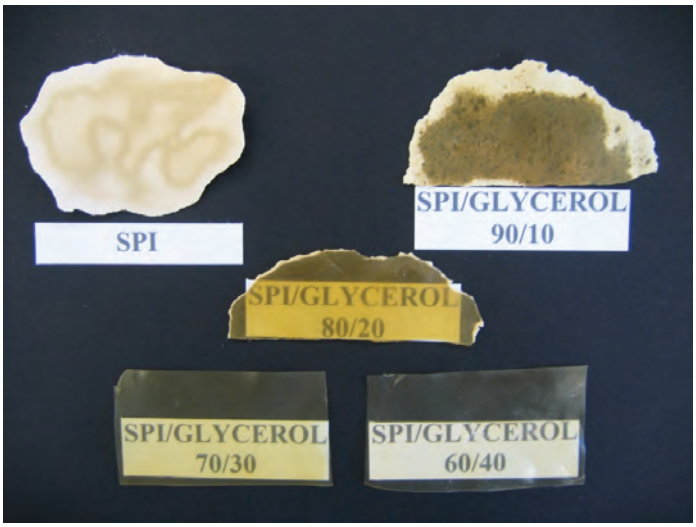


Figure 3.6. Visual aspect of SPI-based films obtained by compression with different glycerol contents.

Compression			
System	**E (MPa)**	**σ (MPa)**	**ε (%)**
SPI30	114.2±6.3[a]	7.8±0.5[a]	132.4±15.7[a]
SPI40	33.6±2.4[b]	3.8±0.2[b]	147.5±20.4[b]
SPI50	15.4±2.9[c]	2.9±0.4[c]	136.4±16.6[c]
Casting			
SPI30	112.4±5.3[a]	4.1±0.4[d]	105.4±13.3[d]
SPI40	23.6±2.6[d]	1.6±0.3[e]	145.5±22.6[b]
SPI50	12.4±3.7[e]	1.5±0.2[f]	170.2±18.5[e]
Freeze-drying			
SPI30	120.5±5.3[f]	7.7±0.6[a]	140.6±13.3[f]
SPI40	35.6±3.4[b]	3.9±0.3[b]	163.3±11.6[g]
SPI50	16.3±3.7[c]	2.8±0.3[c]	186.1±14.4[h]

Table 3.1. Mechanical properties of SPI-based films processed by compression, casting, and freeze-draying followed by compression with different glycerol contents. [a–h] Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Tukey's multiple range test.

The hydroxyl groups of glycerol could interact with amine and acid groups in the protein, so decreasing inter- and intramolecular interactions between protein chains, such as hydrogen bonds, and thus improving the motion ability of protein macromolecules, which results in the flexibility of materials. As a result, the behaviour of the films based on SPI changes from brittle to flexible, as it can be seen in **Figure 3.7.**

Soy proteins have polar and non-polar side chains, which promote strong inter- and intramolecular interactions, such as hydrogen bonding and dipole-dipole. The strong charge and polar interactions between side chains of soy protein molecules restrict segment rotation and molecular mobility, which lead to an increase of modulus (E) and tensile strength (σ). According to Krochta (Sothornvit and Krochta, 2001; Sothornvit et al., 2007), two kinds of events occur during the film formation. Firstly, during the heating phase, protein structure is disrupted, some native disulfide bonds are cleaved, and sulfhydryl and hydrophobic groups are exposed. Then, during the film drying phase, new hydrophobic interactions occur and also new hydrogen bonds are formed.

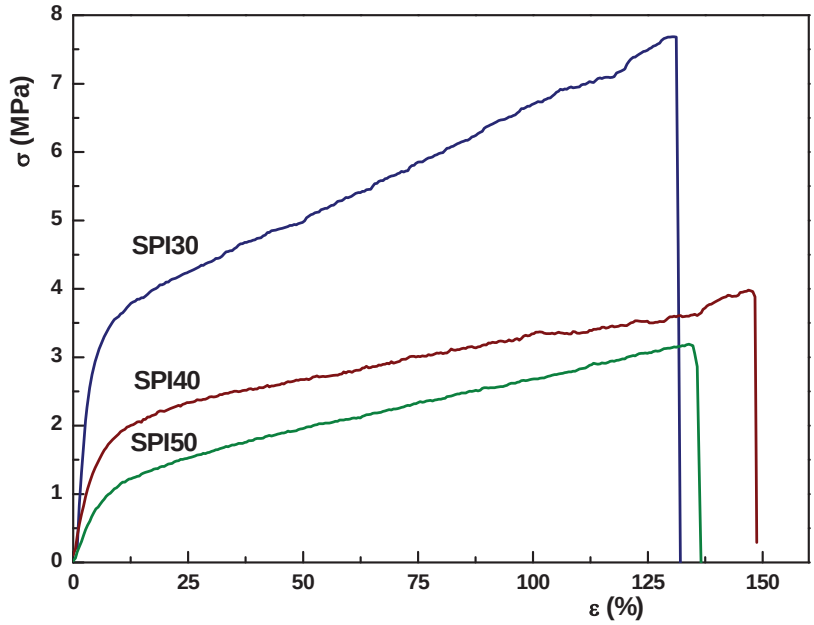


Figure 3.7. Mechanical properties of the films based on SPI with different glycerol content processed by compression.

There are several typical inter- and intramolecular interactions, such as hydrogen bond, disulfide-bond, dipole-actions, charge-charge, and hydrophobic interactions, in soy protein that are characteristic of natural proteins. According to the amino acid composition of SPI, hydrogen bonding occurs among -NH_2 (in arginine and lysine), -NH- (in proline and histidine), -OH (in tyrosine, threonine, and serine), -COOH (in glutamic acid), and peptide bonds. It seems that the density and strength of the interactions are greatly different at specific locations in SPI molecules and, as a result, soy protein molecules contain different regions with distinct abilities to accept glycerol molecules (Knubovents et al., 1999). The use of plasticizers to break intermolecular linkage that stabilizes the protein in their primitive structure makes the protein chains mobile. The orientation and restructuring of the chains as well as the formation of new intermolecular linkage stabilize the three-dimensional network formed.

Increasing the amount of glycerol causes a decrease of tensile strength (σ) and an increase of elongation at break (ε) due to the fact that glycerol reduces the interactions between protein chains, so increasing chain mobility (Osés et al., 2009; Sothornvit et al., 2007; Wang et al., 1996; Zhang et al., 2001). Nevertheless, when the amount of glycerol added is 50% by weight no further improvement of elongation is observed. Taking the above in consideration, the effect of the processing method in the mechanical properties of the SPI40 films is shown in **Figure 3.8**.

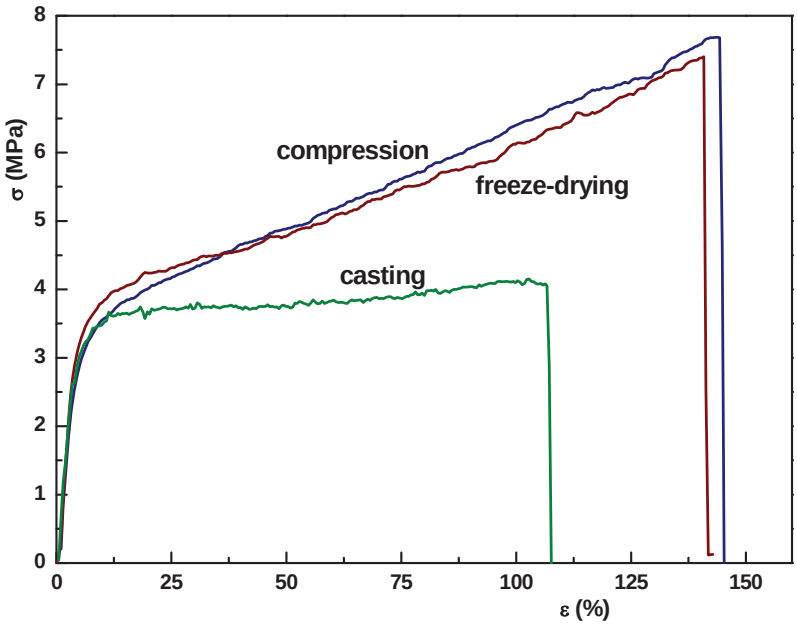


Figure 3.8. Effect of the processing method in the mechanical properties for SPI30 system.

Films obtained by compression, as well as the ones freeze-dried and further hot-pressed, show similar tensile strength and elongation at break. No difference has been found between the films prepared by manual mixing and the ones prepared by dispersion in water while heating, so it can be said that the diffusion of glycerol in the protein is independent from the system employed

to mix the protein and the plasticizer. In contrast, the films obtained by casting show the worst mechanical behaviour, in other words, the lower resistance and elongation at break.

3.4. Conclusions

SPI films with glycerol content between 30 and 40% by weight are the most highly recommended in order to improve mechanical properties. A lower amount of plasticizer produced brittle films and a higher content resulted in sticky films. The optimum film-processing method was compression which enhanced both tensile strength and elongation at break. Moreover, this method allowed a much shorter period of time for film preparation and the use of conventional techniques which are more convenient for industrial applications than casting.

EFFECT OF PROCESSING CONDITIONS

Published as: Guerrero P., de la Caba, K., 2010. Thermal and mechanical properties of soy protein films processed at different pH by compression. *Journal of Food Engineering* 100 (2), 261-269.

4. Effect of processing conditions

4.1. Summary

Functional properties reflect the intrinsic physical attributes of the protein per se (composition, conformation, structure) as affected by interactions with additives and the environment. Therefore, the knowledge of fundamental properties of proteins is essential for understanding the functional ones, for modifying proteins to acquire needed functionality, and for predicting potential applications. The behaviour of proteins is determined by its amino acid sequence and composition, molecular size conformation, charge distribution, and the extent of inter- and intramolecular bonding but also processing conditions. In globular proteins, the nature of the inter- and intramolecular forces is responsible for molecular stability under certain conditions (temperature, pH), which govern functional properties. Both covalent and non-covalent forces (hydrophobic interactions, hydrogen bonding, electrostatic attractions) are involved in protein-protein, and protein-solvent interactions which influence the overall functional properties.

There are two processes to prepare protein films. The most commonly used process is solution casting, but hot-pressing moulding, which has not been widely used in the case of soy protein, is a more convenient one for industrial scale. In the previous chapter, it was shown that SPI-based films prepared by compression had better mechanical properties than the ones prepared by casting (Guerrero et al., 2010), so in this chapter, we analysed the final properties of glycerol-plasticized soy protein films prepared at different pHs and processed by freeze-drying followed by compression. The effect of glycerol content was also studied. Results were related to data obtained by infrared analysis (FTIR), differential scanning calorimetry (DSC), and thermo-gravimetric analysis (TGA).

4.2. Film preparation

SPI was dispersed in distilled water and heated at 80 ºC at 150 rpm on a magnetic stirrer for 30 min and then glycerol was added to the dispersion to obtain SPI/glycerol mixtures with the desired composition, as described in the previous chapter. The dispersions were maintained at 80 ºC for other 30 min under stirring at 150 rpm. The pH of the dispersions was appropriately adjusted with HCl (0.1 M) and NaOH (0.1 M). These samples were freeze-dried using Alpha 1-4 LD freeze-dryer (Martin Chirst). The powder obtained was thermally compacted using a caver laboratory press (Atlas^{TM}). SPI was placed between two sheets of aluminium (0.2 mm thick and 100 mm diameter). These sheets were placed between the platens of the press, which had been previously heated to 150 ºC. A pressure of 12 MPa was applied for 2 min. The platens were allowed to cool for 3 min before removing film samples.

Sample	Glycerol (wt %)	pH	Visual aspect
SPI-1.4	0	1.4	Inhomogeneous, transparent, brown brittle film
SPI-4.6	0	4.6	Inhomogeneous, opaque, beige brittle film
SPI-7.5	0	7.5	Inhomogeneous, opaque, beige brittle film
SPI-10.0	0	10.0	Inhomogeneous, opaque, slightly green brittle film
SPI-11.0	0	11.0	Inhomogeneous, opaque, slightly green brittle film
SPI30-1.4	30	1.4	Homogeneous, transparent, dark yellow brittle film
SPI30-4.6	30	4.6	Homogeneous, transparent, dark yellow brittle film
SPI30-7.5	30	7.5	Homogeneous, transparent, slightly yellow flexible film
SPI30-10.0	30	10.0	Homogeneous, transparent, slightly yellow flexible film
SPI40-1.4	40	1.4	Homogeneous, transparent, dark yellow brittle film
SPI40-4.6	40	4.6	Homogeneous, transparent, dark yellow brittle film
SPI40-7.5	40	7.5	Homogeneous, transparent, slightly yellow flexible film
SPI40-10.0	40	10.0	Homogeneous, transparent, slightly yellow flexible film

Table 4.1. Sample references for the compositions and conditions employed in this study.

4.3. Results and discussion

Films prepared without glycerol were not homogenous and were extremely brittle, so that it was no possible to cut samples for mechanical analysis. They were also opaque, except SPI-1.4, which was brown but

transparent, as it can be seen in **Figure 4.1**.

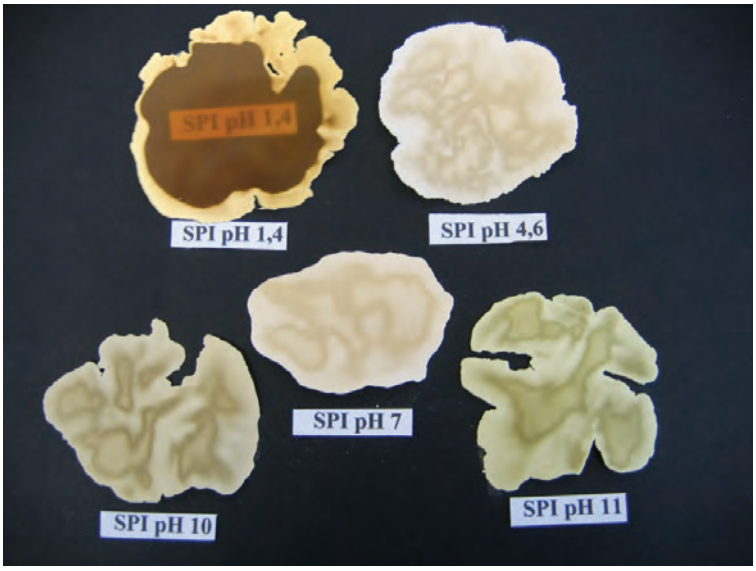


Figure 4.1. SPI films prepared at different pHs, freeze-dried and hot-pressed.

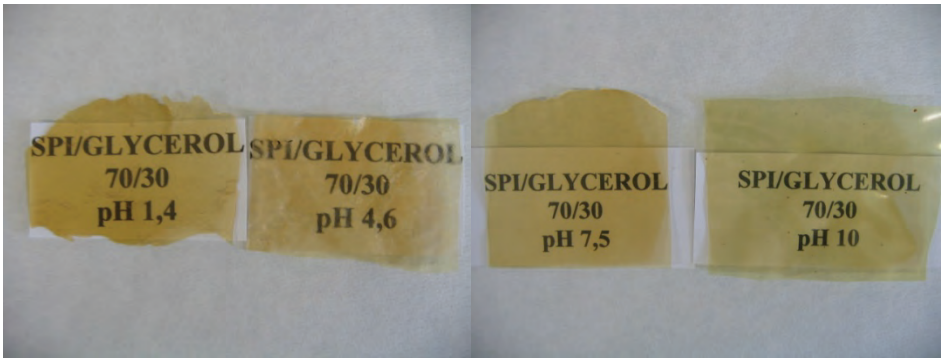


Figure 4.2. SPI/glycerol films prepared at different pHs, freeze-dried and hot-pressed.

In the case of SPI/glycerol systems, all the films obtained at different pHs were homogeneous, transparent and flexible. The colour of the films changed from dark yellow to slightly yellow when the pH was increased, as it is shown in **Figure 4.2**

4.3.1. FTIR analysis

The FTIR spectra of SPI at different pHs are shown in **Figure 4.3.** As cited in the previous chapter, the main absorption peaks related to SPI correspond to C=O stretching at 1630 cm^{-1} (amide I), N-H bending at 1530 cm^{-1} (amide II) and C-N stretching (amide III) at 1230 cm^{-1}.

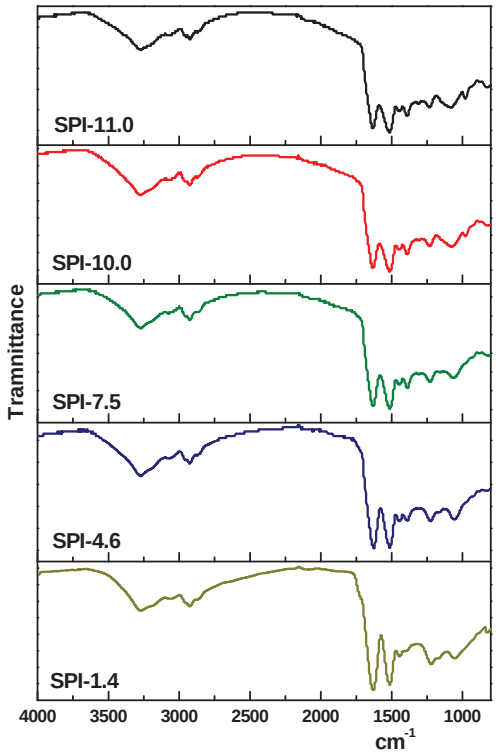


Figure 4.3**.** FTIR spectra of SPI films processed at different pHs by freeze-drying followed by compression.

It can be seen that the band at 1230 cm^{-1} does not show any change as a function of the pH employed. However, the intensity of the band at 1630 cm^{-1} (amide I) is higher than the one at 1530 cm^{-1} (amide II) for SPI-1.4. These two bands have the same intensity at the isoelectric pH, but at basic pHs the intensity of the band at 1530 cm^{-1} (amide II) becomes higher than the one at

1630 cm^{-1} (amide I). The conditions used for film preparation influence the protein structure and it seems that basic pHs favour or induce a certain degree of protein unfolding.

Spectral data for SPI plasticized with 30 and 40% by weight of glycerol are shown in **Figure 4.4**. Comparing these spectra with the previous ones corresponding to pure SPI at different pHs, it can be seen that no changes take place in the characteristic peaks of both protein and glycerol.

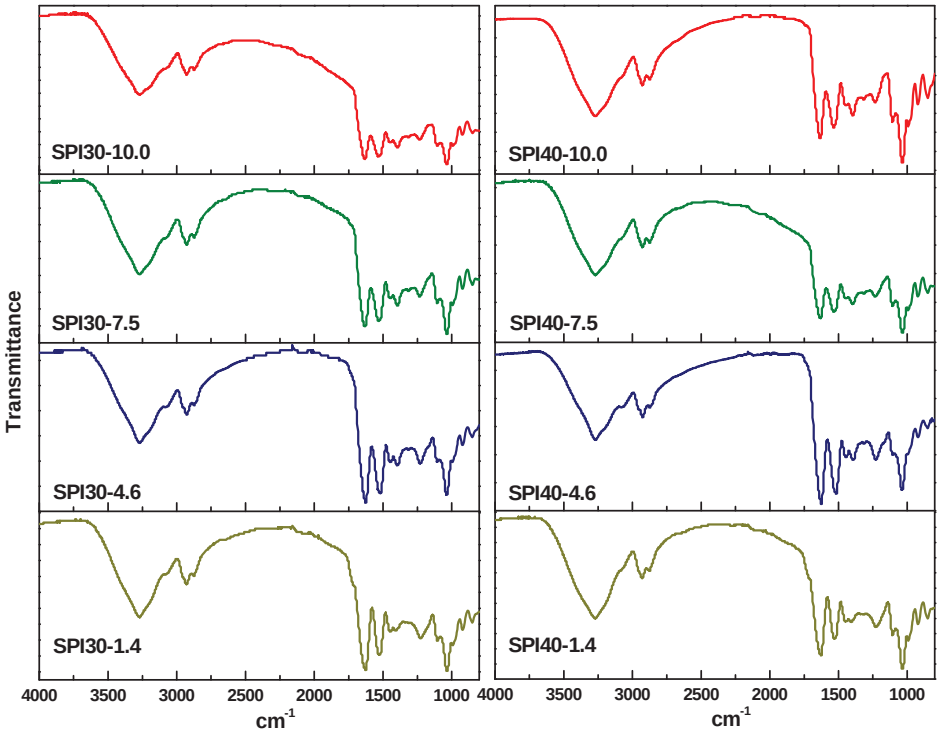


Figure 4.4. FTIR spectra of SPI30 and SPI40 films processed at different pHs by freeze-drying followed by compression.

This fact indicates that glycerol does not react with the protein through covalent linkages. In the case of SPI/glycerol systems, the intensity of the band at 1630 cm^{-1} (amide I) is always higher than the one at 1530 cm^{-1} (amide II) but

the difference of intensity in these two bands become smaller when the pH is increased, which could indicate that N-H groups in SPI and O-H groups in glycerol are certainly able to form inter- and intramolecular hydrogen bonding at basic pHs when the protein unfolding is optimal.

4.3.2. Thermal properties

Differential Scanning Calorimetry (DSC) has been widely used to characterize the thermal properties of food proteins, including heat-induced denaturation.

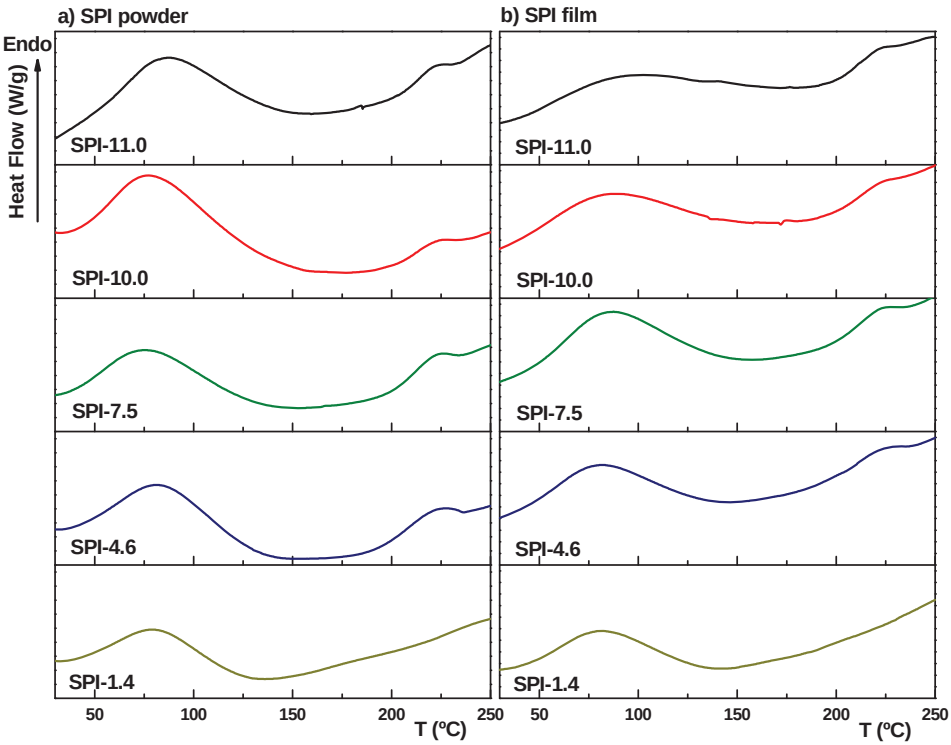


Figure 4.5. DSC thermograms at different pHs a) for SPI freeze-dried powder and b) for SPI films processed by freeze-drying followed by compression.

The denaturation process is an intramolecular change involving the destruction of internal order, and in some cases, the complete unfolding of

protein chains with the formation of so-called “random coils”. Heating can change soybean protein from its native state to a denatured one, accompanied by unfolding and disruption of the intramolecular bonding (Kitabatake et al., 1990), which is observable as an endothermic peak. In **Figure 4.5** the two denaturation peaks of SPI can be observed: the one at 77 ºC, corresponding to the low molecular weight 7S globulin, and the one at 227 ºC, related to the high molecular weight 11S globulin.

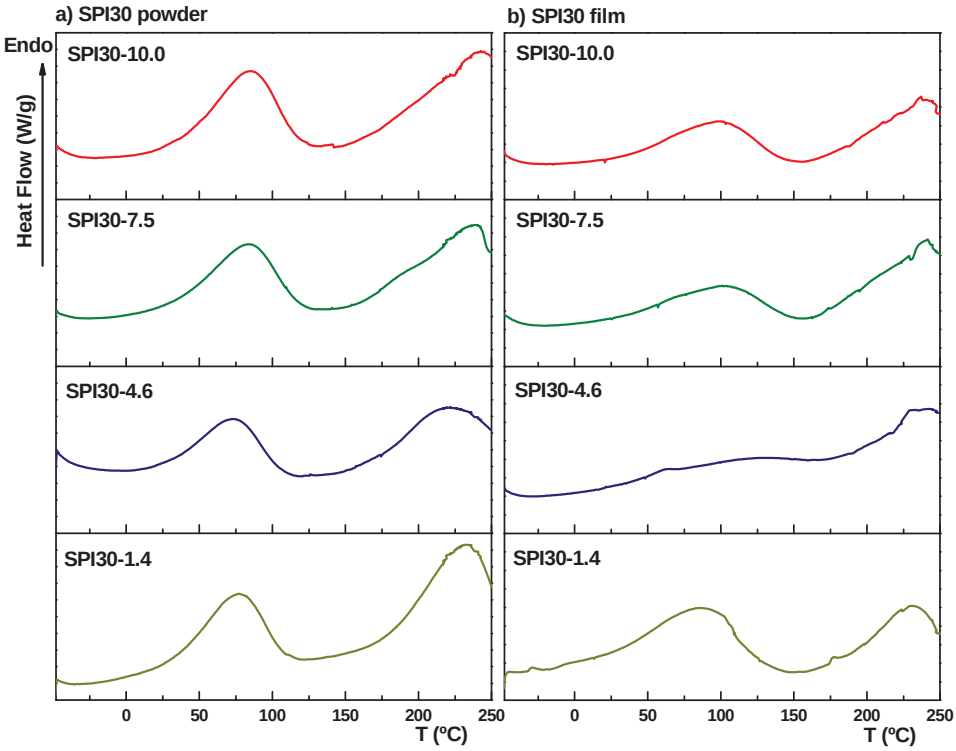


Figure 4.6**.** DSC thermograms at different pHs a) for SPI30 freeze-dried powder and b) for SPI30 films processed by freeze-drying followed by compression.

Denaturation temperature of 11S globulin for the plasticized systems, 240 ºC, was higher than that for the unplasticized ones, as it is shown in

Figure 4.6. Comparing **Figure 4.5** with **Figures 4.6** and **4.7**, it can be seen that the endothermic peak associated to the 7S globulin almost disappeared, which indicates that the process of compression (heat and pressure) causes denaturation of the lower molecular weight globulin fraction. DSC studies showed that proteins were extensively denatured in SPI/glycerol films at the isoelectric point and were partially denatured in SPI/glycerol films at the rest of pHs.

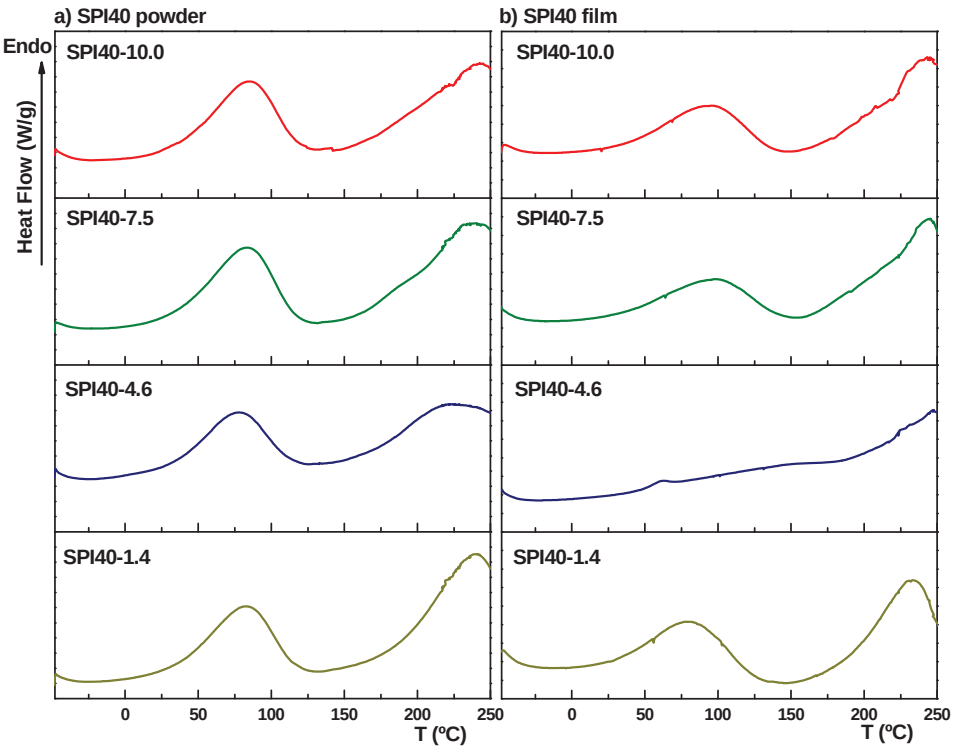


Figure 4.7. DSC thermograms at different pHs a) for SPI40 freeze-dried powder and b) for SPI40 films processed by freeze-drying followed by compression.

The effect of pH on the thermal degradation of soy protein films plasticized with different contents of glycerol was investigated by

thermogravimetry. **Figure 4.8** shows the weight loss as a function of temperature for pure SPI processed at different pHs. It can be seen that the weight loss starts to become significant above 225 ºC independent of the pH employed. For the plasticized films, **Figures 4.9** and **4.10** show that the weight loss starts to become significant above 200 ºC, as it was shown in the third chapter. This result is also independent of the pH used in the processing.

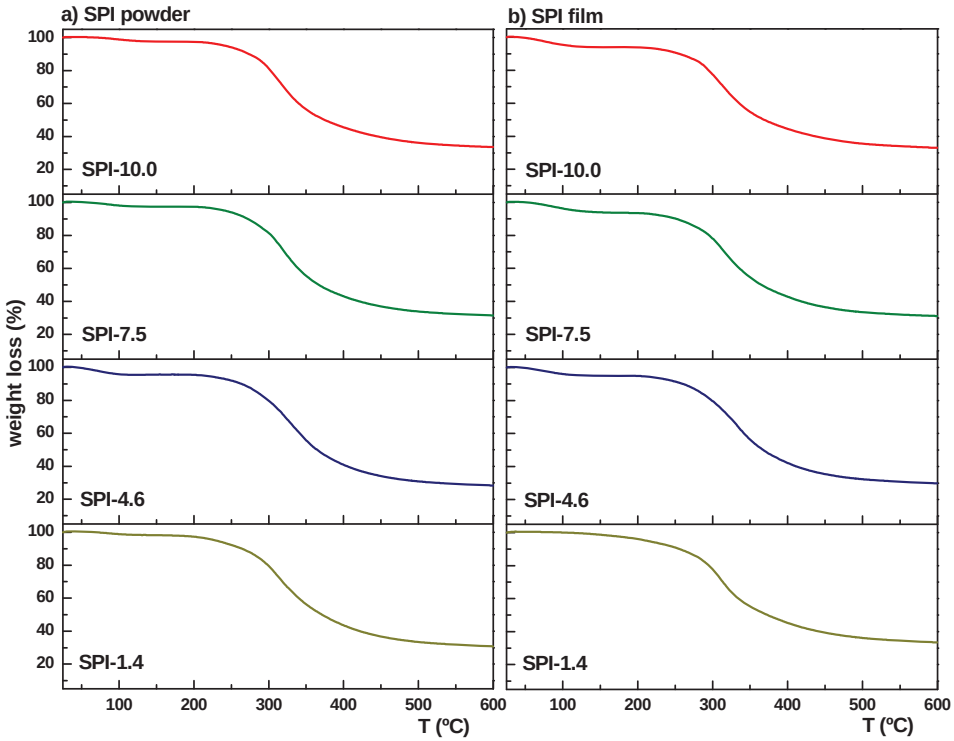


Figure 4.8**.** Thermo-gravimetric analysis at different pHs a) for SPI freeze-dried powder and b) for SPI films processed by freeze-drying followed by compression.

Comparing **Figures 4.8-4.10**, it can be said that the behaviour is similar after compression and when glycerol content is increased (**Figures 4.9** and **4.10**), although the two-step degradation is more clearly observed for SPI40 systems.

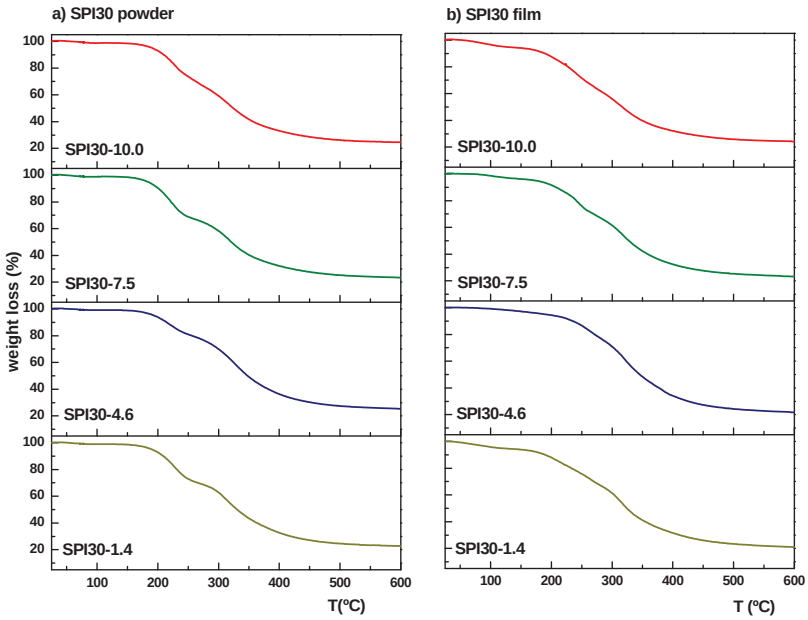


Figure 4.9. Thermo-gravimetric analysis at different pHs a) for SPI30 freeze-dried powder and b) for SPI30 films processed by freeze-drying followed by compression.

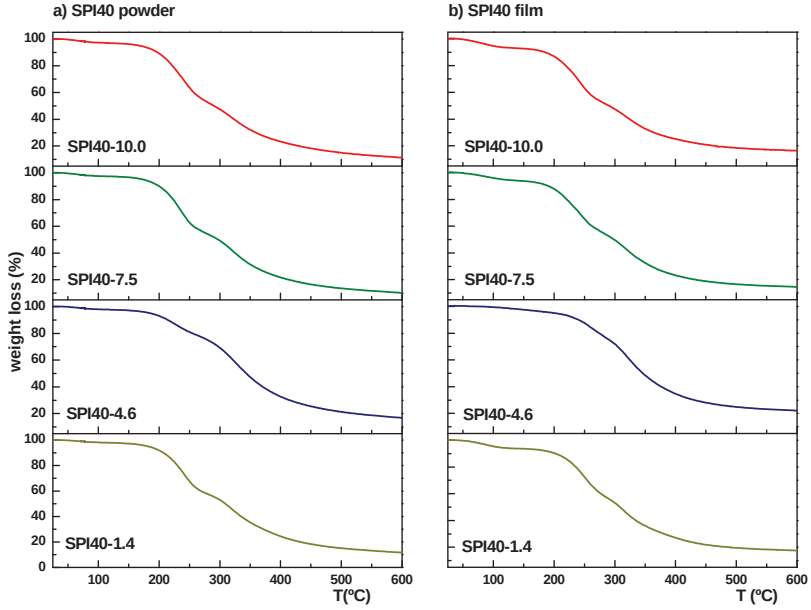


Figure 4.10**.** Thermo-gravimetric analysis at different pHs a) for SPI40 freeze-dried powder and b) for SPI40 films processed by freeze-drying followed by compression.

4.3.3. Mechanical properties

As it was shown in the previous chapter, glycerol is compatible with soybean protein. Because of its small molecular size, it fits easily into the protein chains and establishes hydrogen bonds with the polar groups. As a result, the protein-protein interactions decreased owing to the increased plasticizer-protein interactions and films with both 30 and 40% by weight of glycerol are flexible and present good mechanical properties.

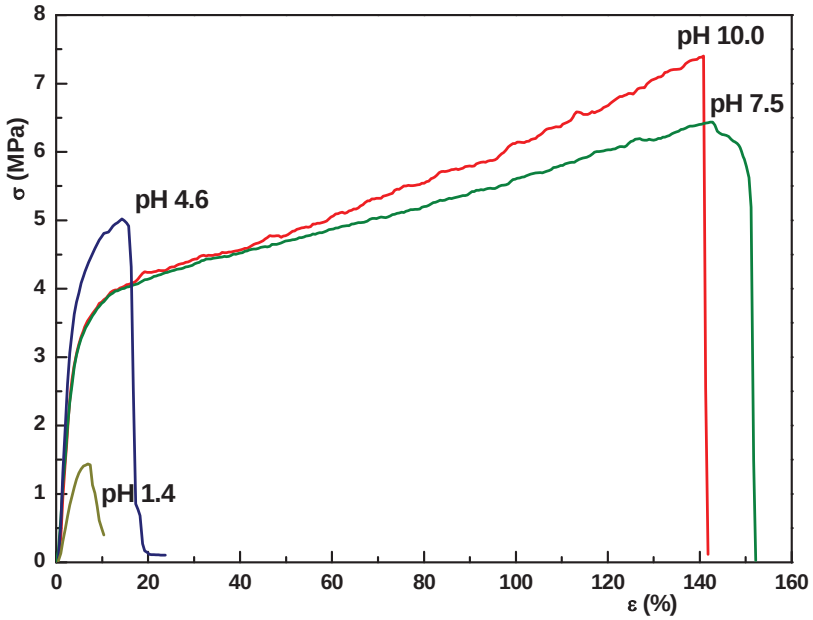


Figure 4.11**.** Tensile stress-strain curves for SPI30 films processed at different pHs.

Mechanical behaviour for SPI30 system at different pHs is shown in **Figure 4.11**. It can be seen that both elongation at break and tensile strength increased when the pH of initial dispersion was increased. Films prepared at acid pHs showed the most brittle behaviour, while the ones at pH 7.5 and pH 10.0 showed the best mechanical properties: the highest elongation and tensile strength. It is believed that alkaline pHs promote formation of inter- and intramolecular interaction (Cheftel et al., 1985).

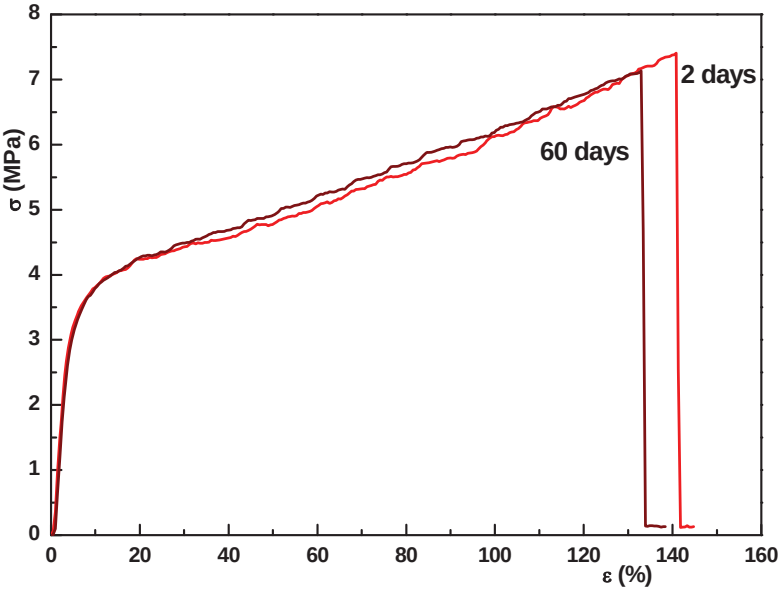


Figure 4.12. Tensile stress-strain curves for SPI30 films processed at pH 10.0 and stored at 25 ºC and 50% RH for 60 days.

Presumably, such interactions contributed to the increased toughness observed at the basic pHs employed in this study. It is worth to note that these interactions are stable along time because the mechanical properties of samples do not change after being stored at 25 ºC and 50% relative humidity for 60 days, as it is shown in **Figure 4.12**.

4.4. Conclusions

The use of freeze-drying allowed us to modify protein structure at different pHs and process films by compression. It was probed that the best mechanical properties were obtained when basic pHs were used due to a major unfolding of the protein which allowed the exposition of the polar groups to be able to interact with small glycerol molecules. The differences in the protein-glycerol interactions as a function of pH were seen by FTIR, which showed changes in the intensity of bands related to amide I and II. The effect

of heat and pressure after freeze-drying was also observed by DSC with the disappearance of the peak corresponding to low molecular weight globulins, which indicates denaturation of protein. Moreover, it is worth to note that final properties remained invariable after two months of storage, which is of crucial importance for packaging purposes.

EFFECT OF GELATIN ADDITION

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5. Effect of gelatin addition

5.1. Summary

There are different strategies to improve the functional properties of films: plasticizers addition, processing optimization and blending. Once optimized plasticizer content and processing conditions in the two previous chapters, SPI-based films, blended with gelatin, and plasticized with glycerol at pH 10, were prepared by compression moulding.

Gelatin is a soluble protein obtained by partial hydrolysis of collagen, the main fibrous protein constituent in bones, cartilages and skins; therefore, the source of collagen is an intrinsic factor influencing the properties of gelatins (Gómez-Guillén, et al., 2011). Gelatin quality for a particular application depends largely on its rheological properties (Stainsby, 1987). Apart from basic physicochemical properties, such as composition parameters, solubility, transparency and colour, the main attribute that best define the overall commercial quality of gelatin is the gel strength, which is expressed in the normalized bloom value (Wainewright, 1977).

In this chapter, the influence of gelatin type and content on mechanical properties and water uptake were analyzed and results were explained in relation to the interactions between soy protein and gelatin observed by FTIR analysis. Appearance of films was also analyzed and it was observed that transparency and colour were maintained when gelatin was added.

5.2. Film preparation

SPI-based films with different gelatin contents (5, 10, and 15% by weight on SPI dry basis) were prepared by dispersing 5 g of SPI and the amount of gelatin required to obtain the desired percentage in 100 mL distilled water. Dispersions were heated at 80 ºC for 30 min and stirred at 150 rpm. Then, glycerol was added as plasticizer at two constant SPI/glycerol ratios:

70/30 and 60/40 (w/w), which were chosen on the basis of previous experiments in which the effect of glycerol content was analysed (Guerrero et al, 2010). Dispersions were maintained at 80 ºC for other 30 min under stirring at 150 rpm. The pH of the dispersions was appropriately adjusted to pH 10 with NaOH (0.1 M).

These samples were freeze-dried using Alpha 1-4 LD freeze-dryer (Martin Chirst). The powder obtained was thermally compacted using a caver laboratory press (Atlas^{TM}). 1.7 g of powder was placed between two sheets of aluminium (0.2 mm thick and 100 mm diameter). These sheets were placed between the platens of the press, which had been previously heated to 150 ºC. A pressure of 12 MPa was applied for 2 min. The platens were allowed to cool to room temperature before removing film samples.

SPI-based films were designed as a function of gelatin content (5, 10, or 15%), type of gelatin (Va for bovine gelatin, and Fi for fish gelatin), and bloom index (100, 140, or 200). For example: 15Va200 film has 15% by weight of bovine gelatin with a bloom index of 200. Gelatin-free films were also prepared as control films and designed as SPI30 (for SPI/glycerol ratio of 70/30) and SPI40 (for SPI/glycerol ratio of 60/40).

5.3. Results and discussion

SPI-based films processed by compression molding are shown in **Figure 5.1.** As it can be seen, films were flexible and transparent. All films had similar thickness (60-80 μm). In the present chapter, all films were plasticized with glycerol and modified with gelatin from different sources.

5.3.1. MC and TSM values

Packaging films should maintain moisture levels within the packaged product. Therefore, the knowledge of water solubility and moisture content of the film is very important for food packaging applications. **Table 5.1** shows the

moisture content and total soluble matter for control films and for films with gelatin. MC values for films with gelatin are lower ($P < 0.05$) and TSM values are slightly higher ($P < 0.05$) than the ones for control films.

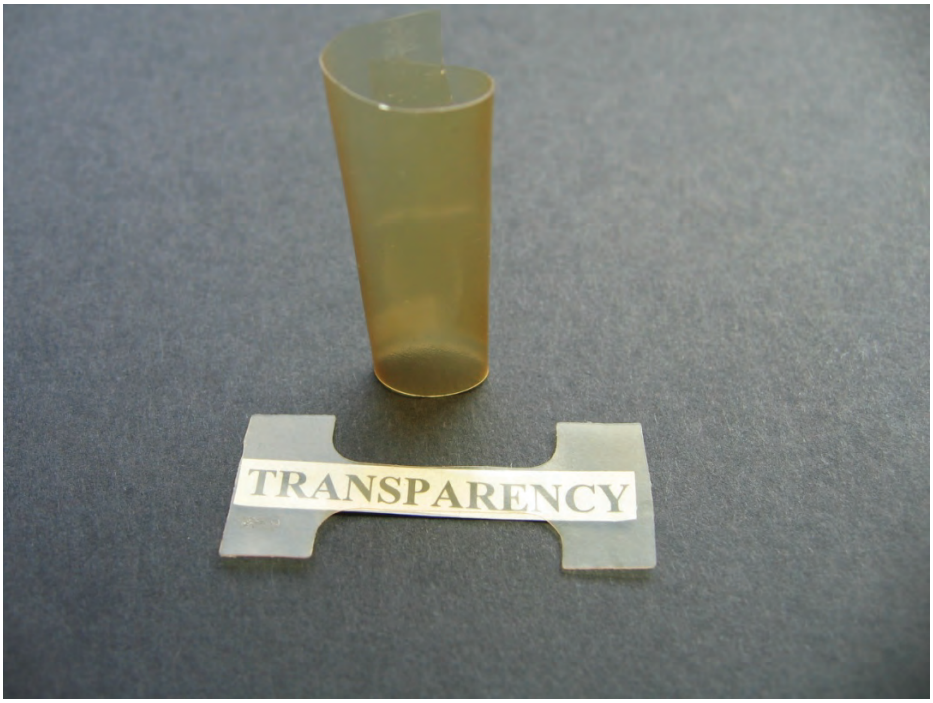

Figure 5.1. Visual aspect of SPI-based films modified with 15% bovine gelatin and processed by compression moulding technique.

These results indicate that gelatin could interact with polar groups in SPI, thus hydrophilic sites along protein chains are not exposed to water molecules, resulting in a lower water uptake. This behaviour was similar for all systems, and did not depend on the type or concentration of gelatin used in this study.

a)	MC (%)	TSM (%)
SPI30	19.66±0.74^{a}	30.56±0.36^{a}
5Va100	18.28±0.49^{bd}	32.15±0.31^{b}
10Va100	17.98±0.51^{b}	32.17±0.27^{b}
15Va100	17.81±0.38^{b}	32.23±0.59^{b}
5Va200	17.27±0.35^{c}	33.53±0.46^{c}
10Va200	17.35±0.20^{c}	33.46±0.37^{c}
15Va200	17.43±0.37^{bc}	33.44±0.42^{c}
5Fi140	17.37±0.30^{bc}	32.73±0.27^{bc}
10Fi140	17.46±0.48^{bc}	32.46±0.54^{b}
15Fi140	17.15±0.43^{c}	32.89±0.20^{bc}
5Fi200	18.13±0.11^{cd}	32.47±0.22^{ab}
10Fi200	18.50±0.43^{cd}	32.20±0.27^{b}
15Fi200	18.01±0.39^{cd}	32.04±0.37^{b}
b)		
SPI40	28.44±0.64^{a}	33.49±0.30^{a}
5Va100	27.69±0.30^{b}	35.51±0.23^{b}
10Va100	27.54±0.27^{b}	35.24±0.22^{b}
15Va100	27.74±0.10^{b}	35.48±0.25^{b}
5Va200	26.78±0.28^{c}	34.99±0.37^{bc}
10Va200	26.28±0.28^{c}	35.19±0.19^{bc}
15Va200	26.66±0.39^{c}	35.67±0.10^{b}
5Fi140	27.29±0.51^{bd}	34.88±0.19^{c}
10Fi140	27.68±0.31^{b}	34.98±0.12^{bc}
15Fi140	27.64±0.29^{b}	35.05±0.10^{bc}
5Fi200	27.72±0.22^{b}	34.50±0.30^{c}
10Fi200	27.69±0.36^{b}	34.96±0.19^{bc}
15Fi200	27.57±0.21^{b}	34.85±0.33^{c}

Table 5.1. Moisture content and total soluble matter of SPI-based films with a) 30% and b) 40% glycerol. $^{a-c}$ Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Duncan´s multiple range test.

TSM values for control films were similar to those reported by other authors (Kunte et al., 1997; Struchell and Krochta, 1994; Denavi et al., 2009a). It is also worth to note that the water insoluble fraction of the films maintained the initial structural integrity, as it is shown in **Figure 5.2**, where the influence of the gelatin addition on the swelling of samples can be clearly seen. The addition of gelatin caused a higher swelling, which might be due to the fact that

gelatin could swell more strongly in water than SPI, as it was also observed by other authors (Cao et al., 2007).

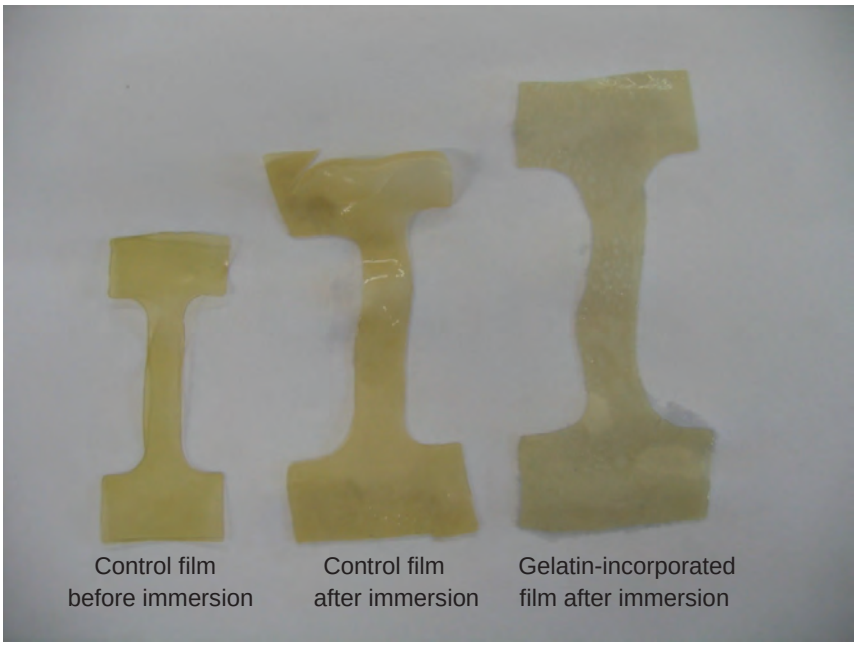

Figure 5.2. Swelling of SPI-based films without gelatin and with 15% bovine gelatin after immersion in water for 24 h.

5.3.2. Light absorption

Light transmission values at selected wavelengths from 200 to 800 nm and transparency for control films and films with gelatin in comparison to some commonly used synthetic films, oriented polypropylene (OPP) and low density polyethylene (LDPE), are shown in **Table 5.2.** It can be seen that gelatin addition did not diminish transparency ($P < 0.05$) in relation with control films. The good values of transparency were maintained independently of the type or concentration of gelatin used in this work. Moreover, transparency values of the films with gelatin were similar to the values measured for oriented

polypropylene (OPP) and better than the ones obtained for low-density polyethylene (LDPE), which are commercial films used for packaging purposes.

a)	200 nm	280 nm	350nm	400 nm	800 nm	A_{600}/mm
SPI30	0.00523	0.01166	0.52031	6.28901	53.28860	1.74946[a]
5Va100	0.01712	0.02478	1.73835	10.18630	65.78290	1.38361[b]
10Va100	0.00419	0.01257	0.95105	11.07280	63.88750	1.39092[b]
15Va100	0.01700	0.02610	1.55307	10.34610	62.11730	1.44631[bc]
5Va200	0.01366	0.01494	1.04194	10.11110	56.08600	1.36293[b]
10Va200	0.01220	0.00523	0.99835	9.75339	59.27380	1.37042[b]
15Va200	0.00405	0.00871	0.83490	9.94756	60.99910	1.37984[b]
5Fi140	0.00847	0.01539	0.78134	9.86112	61.02490	1.51218[c]
10Fi140	0.00832	0.01129	0.75672	9.77924	60.42470	1.54108[c]
15Fi140	0.01193	0.01798	0.84580	9.30012	60.87530	1.56761[c]
5Fi200	0.00766	0.01381	0.90464	9.62940	60.63890	1.38927[b]
10Fi200	0.01201	0.01151	0.66955	9.67572	59.89240	1.37054[b]
15Fi200	0.00397	0.00899	0.66993	9.72639	57.15360	1.38520[b]
OPP	4.21646	71.78940	81.08810	83.32960	88.71360	1.56641
LDPE	0.45178	27.64510	35.76000	39.97560	56.32850	4.26348

b)	200 nm	280 nm	350nm	400 nm	800 nm	A_{600}/mm
SPI40	0.01106	0.00966	5.36301	22.37000	68.83820	1.62871[a]
5Va100	0.01566	0.02685	2.12358	14.71715	59.77360	1.41371[b]
10Va100	0.00939	0.01434	2.20321	13.64810	60.07900	1.48723[bc]
15Va100	0.00885	0.01859	1.28850	13.60910	63.81970	1.49342[bc]
5Va200	0.01515	0.01147	2.79557	16.18990	62.97190	1.57533[a]
10Va200	0.01034	0.01101	2.64565	16.08070	62.74800	1.58029[a]
15Va200	0.00374	0.00726	2.12987	15.17027	62.08450	1.60413[a]
5Fi140	0.00710	0.01855	2.95604	17.34710	68.41070	1.39976[b]
10Fi140	0.00136	0.00953	2.67874	15.55150	64.25200	1.44962[b]
15Fi140	0.01242	0.02290	2.52111	15.50710	60.83960	1.50872[bc]
5Fi200	0.08512	0.01015	3.68341	17.95650	64.39020	1.39374[b]
10Fi200	0.00561	0.01709	3.17704	15.02680	64.14970	1.41037[b]
15Fi200	0.00790	0.01500	2.79835	15.56990	64.64380	1.46744[bc]
OPP	4.21646	71.78940	81.08810	83.32960	88.71360	1.56641
LDPE	0.45178	27.64510	35.76000	39.97560	56.32850	4.26348

Table 5.2**.** Light transmission (%) and transparency (A_{600}/mm) values for SPI-based films with a) 30 and b) 40% glycerol, compared with the values of films based on synthetic polymers. [a-c]Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Duncan´s multiple range test.

It is also noteworthy that control films showed excellent barrier properties to UV light in the range of 200-280 nm, which were not affected by gelatin addition, regardless gelatin content and type. Films based on SPI had excellent barrier properties to UV light in the range of 200-280 nm due to tyrosine (Tyr, 3.8% in SPI), phenylalanine (Phe, 5.2% in SPI) and tryptophan (Trp, 1.1% in SPI), which are well known to be sensitive chromophores that absorb the light at the wavelength below 300 nm (Li et al., 2004). The aromatic amino acid content might also play an important role in UV barrier properties. Moreover, these values were much better than the ones of the synthetic films analysed in this study, which showed no barrier property to UV light. Therefore, SPI based films would effectively prevent UV light transmission, whilst the synthetic films analysed in this study would not. These results suggest the potential preventive effect of SPI films on the retardation of product oxidation induced by UV light.

5.3.3. Colour

The colour characteristic can be an important factor for consumer acceptance of films in packaging applications. L*, a*, and b* colour values and the total colour difference (ΔE^*) values of the films with gelatin are shown in **Table 5.3** related to the values of control films. It can be seen that lightness (L*) slightly increased ($P < 0.05$) when gelatin was added, whilst redness (a*) and yellowish (b*) diminished ($P < 0.05$). These results were not dependent on gelatin's source or bloom. On the other hand, when glycerol content was increased, b* (yellowness) values diminished and L* (whiteness) values increased. These results showed that the addition of gelatin and glycerol improved the visual aspect of films, diminishing the typical yellowish of SPI proteins.

a)	L*	a*	b*	ΔE*
SPI30	86.45^{a}	-0.69^{a}	33.85^{a}	
5Va100	87.98^{b}	-1.08^{b}	31.09^{b}	3.18^{a}
10Va100	88.66^{bc}	-1.22^{c}	30.95^{b}	3.68^{b}
15Va100	88.70^{bc}	-1.46^{d}	30.90^{b}	3.79^{b}
5Va200	88.05^{b}	-1.12^{b}	31.16^{bc}	3.16^{a}
10Va200	88.55^{bc}	-1.28^{c}	31.05^{b}	3.55^{ab}
15Va200	88.70^{bc}	-1.28^{c}	30.97^{b}	3.70^{b}
5Fi140	87.95^{b}	-1.15^{b}	31.23^{c}	3.05^{a}
10Fi140	88.52^{bc}	-1.55^{d}	31.19^{bc}	3.48^{a}
15Fi140	88.73^{bc}	-1.43^{d}	31.09^{ab}	3.66^{b}
5Fi200	88.06^{b}	-1.10^{b}	31.07^{b}	3.24^{a}
10Fi200	88.61^{bc}	-0.98^{b}	31.03^{b}	3.56^{ab}
15Fi200	88.71^{bc}	-1.22^{c}	30.01^{b}	3.67^{b}
b)				
SPI40	89.79^{a}	-1.39^{a}	27.09^{a}	
5Va100	91.29^{b}	-1.66^{b}	24.07^{b}	3.38^{a}
10Va100	91.04^{b}	-1.76^{bc}	23.51^{c}	3.81^{b}
15Va100	90.47^{c}	-1.73^{bc}	23.62^{c}	3.55^{a}
5Va200	91.26^{b}	-1.72^{bc}	23.59^{c}	3.81^{b}
10Va200	90.37^{c}	-1.68^{b}	23.43^{c}	3.72^{b}
15Va200	90.46^{c}	-1.74^{bc}	23.57^{c}	3.60^{ab}
5Fi140	90.33^{c}	-1.64^{b}	23.89^{cd}	3.25^{a}
10Fi140	90.41^{c}	-1.71^{bc}	23.55^{c}	3.61^{ab}
15Fi140	90.78^{c}	-1.85^{c}	23.32^{c}	3.92^{bc}
5Fi200	90.89^{c}	-1.63^{b}	23.64^{c}	3.63^{ab}
10Fi200	91.46^{b}	-1.81^{c}	23.72^{cd}	3.78^{b}
15Fi200	91.50^{b}	-1.92^{d}	23.53^{c}	3.95^{bc}

Table 5.3. Colour mean values (L*, a*, b*, and ΔE*) of SPI-based films with a) 30% and 40% glycerol. $^{a-c}$Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Duncan´s multiple range test.

5.3.4. Contact angle values

Water contact angle values are good indicators of the degree of hydrophilicity of films, being higher when hydrophilicity is lower, so the final state of the water drop on the film surface can be taken as an indication of surface wettability.

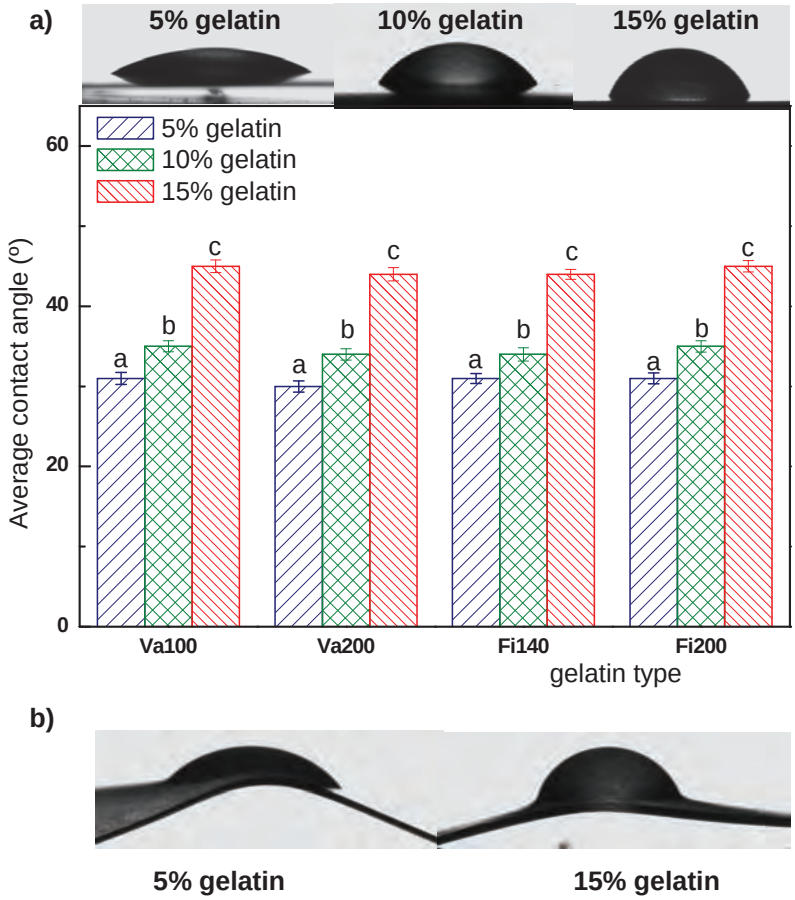

Figure 5.3. Contact angle results for gelatin-incorporated SPI-based films at different times a) t = 0, and b) t = 2 min. [a-c]Columns with the same letter are not significantly (P > 0.05) different through the Duncan´s multiple range test.

To understand the effect of gelatin on film wettability, contact angles have been investigated for films with different content and type of gelatin and have been related to control films, as it is shown in **Figure 5.3,** where we can distinguish the water absorption process by the bending of the film.

In this study, contact angle increased with gelatin addition. This behavior indicates that SPI-gelatin interactions could result in a higher

exposure of hydrophobic groups of SPI, increasing hidrophobicity of the films. The results on the outer surface were lower than 50º in all cases. Similar contact angles values were obtained with the different types of gelatin used in this work. Moreover, contact angles on SPI films decreased with contact time, indicating the water absorption by the film (Extrand and Kumagai, 1997).

5.3.5. FTIR analysis

FTIR spectra of pure SPI and pure gelatin are shown in **Figure 5.4**. The FTIR spectrum of pure gelatin exhibited similar absorption peaks to SPI, but the amide I band was found at 1628 cm^{-1} (Denavi et al., 2009a; Kong and Yu, 2007; Li et al., 2004; Muyonga et al., 2004; Plepis, et al., 1996). The absorption peak at 1332 cm^{-1} is attributable to CH_2 wagging of proline, the band at 1198 cm^{-1} corresponds to N-H bending, and the peak at 1028 cm^{-1} is related to C-O stretching (Jackson et al., 1995). When gelatin was added, the absorption peaks of pure gelatin at 1332, 1198, and 1028 cm^{-1} disappeared, indicating that the gelatin could undergo conformational changes that produced a certain degree of interaction between gelatin and SPI, such as hydrogen bonds, dipole-dipole and hydrophobic interactions, characteristic of natural proteins.

FTIR spectra of films with gelatin before and after immersion in water are shown in **Figure 5.5**. It can be seen that no change take place in the characteristic peaks of protein, but the difference in the intensity between the bands at 1630 cm^{-1} (amide I) and 1530 cm^{-1} (amide II) becomes higher after immersion, suggesting that only small peptides and non-protein materials present in the composition of commercial proteins were solubilised in water. However, the bands at 850, 925, 995, 1045 and 1117 cm^{-1}, corresponding to C-C and C-O bonds in glycerol, disappeared after immersion in water for 24 h, indicating migration of glycerol from the film and solubilisation in water.

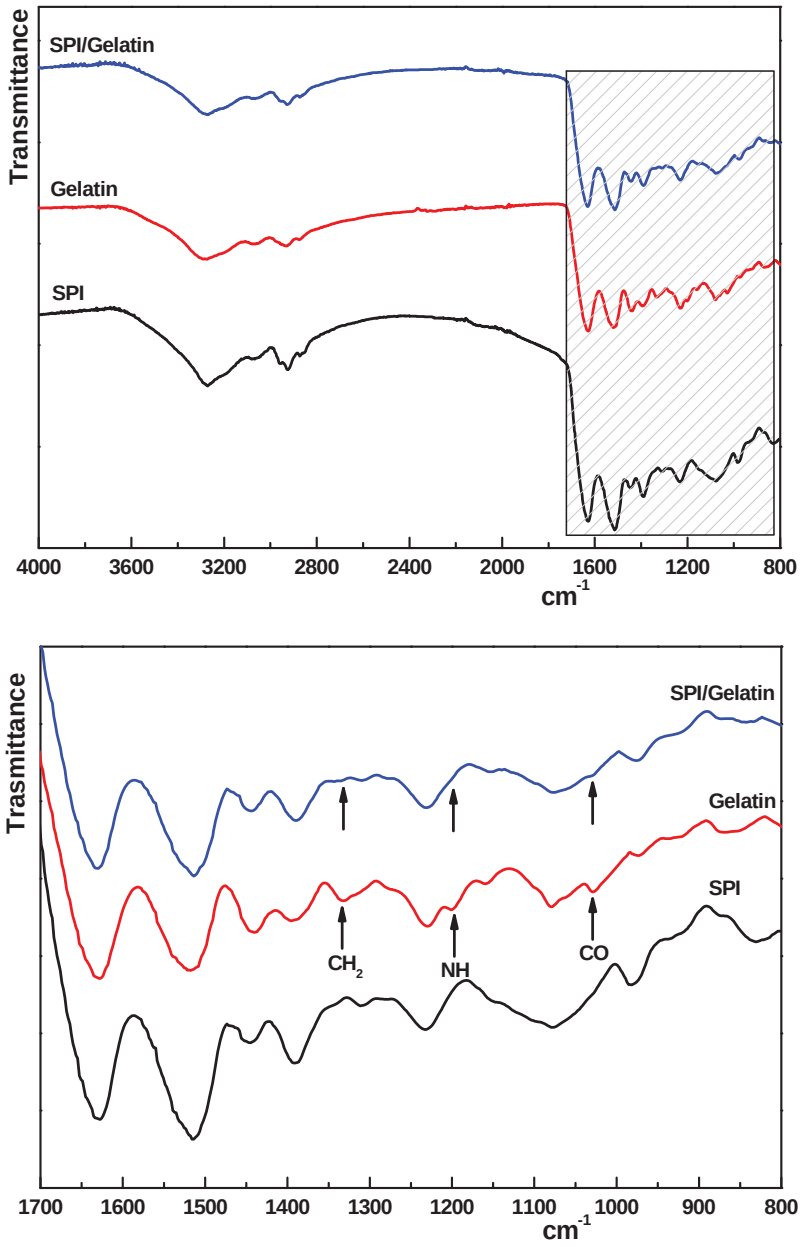

Figure 5.4. Infrared spectra of SPI, gelatin, and films based on SPI with 15% bovine gelatin.

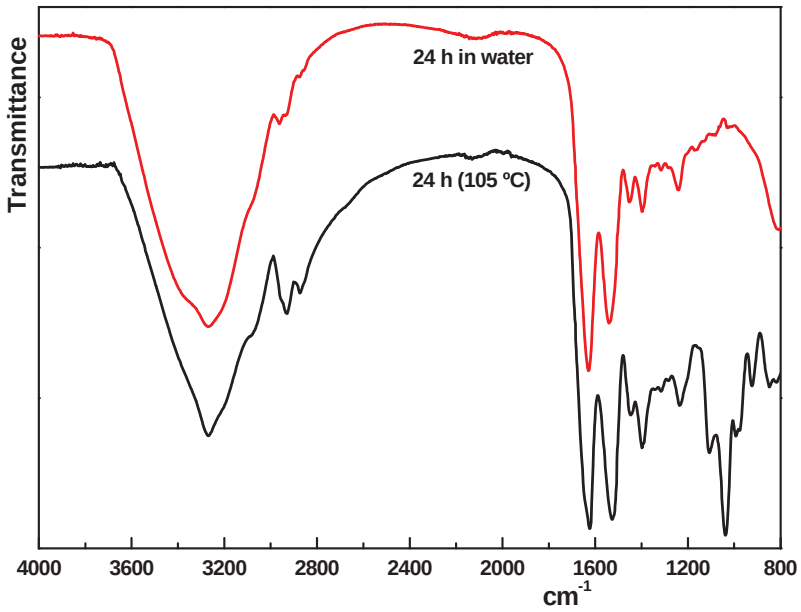

Figure 5.5. Infrared spectra of films based on SPI with 15% bovine gelatin before and after immersion in water for 24 h.

5.3.6. Mechanical properties

Mechanical properties are largely associated with distribution and density of intermolecular and intramolecular interactions in the network. According to Chambi and Grosso (2006), these interactions will be dependent of arrangement and orientation of polymer chains. The effect of gelatin addition on mechanical properties of SPI-based films is shown in **Table 5.4**. When gelatin was added, tensile strength (σ) increased ($P < 0.05$), while elongation at break (ε) values did not show substantial changes ($P < 0.05$) and remained always higher than 130%.

Compared to control films, the tensile strength of gelatin-incorporated SPI films increased up to a value of 11 MPa with the addition of 15% bovine gelatin with bloom 200. This fact may be attributed to protein-protein interactions, which are determined by hydrogen bonds, electrostatic and/or

hydrophobic interactions (Cheftel et al., 1985; Sothornvit and Krochta, 2001).

a)	σ (MPa)	ε (MPa)
SPI30	7.24±0.50[a]	139.28±5.91[a]
5Va100	8.40±0.24[b]	139.59±6.15[a]
10Va100	8.58±0.37[b]	136.72±6.95[c]
15Va100	8.85±0.32[b]	138.95±7.05[a]
5Va200	10.22±0.47[c]	138.39±7.25[a]
10Va200	10.49±0.49[c]	145.93±6.69[d]
15Va200	11.31±0.22[d]	141.74±7.75[d]
5Fi140	8.52±0.38[b]	135.98±6.79[c]
10Fi140	9.26±0.39[e]	143.82±5.23[d]
15Fi140	9.31±0.26[e]	139.38±6.07[b]
5Fi200	7.62±0.45[a]	137.71±4.45[ac]
10Fi200	9.52±0.39[e]	143.39±6.91[d]
15Fi200	9.55±0.47[e]	142.40±3.75[d]
b)		
SPI40	3.90±0.31[a]	163.54±5.93[a]
5Va100	3.45±0.42[b]	142.49±8.17[b]
10Va100	3.56±0.31[b]	137.41±8.02[c]
15Va100	4.46±0.33[c]	140.70±7.81[b]
5Va200	4.46±0.30[c]	143.67±6.41[b]
10Va200	4.65±0.15[cd]	139.21±8.13[c]
15Va200	4.93±0.35[d]	140.62±7.07[b]
5Fi140	4.04±0.12[a]	141.14±7.78[b]
10Fi140	4.49±0.26[c]	143.25±6.95[b]
15Fi140	4.88±0.21[d]	149.53±6.56[d]
5Fi200	4.35±0.14[c]	146.56±7.96[d]
10Fi200	4.85±0.26[d]	142.74±6.90[b]
15Fi200	5.16±0.29[e]	148.18±8.11[d]

Table 5.4. Tensile properties of SPI-based films with a) 30% and b) 40% glycerol. [a-d]Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Duncan´s multiple range test.

These interactions are ultimately influenced by both the sequence of amino acid residues and by the three dimensional size of the entire network. The gelatin can reacquire part of the triple helix structure with a high degree of organization, and consequently films produced with gelatin can show more

organized networks compared to those made from SPI (Cao et al., 2007; Denavi et al., 2009a). These results are in good agreement with the reduction of TSM values observed by immersion in water tests and with the disappearance of the band corresponding to the proline shown in FTIR spectra.

In this study, tensile strength values were higher for films with bovine gelatin than the ones for fish gelatin. This difference could be due to a higher content of the imino acids Pro and Hyp in bovine gelatins, which would impart a higher degree of molecular rigidity (Eysturskaro et al., 2009; Gómez-Estaca et al., 2009). On the other hand, tensile strength values were also influenced by bloom index. In general, the bloom index is related to α and β fractions and to the molecular weight of these fractions, providing films with different renaturation degrees and mechanical properties (Segtnan and Isaksson, 2004). The results of this work indicate that tensile strength values were higher for films with gelatin with a higher bloom index. These results might be due to the higher molecular weight associated to higher bloom index, which could lead to a more perfect network due to a greater amount of triple helix structure (Bigi et al., 2004).

When gelatin was added, the increase of tensile strength was associated, as expected, with a decrease of elongation at break for the films with 40% glycerol. However, the addition of gelatin caused an increase of tensile strength and maintained elongation at break for the films with 30% glycerol, indicating that adding more than 30% glycerol would not favour SPI-gelatin interactions, responsible for better mechanical properties. As consequence, 30% glycerol can be considered the optimal plasticizer content for these systems.

5.4. Conclusions

Mechanical properties of SPI-based films have been improved by the addition of gelatin, especially when 15% bovine gelatin with 200 bloom index

was added in the system plasticized with 30% glycerol, showing an increase in tensile strength with the maintenance of elongation at break. These results have been related to the changes of the bands associated with gelatin in FTIR spectra and to the decrease of total soluble matter values carried out by immersion in water testing, which are indicative of SPI-gelatin interactions. Moreover, gelatin-incorporated SPI-based films showed excellent barrier properties to UV light, suggesting the potential preventive effect of SPI films on the retardation of product oxidation induced by UV light, and transparency values similar to the ones measured for OPP and better than the ones obtained for LDPE, which are commercial films used for packaging purposes.

EFFECT OF ACIDS AND OILS

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6. Effect of acids and oils

6.1. Summary

Soy protein isolate films present adequate properties to be used as food packaging, such as they have very low aroma and oxygen permeability (Gennadios et al., 1993; Ghorpade et al., 1995), which makes them useful for applying to sensitive to oxygen products or for preserving flavours. Nevertheless, their high water vapour permeability is an obstacle to use them in food packaging (Rhim et al., 2000). Many attempts have been made to modify the relatively high moisture sensitivity of SPI films (Park et al., 2001). Lipids can be used to improve water resistance (Rhim et al., 1999). However, as SPI contains 58% polar amino acids that cause its hydrophilicity, its moisture sensitive is difficult to eliminate (Rhim and Lee, 2004).

In the previous chapter, SPI-gelatin films were prepared and it was shown that tensile strength and elongation at break increased at the same time by increasing gelatin ratio, as well as films became more transparent and easier to handle. However, it was observed that gelatin was not able to provide a significant moisture barrier.

Many attempts have been made to modify the poor surface hydrophobicity of biofilms. In this chapter, epoxydized soybean oil (ESO), virgin extra olive oil variety picual (OO) or lactic acid (LA) were added to SPI in order to improve the hydrophobic character of the films prepared by compression. Therefore, the aim of this chapter was to improve contact angle values, maintaining water vapour permeability, optical, and mechanical properties obtained in the control films previously prepared (Guerrero et al., 2011). Results were related to interactions between protein and additives analyzed by FTIR analysis. Thermal behaviour and water uptake were also analyzed as well as appearance, transparency and colour.

6.2. Film preparation

Glycerol and gelatin contents in the blends were selected as 30% (Guerrero et al., 2010) and 15% by weight on SPI dry basis (Guerrero et al., 2011) respectively, based on the results of the previous chapters. 5 g of SPI and the amount of gelatin and the acid or oil required to obtain the desired percentage were dispersed in 100 mL distilled water. Dispersions were heated at 80 ºC for 30 min and stirred at 150 rpm. Then, glycerol was added as plasticizer. Dispersions were maintained at 80 ºC for other 30 min under stirring at 150 rpm. The pH of the solutions was appropriately adjusted to pH 10 with NaOH (0.1 M).

These samples were freeze-dried using Alpha 1-4 LD freeze-dryer (Martin Chirst), and the obtained powder was thermally compacted using a caver laboratory press (Atlas^{TM}). The powder was placed between two sheets of aluminium with a 100 mm diameter. These sheets were placed between the platens of the press, which had been previously heated to 150 ºC. A pressure of 12 MPa was applied for 2 min and the platens were allowed to cool to room temperature before removing film samples

Different acid and oil contents (5, 10, and 15% by weight on SPI dry basis) were added and the samples were designed as 5LA, 10LA, and 15LA; 5ESO, 10ESO, and 15ESO; and 5OO, 10OO, and 15OO.

6.3. Results and discussion

At macroscopic scale, films had uniform appearance and there was no pore on the surface. All films were transparent with a yellowish hue, typical for SPI-based films, and had similar thickness, in the range of 60-80 μm. Films prepared with 15% ESO or 15% OO exuded, indicating an excess of oil content in the composition of the film.

6.3.1. FTIR analysis

The FTIR spectra of the pure acid and oils are shown in **Figure 6.1**, where the main absorption peaks for LA are at 3368 cm^{-1} (O-H), 1716 cm^{-1} (C=O), 1465 cm^{-1} (CH_3 antisymmetric deformation), and 1122 cm^{-1} (C-O antisymmetric stretch). The main absorption peaks for ESO are at 2923 and 2853 cm^{-1} (C-H stretching), 1749 cm^{-1} (C=O), 1460 cm^{-1} (CH_3 antisymmetric deformation), 1156 cm^{-1} (C-O antisymmetric stretch) and 841 cm^{-1} (epoxy). The main absorption peaks for OO appear at 2924 and 2853 cm^{-1} (C-H stretching), 1747 cm^{-1} (C=O), 1465 cm^{-1} (CH_3 antisymmetric deformation) and 1163 cm^{-1} (C-O antisymmetric stretch).

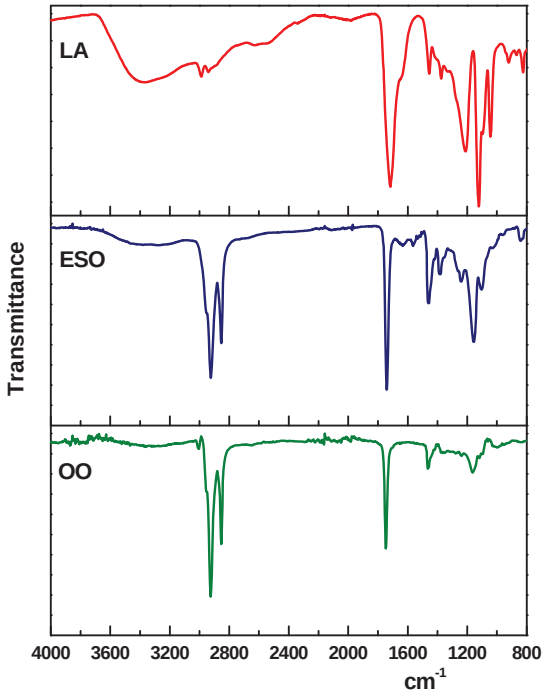

Figure 6.1. FTIR spectra of lactic acid (LA), epoxydized soybean oil (ESO), and olive oil (OO).

FTIR spectra for the films prepared with 10% acid or oil related to the control film are shown in **Figure 6.2**. It can be seen that the band at 1716 cm^{-1}, corresponding to the carboxyl group of LA, disappeared, and the relative intensity between the bands at 1633 and 1514 cm^{-1}, corresponding to the amide I and II became similar, indicating some interaction between the carboxyl group of LA and the protein. This fact did not take place in the case of ESO or OO oils' addition due to the fact that the much higher volume of the oils' molecules prevents these big molecules to effectively get into the protein chains to interact with polar groups.

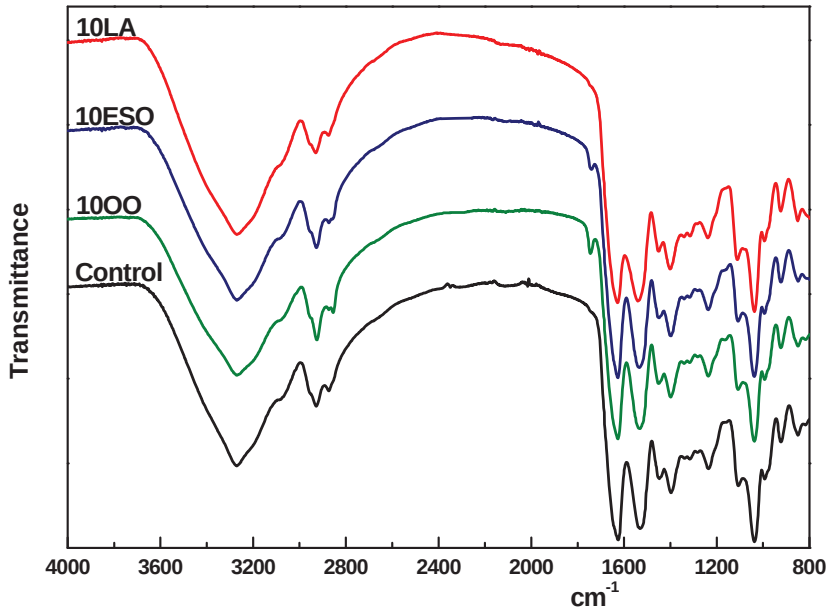

Figure 6.2. FTIR spectra of the films with 10% lactic acid (10LA), 10% epoxydized soybean oil (10ESO), and 10% olive oil (10OO) related to the control film (control).

6.3.2. DSC analysis

DSC has been used to characterize thermal properties of SPI-based films, including heat-induced denaturation. As it was shown in previous

chapters, heating changes soy protein from its native state to a denatured one, which can be seen as an endothermic peak in DSC thermograms.

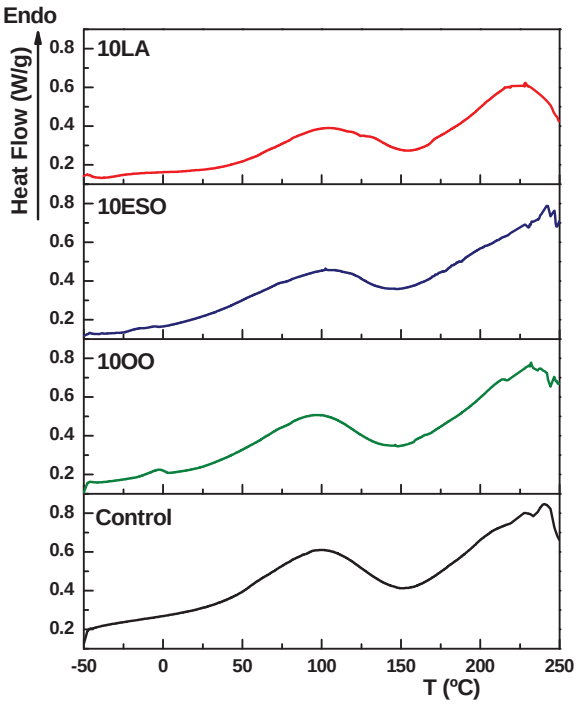

Figure 6.3. DSC thermograms of the films with 10% lactic acid (10LA), 10% epoxydized soybean oil (10ESO), and 10% olive oil (10OO) related to the control film (control).

The two denaturation peaks of SPI can be observed in **Figure 6.3**: the one at 99 ºC, corresponding to the low molecular weight 7S globulin, and the one at 227 ºC, related to the high molecular weight 11S globulin. The denaturation temperature of 7S globulin was shifted to higher values than those usually expected due to the low water content of the films (Denavi et al., 2009a; Guerrero and de la Caba, 2010). The enthalpy of denaturation did not change significantly, only the thermogram for the film with OO showed a small endothermic peak at -2 ºC, which can be attributed to the melting peak of olive oil variety picual (Jiménez and Beltrán, 2003). These results indicate that the addition of the acid or oils did not influence the denaturation process of the soy

protein used in this work and that a significant amount of SPI remained in its native conformation with globular structure.

6.3.3. TGA analysis

TGA analysis was performed to distinguish the evaporation effects caused by different volatile matter while the sample was heated. The TGA and DTG curves for the control film and the ones with different contents of acid or oils are shown in **Figure 6.4**.

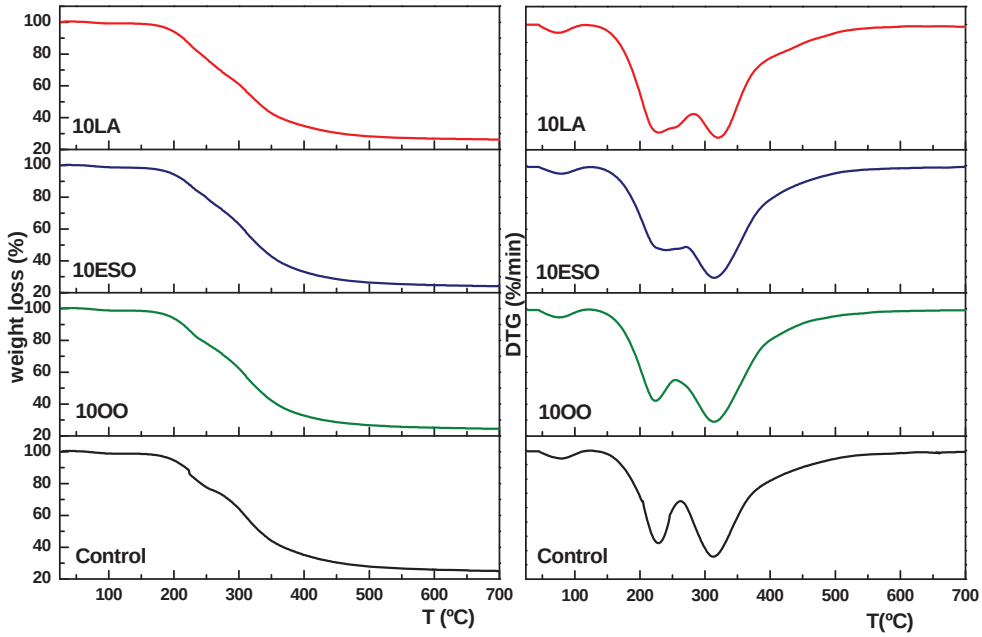

Figure 6.4. TGA and DTG curves of the films with 10% lactic acid (10LA), 10% epoxydized soybean oil (10ESO), and 10% olive oil (10OO) related to the control film (control).

All films presented similar behaviour with three main stages of mass loss. The first stage, observed up to 100 ºC was related to the loss of water, which indicates the existence of residual moisture in the films. The second stage, the weight loss at 228 ºC, was attributed to the evaporation of glycerol.

This temperature is higher than the boiling point of glycerol (182 ºC), which indicates that some kind of interaction (hydrogen bonds) exists between SPI and glycerol. Moreover, when LA was added, it appeared a shoulder at 285 ºC, which can not be related to LA (boiling point at 122 ºC), so the shoulder could indicate interactions between the small molecules of acid and glycerol. The third stage was associated with SPI degradation and appeared at 312 ºC. In addition, a slight increase in this temperature from 312 ºC, for ESO and OO, to 324 ºC for LA was observed, which can be due to the fact that polar side groups in SPI (such as hydroxyl, carbonyl, and amine groups) can interact with LA, in accordance with the spectra in **Figure 6.2**, where the relative intensity between the bands related to amide I and amide II changed when comparing with the spectrum for the control film.

6.3.4. XRD analysis

XRD was used to determine the structure for the films with the acid and oils related to the control film, as it is shown in **Figure 6.5.**

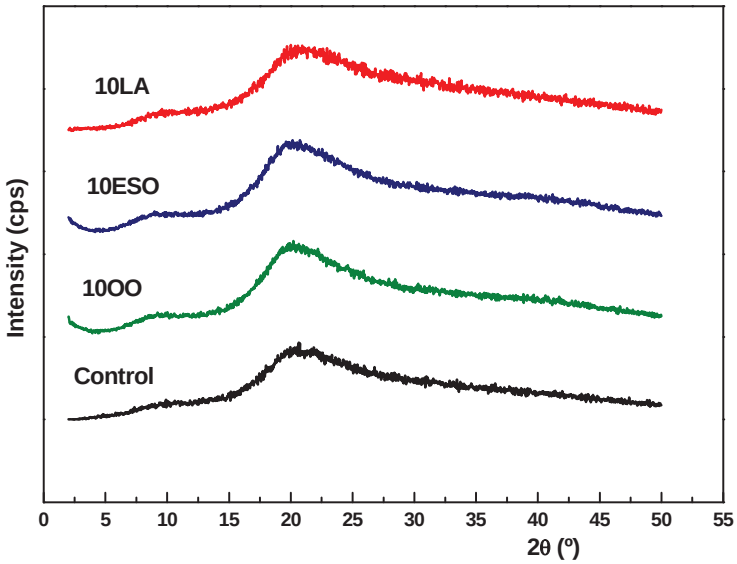

Figure 6.5. XRD patterns of the films with 10% lactic acid (10LA), 10% epoxydized soybean oil (10ESO), and 10% olive oil (10OO) related to the control film (control).

XRD data show that the diffraction patterns of the control film exhibited a dominant amorphous halo, a broad band with a maximum at $2\theta = 21^{o}$, characteristic for pure SPI, which has 7S and 11S amorphous globulins as the main components. The intensity of this band is similar for all the compositions analyzed in this study, indicating that the addition of the acid or oils did not affect the amorphous structure of the SPI based-films.

6.3.5. Light absorption

Light transmission values at wavelengths from 200 to 800 nm and transparency are shown in **Table 6.1** for the control film and for the films with different contents of ESO, OO and LA, in comparison to some commonly used synthetic films: oriented polypropylene (OPP) and low density polyethylene (LDPE).

	200nm	280nm	350nm	400nm	800nm	A_{600}/mm
Control	0.00405	0.00871	0.83490	9.94756	60.99910	1.37984
5ESO	0.00328	0.07744	1.35830	9.16324	43.83210	2.75188[a]
10ESO	0.00477	0.01346	0.84331	7.89936	23.83950	3.96793[b]
15ESO	0.00503	0.04221	0.93287	4.99665	20.42090	4.24480[c]
5OO	0.00152	0.02307	0.38224	3.35888	23.17980	3.94721[d]
10OO	0.00495	0.03679	0.43480	3.62150	20.31270	4.75126[e]
15OO	0.00650	0.04212	0.44859	3.50808	17.62850	4.86596[f]
5LA	0.00874	0.04338	1.00069	9.38247	53.32980	1.65647[g]
10LA	0.00604	0.06116	1.12644	9.83722	50.37090	2.16236[h]
15LA	0.00338	0.08837	1.11497	9.77667	48.15740	2.26684[i]
OPP	4.21646	71.78940	81.08810	83.32960	88.71360	1.56641
LDPE	0.45178	27.64510	35.76000	39.97560	56.32850	4.26348

Table 6.1. Light transmission (%) and transparency (A_{600}/mm) values for the control film and for the films with different contents of ESO, OO and LA, compared with the ones for commercial films based on synthetic polymers. [a-i]Two means followed by the same letter in the same column are not significantly ($P > 0.05$) different through the Tukey´s multiple range test.

The addition of ESO and OO produced a sharp decrease in transparency related to the control film. This reduction in transparency

increased ($P < 0.05$) when the content of ESO and OO was increased. In contrast, the addition of LA caused a slight decrease ($P < 0.05$) in transparency values. Transparency of films is an auxiliary criterion to judge the compatibility of the components (Liu and Zhang, 2006), so the results could indicate that the interaction of the small molecule of LA with protein and glycerol, shown by FTIR and TGA results, improved the transparency of the films. Moreover, transparency values for the films with LA were similar to the values measured for OPP, which is a commercial film used for packaging purposes.

As it was shown in the previous chapter for gelatin-incorporated films, films prepared with LA, ESO, or OO showed excellent barrier properties to UV light in the range of 200-280 nm, which were not affected by the acid or oils' content. This behavior emphasizes the potential use of these films for preventing food oxidation induced by UV light.

6.3.6. Optical properties

Optical properties of films are relevant since they have direct impact on the appearance of the packaged product. In order to evaluate the differences between control films and those prepared with different contents of acid or oils, L*, a*, b*, C^*_{ab}, h^*_{ab}, and WI were calculated from spectral distribution of the films on a standard white plate. ΔE^* values with respect to the control film were also measured. All values are shown in **Table 6.2**.

Control films were smooth and transparent with a greenish and yellowish colour. The lightness (L*) remained constant ($P < 0.05$) with the addition of the acid or oils. However, greenness (a*) significantly decreased ($P < 0.05$) and yellowness (b*) decreased ($P < 0.05$) with the addition of the acid or oils. The decline of yellowness was greater ($P < 0.05$) with the addition of LA. C^*_{ab} values decreased ($P < 0.05$) when the acid or oils were incorporated and the films with the highest values were those containing lower acid or oils' ratios. Concerning h^*_{ab} values, they remained constant ($P < 0.05$) with the

addition of the acid or oils, while the WI increased ($P < 0.05$) when the acid or oils were incorporated. These results show that the addition of the acid or oils slightly improved the visual aspect of the films, diminishing the typical yellowish of SPI-based films. Moreover, ΔE^* values showed an increase ($P < 0.05$) of colour variation as the acid or oil content increased.

	L*	a*	b*	C*	h*	WI	ΔE*
Control	88.70±0.52^{c}	-1.28±0.13^{a}	30.97±0.51^{a}	30.99±0.50^{a}	87.63±0.27^{a}	67.01±0.37^{a}	
5ESO	86.99±0.38^{ab}	-0.07±0.25^{c}	30.34±0.26^{a}	30.34±0.26^{a}	89.56±0.11^{c}	66.98±0.37^{a}	2.22±0.32^{a}
10ESO	88.44±0.44^{c}	-0.17±0.12^{c}	28.01±0.28^{b}	28.01±0.28^{b}	89.64±0.25^{c}	69.69±0.23^{c}	3.20±0.26^{b}
15ESO	89.78±0.46^{de}	-0.28±0.13^{c}	26.86±0.57^{c}	26.86±0.57^{c}	89.40±0.29^{c}	71.26±0.67^{d}	4.38±0.61^{c}
5OO	87.45±0.49^{b}	-0.28±0.12^{c}	28.79±0.72^{b}	28.80±0.72^{b}	89.45±0.25^{c}	68.59±0.84^{b}	2.80±0.33^{ab}
10OO	86.08±0.52^{a}	-0.02±0.29^{c}	27.96±0.17^{b}	27.96±0.17^{b}	89.55±0.31^{c}	68.76±0.31^{bc}	4.21±0.39^{c}
15OO	88.84±0.32^{cd}	-0.07±0.14^{c}	26.04±0.56^{cd}	26.04±0.56^{cd}	89.73±0.20^{c}	71.67±0.60^{de}	5.09±0.52^{cd}
5LA	88.69±0.32^{c}	-0.85±0.07^{b}	28.90±0.68^{b}	28.91±0.68^{b}	88.32±0.12^{b}	68.96±0.71^{bc}	2.14±0.68^{a}
10LA	89.10±0.61^{cde}	-0.81±0.06^{b}	25.62±0.36^{d}	25.64±0.35^{d}	88.18±0.15^{b}	72.14±0.25^{de}	5.41±0.34^{d}
15LA	89.80±0.24^{e}	-0.86±0.04^{b}	25.26±0.52^{d}	25.27±0.52^{d}	88.04±0.11^{ab}	72.75±0.46^{e}	5.84±0.50^{d}

Table 6.2. Optical parameters for the films with different contents of ESO, OO and LA related to the ones for the control film. $^{a-e}$Two means followed by the same letter in the same column are not significantly ($P > 0.05$) different through the Tukey´s multiple range test.

Taking into account that the colour parameters were obtained on a standard plate, the increase of WI and the decrease of C^*_{ab} values with respect to the control film may reflect a loss of colour of the films. In this sense, the

incorporation of the acid or oils contributes to soften the colour of the SPI matrix. The more acid or oil added, the greater the softening effect.

6.3.7. Contact angle values

To understand the effect of the acid and oils on the film wettability, contact angles were investigated for films with different contents of the acid and oils and were related to the control film, as it is shown in **Figure 6.6.**

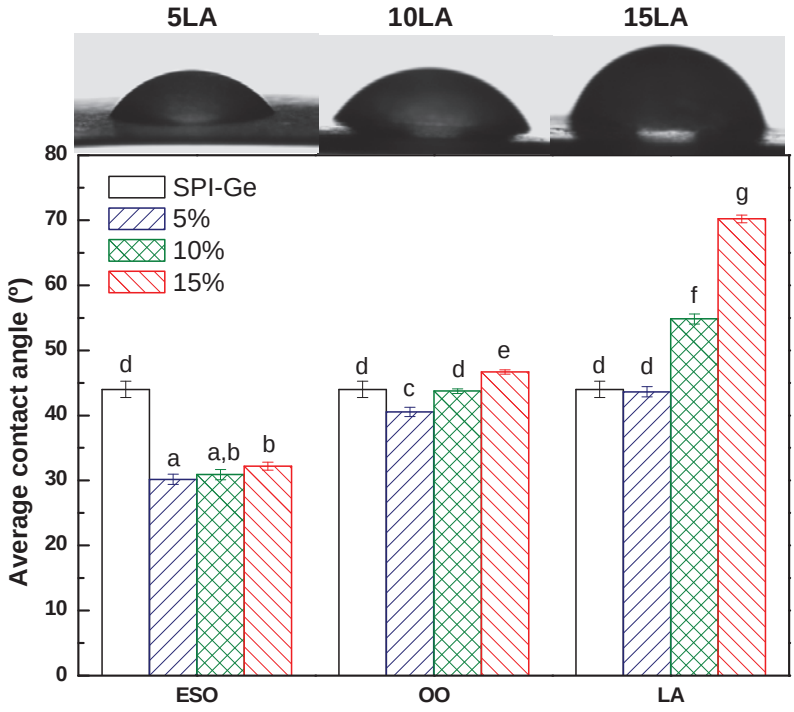

Figure 6.6. Contact angle values of the films with different contents of lactic acid (LA), epoxydized soybean oil (ESO), and olive oil (OO) related to the control film (control). [a-g]Columns with the same letter are not significantly (P > 0.05) different through the Tukey´s multiple range test.

Contact angle values decreased (P < 0.05) with the addition of ESO, remaining constant (P > 0.05) when ESO content was increased. Adding OO caused a slight decrease (P < 0.05) in contact angle with 5% OO, slightly

increasing until similar values to those of the control film when the content of OO was increased. On the other hand, the addition of LA produced an improvement in the contact angle, which increased ($P < 0.05$) when LA content was increased. As it can be seen in **Figure 6.6**, contact angles increased from 44° for the control film to 70º for the films with 15% LA. This behaviour indicates that surfaces of the films differed depending on the type of additive. In the case of oils, some authors (Djagny et al., 2001; Quiroga et al., 2010) stated that, depending on the size of the molecule, large contents of oils led to partial changes of the protein configuration, reducing hydrophilic groups on the surface, so thus increasing the hydrophobic character. This fact could explain the behaviour of the smaller molecule of OO related to ESO. In the case of LA, the interaction of LA with protein and glycerol caused a decrease in polar groups on the surface and an increase of the hydrophobic character of the film. These results are consistent with those observed by FTIR and TGA.

6.3.8. MC, TSM, WVP and OP values

MC, TSM, WVP and OP values for the films with different acid and oil contents related to the control film are shown in **Table 6.3**. MC values are similar for all films, being around 15-17% ($P < 0.05$). TSM values for the films with ESO and OO were similar ($P > 0.05$) but lower than the ones for the control film ($P < 0.05$). On the contrary, TSM values increased ($P < 0.05$) for the films with LA, which can be related with the interactions promoted by LA. However, it is worth to note that the water insoluble fraction of the films maintained the initial structural integrity.

The WVP values for the films with the acid or oils were similar ($P > 0.05$) to the ones for the control film. As it is well known, the water vapour transmission behaviour for natural polymers is a two-step process, namely water sorption and water diffusion. Hydrophobic groups on the surface decrease the initial step of sorption. On the other hand, hydrogen bonding between natural polymers and additives may increase free volume, enhancing

the process of water passage through the film (Cuq et al., 1997; Sobral et al. 2001; Su et al., 2010). Permeability involves a thermodynamic factor (solubility) and kinetic factor affected by molecular mobilities (diffusion). Taking the above into consideration and the results of contact angle measurements, WVP values could indicate that the rate of water diffusion through the film was higher in the films with LA, which had the higher contact angle values and so the most hydrophobic surface. This could be explained by the fact that the small size of LA enhanced the interaction with the protein, so decreasing the interaction between protein chains and increasing free volume, which results in a less dense network and facilitates the diffusion and also solubilisation in water that caused the increase of TSM values.

	MC (%)	TSM (%)	WVP x 10^{12} (g/cm·s·Pa)	OP (cm^3·µ/m^2·d·KPa)
Control	17.43±0.37^{b}	33.42±0.42^{b}	5.62±0.14^{ab}	8.01±0.37^{a}
5ESO	14.95±0.12^{a}	31.40±0.54^{a}	4.91±0.33^{a}	16.43±0.58^{a}
10ESO	16.62±0.55^{ab}	30.05±0.13^{a}	5.19±0.27^{ab}	371±11^{c}
15ESO	16.59±0.56^{ab}	30.62±0.44^{a}	5.82±0.46^{ab}	6914±135^{d}
5OO	15.58±0.74^{a}	30.73±0.63^{a}	5.57±0.36^{ab}	15.40±0.76^{a}
10OO	16.12±0.68^{ab}	31.00±0.68^{a}	5.71±0.35^{ab}	259±7^{b}
15OO	15.95±0.83^{ab}	30.69±0.42^{a}	5.83±0.45^{ab}	473±1^{c}
5LA	16.96±0.73^{b}	35.29±0.61^{c}	5.53±0.37^{ab}	4.43±0.57^{a}
10LA	17.51±0.48^{b}	38.32±0.41^{d}	5.83±0.37^{ab}	4.56±0.34^{a}
15LA	17.42±0.49^{b}	40.88±0.20^{e}	6.13±0.38^{b}	4.78±0.43^{a}

Table 6.3. Moisture content (MC), total soluble matter (TSM), water vapour permeability (WVP) and oxygen permeability (OP) values for the films with different contents of ESO, OO and LA related to the ones for the control film. $^{a-e}$Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Tukey´s multiple range test.

On the other hand, OP values remained constant (P > 0.05) with LA content due to LA-protein interactions, and increased (P < 0.05) with ESO and OO content owing to the increase of hydrophobic components that facilitate oxygen diffusion, as it was also shown by others authors (Bertan et al., 2005;

Gennadios et al., 1993).

6.3.9. Mechanical properties

Puncture strength and deformation are shown in **Figure 6.7a** and **6.7b**, respectively.

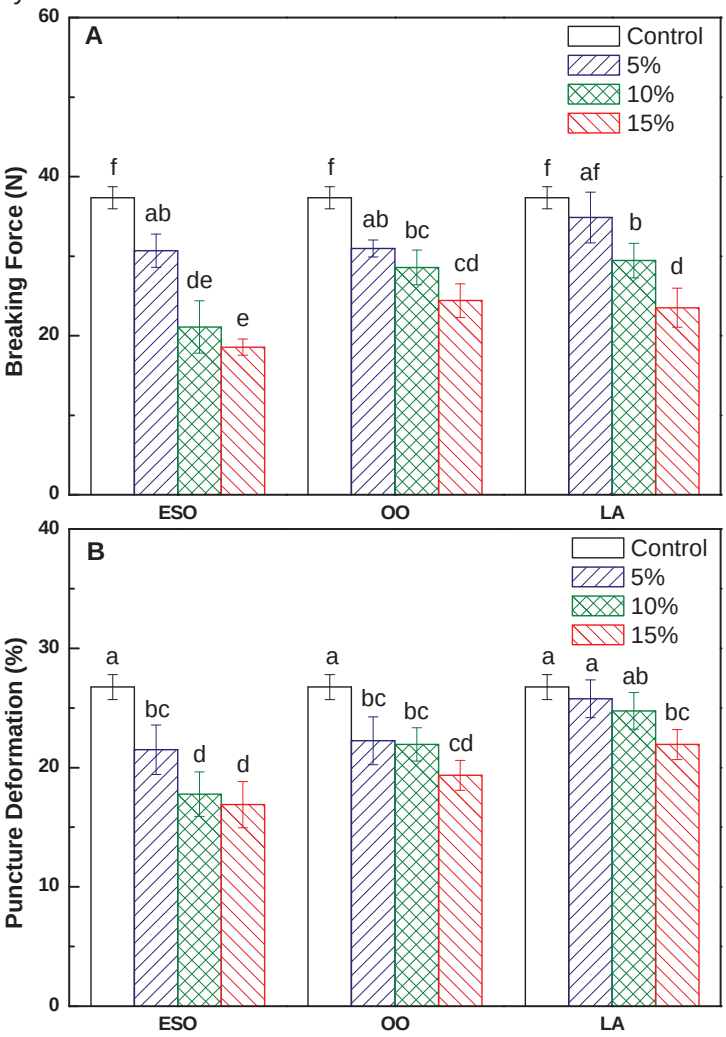

Figure 6.7. Puncture strength (a) and deformation (b) of the films with different contents of ESO, OO, and LA related to the control film (control). [a-f]Columns with the same letter are not significantly (P > 0.05) different through the Tukey´s multiple range test.

The addition of the acid or oils reduced both puncture strength ($P < 0.05$) and puncture deformation ($P < 0.05$). LA was the additive that showed the best performance to puncture breaking related to the control film, indicating that LA provides more flexible structures than ESO or OO. Oils could lead to discontinuities in the protein matrix (Fabra et al., 2008) that make it less resistant to fracture. In the case of LA addition, the decrease in puncture deformation did not show significant differences ($P > 0.05$). It is also noteworthy that the control film had average puncture strength value of 37 N, higher than those indicated by other authors (Gómez-Guillén et al., 2007; Sobral et al., 2001) for films with similar thicknesses.

6.4. Conclusions

The addition of LA produced an increase in contact angle values, indicating an increase of the hydrophobic character of the film, maintaining the WVP obtained for the control film. These results were in good agreement with the changes of bands observed by FTIR, which indicated LA-SPI interactions, and with the changes seen by TGA, which indicated LA-glycerol interactions. The strong charge and polar interactions between side chains in soy protein restrict molecular mobility, but glycerol and LA can reduce inter- and intramolecular hydrogen bonding in protein chains and plasticize SPI films, resulting in good puncture values. Moreover, the decrease in the yellowness of the films, maintaining excellent barrier properties to UV light, is very important for packaging applications.

EXTRUSION

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7. Extrusion

7.1. Summary

Extrusion is generally defined as a process to mix, homogenize, and shape material by forcing it through a specifically designed opening. It is widely used in the plastics industry and most of synthetic polymers are produced by this way. However, most protein films are processed by solution casting, so there are few reports related to proteins extrusion. Despite of the increasing use of this technology, extrusion process is still a complicated multi-input-output system that has to be mastered. Process parameters (screw speed, moisture content, barrel temperature, screw configuration, die dimension, and raw material characteristics) have effects on processability and on the final properties of products, so it is imperative to research the correlation between them in order to better understand the effect of extrusion process on the characteristics of products.

In a typical extrusion operation, the two main sources of energy associated with enthalpy change of the extrudate are convection heat transfer between the barrel and the material, and viscous dissipation of the mechanical energy into heat inside the material (Harper, 1989; Hu et al., 1993). Specific mechanical energy (SME) is the amount of mechanical energy dissipated as heat inside the material, expressed per unit mass of the material. Specifically, it is the work input from the drive motor into the material being extruded, so it is an important process parameter that influences the final product characteristics and thus provides a good characterization of the extrusion process.

Based on the considerations outlined above and the motivation of the fundamental research and potential industrial applications of biocomposites, the purpose of the present chapter was to investigate the extrusion of SPI with gelatin and sugars at low moisture content to study the effect of moisture,

gelatin and Maillard reaction on system parameters and product properties. In this work, a twin-screw extruder, which has considerably more heat exchange capability than single-screw extruders, was employed, thus making the extrusion process more energy efficient and cost effective.

7.2. Sample preparation

All components (SPI, gelatin, sugars, and glycerol) were mixed in a variable speed Stephan mixer, model UMC 5 (Stephan, UK) for 5 min at 1500 rpm. Different glycerol contents (20, 30 and 40 % w/w) were employed and samples were designed as SPI20, SPI30 and SPI40. Gelatin was selected as 15% by weight on SPI dry basis, based on the results of the fifth chapter. Samples were designed as SPI-GE. Different sugar contents (10, 20, 30 and 40% by weight on SPI dry basis) were also added and samples were designed as SUC10, SUC20, SUC30 and SUC40 in the case of sucrose, and LAC10, LAC20, LAC30 and LAC40 for lactose.

Mixtures were added into the feed hopper and mixed with water in the barrel of the twin-screw extruder. A twin-screw extruder (MPF 19/25 APV Baker) was used in this study. This extruder is capable of generating the high pressures needed to extrude a wide range of products. A schematic diagram of the extruder is shown in **Figure 7.1**.

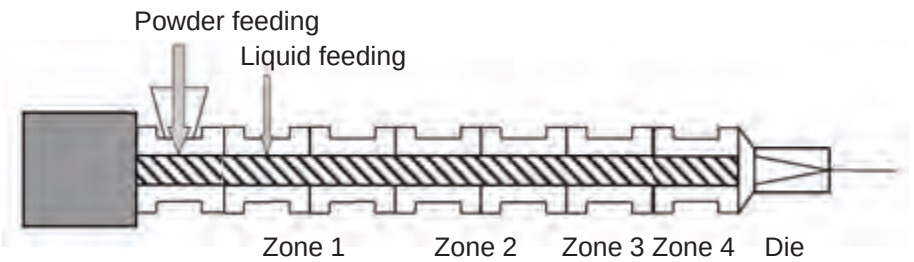


Figure 7.1. Schematic diagram of the twin-extruder employed in this study.

The extruder has 19 mm diameter barrel, a length to barrel diameter ratio of 25:1, and a barrel comprising four controlled temperature zones, resulting in a die temperature of 70 to 100 ºC. The screw configuration used in the present study is shown in **Table 7.1**.

Screw configuration	Screw function
5.0 D	Feed screw
6.0 D x 60º	Forward paddles
5.0 D	Feed screw
5.0 D x 60º	Forward paddles
2.5 D	Feed screw
3.0 D x 60º	Forward paddles
2.0 D	Single lead screw
5.0 D x 60º	Forward paddles
3.0 D	Single lead screw
3.0 D x 60º	Forward paddles
2.0 D	Single lead screw

Table 7.1. Configuration of the twin-screw extruder employed (D = screw diameter).

Based on a series of preliminary experiments, the following optimum processing parameters were used in this work: temperature profile of 70, 80, 95, and 100 ºC along the extrusion direction, feeding rate of 1 kg/h, and screw speed of 250 rpm. All trials were carried out using a water speed of 2.94 g/min (0.15 kg/h). Water was pumped directly into the extruder barrel zone 1 using a peristaltic pump (504U MK, Watson Marlow Ltd.).

The specific mechanical energy was calculated using the expression (Hu et al., 1993):

$$\text{SME (kJ/kg)} = \frac{\text{Screw speed} \times \text{Power (kW)} \times \text{Torque (\%)}}{\text{Max. screw speed} \times \text{Throughput (kg/h)} \times 100}$$

The extruder was operated at a constant speed of 250 rpm. The feed rate of the extruder was adjusted to 1 kg/h, and had a 2 kW/h motor power in practice. Dies were scaled up by maintaining a constant throughput per unit of

orifice area. The extruder was equipped with a single die of 3 mm diameter, giving a throughput per unit area of 0.141 kg/h mm^2.

7.3. Results and discussion

7.3.1. Effect of water and glycerol on extrusion

Firstly, the effect of glycerol on SME values, both with and without water, was analyzed. SME is a parameter that indicates extrusion processability, thus providing good characterization of the extrusion process. SME values also indicate the extent of molecular breakdown or degradation that mechanical force undergoes during extrusion process, so this value is an important parameter influencing final product characteristics such as solubility and expansion index.

When SPI/glycerol mixtures were extruded without water, it was not possible to obtain product from the die due to a high increase in torque, irrespective of the glycerol level employed. When water was added, SME decreased as the content of glycerol was higher, (**Table 7.2**). Based on these results, 20% glycerol was fixed for the extrusion process in order to analyze the effect of the other additives employed in this study.

Sample	Glycerol Content (%)	Water Content (%)	SME (kJ/kg)
SPI20	20	0	∞
SPI20	20	16	1188 ± 22^{a}
SPI30	30	0	∞
SPI30	30	16	972 ± 18^{b}
SPI40	40	0	∞
SPI40	40	16	612 ± 15^{c}

Table 7.2. Effect of water and glycerol on SME values. $^{a\text{-}c}$Two means followed by the same letter in the same column are not significantly (P > 0.05) different through the Tukey´s multiple range test.

The addition of water resulted in accelerating the flow speed of extrudate coming from the extruder. The water content was the most important factor influencing extrusion parameters and product properties. Water played an important role in extrusion processing due to its effect on the heat transfer during extrusion. Increasing water content resulted in increased heat transfer from the extruder barrel to the feed material and consequently decreased viscosity, shear, and friction during extrusion. As the water content was increased, the torque and SME decreased due to a reduction in the force required to extrude wet mass through the die, and this resulted in decreasing the friction between raw material, screw shaft and extruder barrel (Chen et al., 2010). Thus, extruder system parameters were lowered at higher water content and this can be attributed to a water lubricating effect. Increasing water content resulted in the decrease of sample viscosity in the extruder barrel, thus a lower force was required to extrude the molten extrudate through the die, and reduced the conversion ratio of extruder mechanical energy into heat energy, consequently the SME became lower due to a lower viscous material dissipation. As the water content greatly influences final properties such as tensile strength, hardness and chewiness (Chen et al., 2010; Lin et al., 2000), the water content chosen as optimum in this study was 16% due to the fact that the glycerol concentration used also helped to retain moisture.

7.3.2. Effect of gelatin and sugars on extrusion

The effect of gelatin and sugars on SME is shown in **Table 7.2**. SME increased with the incorporation of gelatin due to a frictional increase in the extruder barrel, thereby increasing the force required to extrude wet mass through the die and converting the ratio of extruder mechanical energy into heat energy. Consequently, SME became higher as did temperature in the die and as a result, the viscous dissipation was higher. The product obtained at the extruder die was not continuous, indicating that gelatin could unfold and align during extrusion. As it was shown in a previous work (Guerrero et al., 2011),

this fact may be attributed to protein-protein interactions, which are determined by hydrogen bonds, electrostatic and hydrophobic interactions and are influenced by the amino acid residues, but also by the three dimensional network formed. In the case of gelatin, this protein can reach a high degree of organization and form highly organized networks.

As it can be seen in **Table 7.3**, the incorporation of sugars caused lower SME values compared to those samples without sugars. This change in SME values is due to the fact that torque decreased with increasing sugar content. Sugars in the system could alter the conformation of proteins, interact with them by binding to protein side groups through the Maillard reaction, and influence physicochemical properties (Gu et al., 2009). The Maillard reaction depends on the type of sugar involved. **Figure 7.2** shows the products obtained at the extruder die.

As it can be seen, the colour of the product changed depending on the sugar type employed. Lactose is a reducing disaccharide that can react with protein through the Maillard reaction (**Figure 7.3**).

Sample	Gelatin Content (%)	Sucrose Content (%)	Lactose Content (%)	SME (kJ/kg)
SPI20	0	0	0	1188 ± 22^{a}
SPI-GE	15	0	0	1512 ± 19^{b}
SUC10	15	10	0	828 ± 23^{c}
SUC20	15	20	0	$720\pm22^{d,e}$
SUC30	15	30	0	$684\pm20^{e,f}$
SUC40	15	40	0	648 ± 18^{f}
LAC10	15	0	10	1368 ± 24^{g}
LAC20	15	0	20	900 ± 17^{h}
LAC30	15	0	30	$792\pm15^{c,i}$
LAC40	15	0	40	$756\pm16^{d,i}$

Table 7.3. Effect of gelatin and sugars on SME values. $^{a-j}$Two means followed by the same letter in the same column are not significantly ($P > 0.05$) different through the Tukey´s multiple range test.

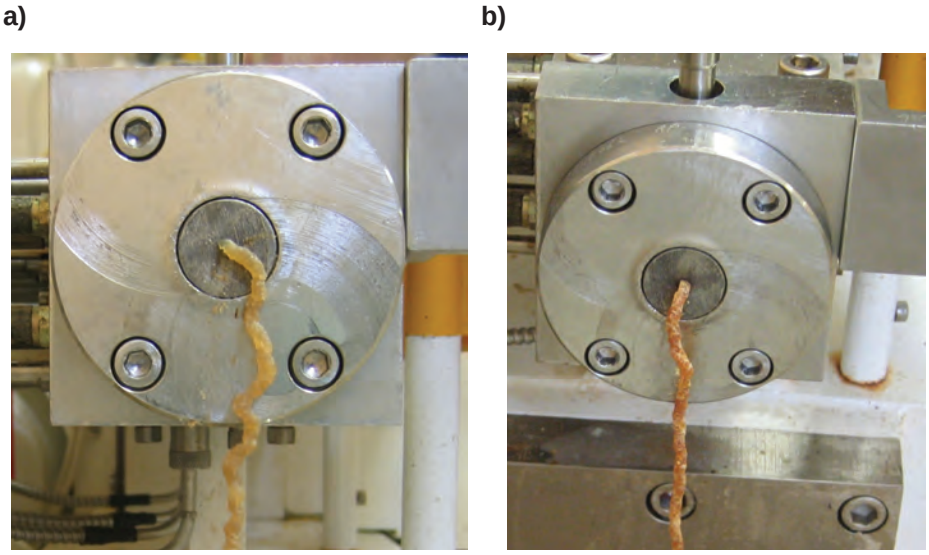


Figure 7.2. Products obtained at the extruder die for a) SPI with sucrose and b) SPI with lactose.

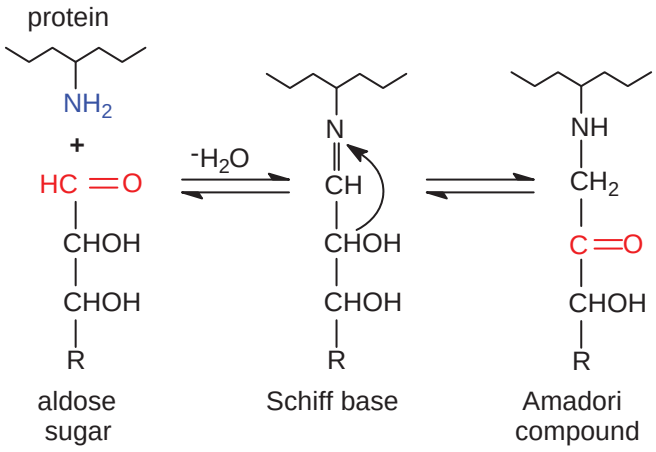


Figure 7.3. Maillard reaction between proteins and sugars.

Reactions that can take place during extrusion are controlled by the composition of samples, temperature, and residence time. However, these

reactions are accelerated during extrusion processing because of shear forces. High barrel temperatures and low moisture contents promote Maillard reaction during extrusion. The early stage of the Maillard reaction involves the formation of the protein-sugar conjugates between the carbonyl group of a reducing carbohydrate with an available amine group in the protein, which leads to a Schiff base with the release of water (Yasir et al., 2007). The Schiff base subsequently cyclises to produce the Armadori compound and, in the final stage, highly coloured, insoluble polymeric compounds, referred as melanoidins, are formed.

7.3.3. Thermal properties of extruded SPI

The two denaturation peaks of SPI, corresponding to the low molecular weight 7S globulin and the high molecular weight 11S globulin, were shown in previous chapters where it was explained that a significant amount of the SPI used in this study remained in its native conformation with globular structure after processing by compression. In the case of extrusion, **Figure 7.4** shows a broad peak for SPI20, indicating that processing conditions did not cause total denaturation of the protein. However, when gelatin was added, this peak disappeared, which is an indication of protein denaturation due to the conformational changes that produce a certain degree of interaction between gelatin and SPI, such as hydrogen bonds, dipole-dipole and hydrophobic interactions, characteristic of natural proteins. These results are in good agreement with the increase of SME with gelatin addition. It is also worth to note that the crystallization peak for pure lactose, which occurs at 124-135 ºC (Drapier-Beche et al., 1997), cannot be observed in the presence of SPI.

When soy protein is extruded under high pressure, high temperature, and low moisture conditions, the sudden release of pressure upon exiting from the die caused instant water evaporation from the extrudates, creating expanded and spongy structures, quite like that of common textured vegetable proteins. According to Kitabatake and Doi (1990), the temperature needed for

texturization in the extruder was the temperature for the denaturation of soy protein, which is dependent on water content. After denaturation, viscosity decreased and the fluidity of soy protein dough might give rise to a certain order under some shearing conditions, which resulted in texturized protein after cooling.

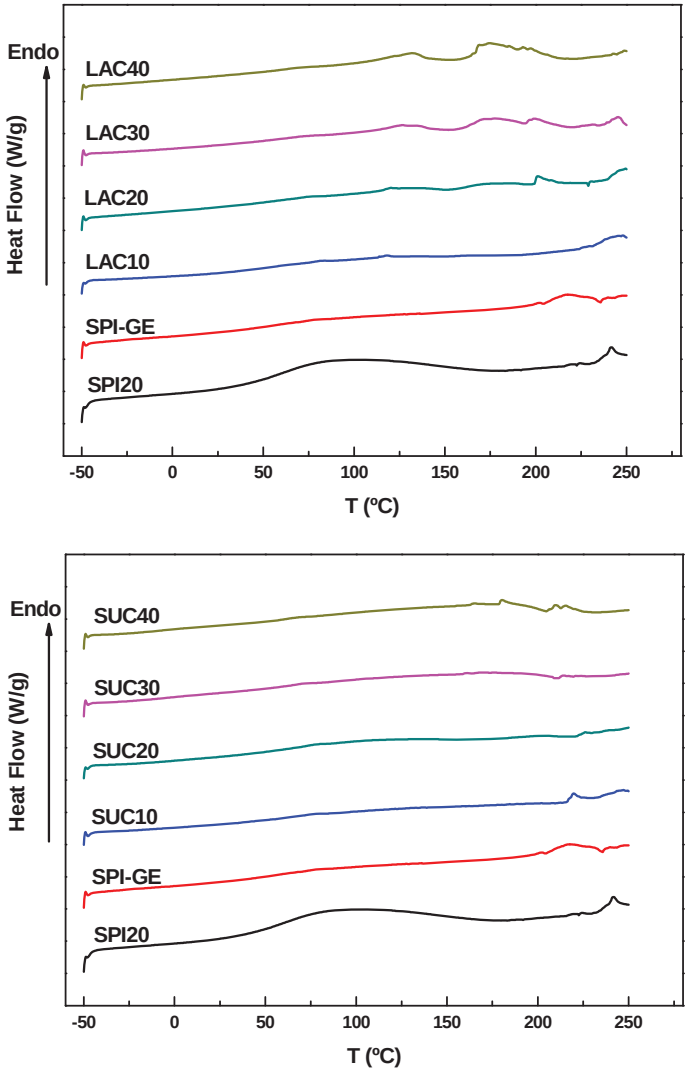


Figure 7.4. DSC thermograms for SPI-based products with different additives.

7.3.4. XRD analysis

XRD was used to determine the structure for the samples with and without sugars. As it was observed in the previous chapter, **Figure 7.5** shows that the diffraction patterns of the SPI samples exhibited a dominant amorphous halo, a broad band with a maximum at 2θ = 20º, which is characteristic for pure SPI.

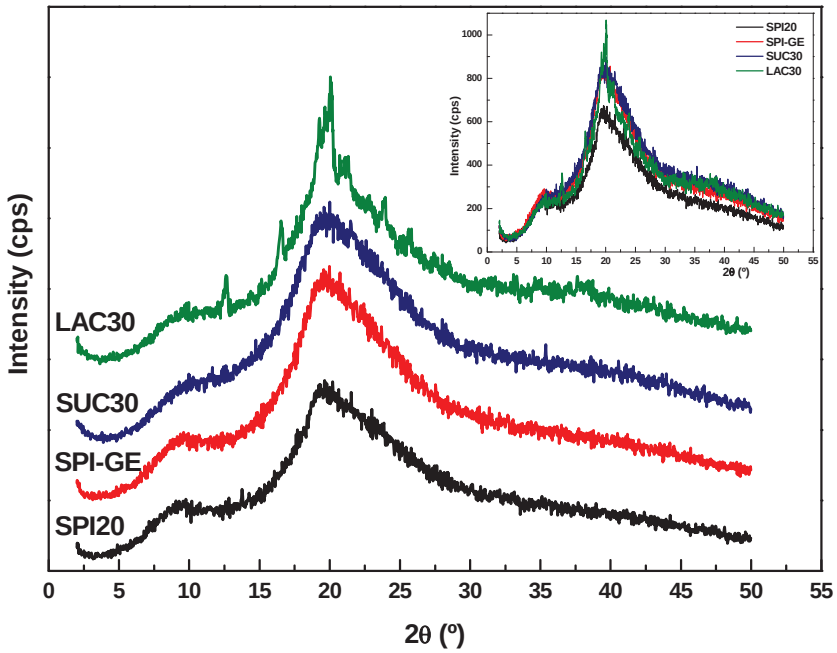


Figure 7.5. XRD patterns for SPI-based products with different additives.

The intensity of this band increased when gelatin was added, indicating a higher order in the structure. The addition of sucrose did not affect the intensity of the peak obtained for SPI-GE; however, the addition of lactose caused a significant increase of crystallinity, indicating that the presence of lactose affected the amorphous structure of the SPI-based samples. It is worth to note that SPI can not totally inhibit the crystallization of lactose, although protein-lactose covalent bonds formed by Maillard reaction prevent crystal

nucleation and growth. These results are in accordance with the ones shown by DSC thermograms, where no crystallization peak was observed for lactose.

7.3.5. FTIR analysis

FTIR spectra of blends with various weight ratios of sugars are shown in **Figure 7.6.**

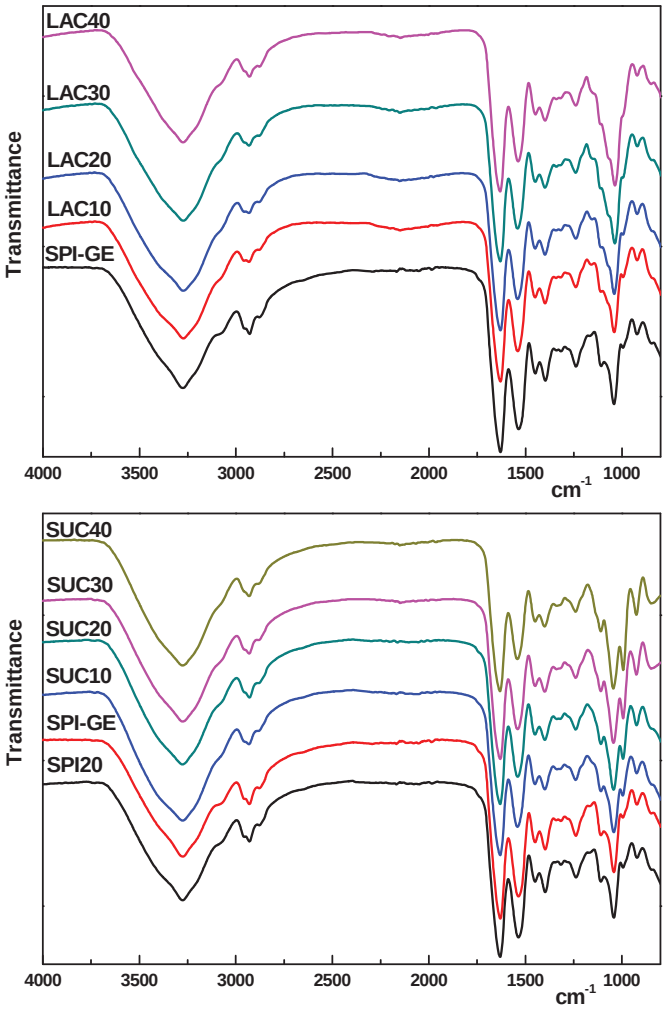


Figure 7.6. FTIR spectra of SPI-based products with different contents of sucrose and lactose.

7.3.6. MC and TSM values

As SPI is water sensitive, materials prepared with SPI need improved water stability for an extended service time. Consequently, increasing water resistance for SPI blends is very meaningful for food packaging applications.

Sample	Gelatin Content (%)	Sucrose Content (%)	Lactose Content (%)	Moisture Content (%)	TSM Method 1 (%)	TSM Method 2 (%)
SPI20	0	0	0	15.8±0.1^{a}	21.5±0.1^{a}	21.5±0.2^{a}
SPI-GE	15	0	0	15.9±0.4^{a}	21.6±0.2^{a}	22.3±0.4^{a}
SUC10	15	10	0	17.4±0.1^{b}	25.9±0.2^{b}	27.7±0.2^{b}
SUC20	15	20	0	16.5±0.2^{c}	31.3±0.5^{c}	32.7±0.2^{c}
SUC30	15	30	0	15.2±0.1^{a}	35.8±0.1^{d}	36.9±0.4^{d}
SUC40	15	40	0	14.2±0.2^{d}	39.7±0.2^{e}	40.6±0.3^{e}
LAC10	15	0	10	20.0±0.2^{e}	18.3±0.1^{f}	22.4±0.2^{f}
LAC20	15	0	20	19.9±0.2^{e}	18.5±0.1^{f,g}	24.8±0.5^{g}
LAC30	15	0	30	19.8±0.2^{e}	19.1±0.1^{f,g}	27.7±0.4^{h}
LAC40	15	0	40	19.5±0.5^{e}	19.3±0.1^{g}	29.9±0.4^{i}

Table 7.4. Effect of gelatin and sugars on MC and TSM values. $^{a-i}$Two means followed by the same letter in the same column are not significantly ($P > 0.05$) different through the Tukey´s multiple range test.

Moisture content and total soluble matter values for SPI blends are shown in **Table 7.4**. MC values for the blends without lactose are lower than those blends with lactose and these MC values are similar ($P > 0.05$) when the lactose content increased, being around 20%. However, when sucrose content increased, MC values decreased significantly ($P < 0.05$), which would indicate that hydrophilic sites were less accessible to water molecules.

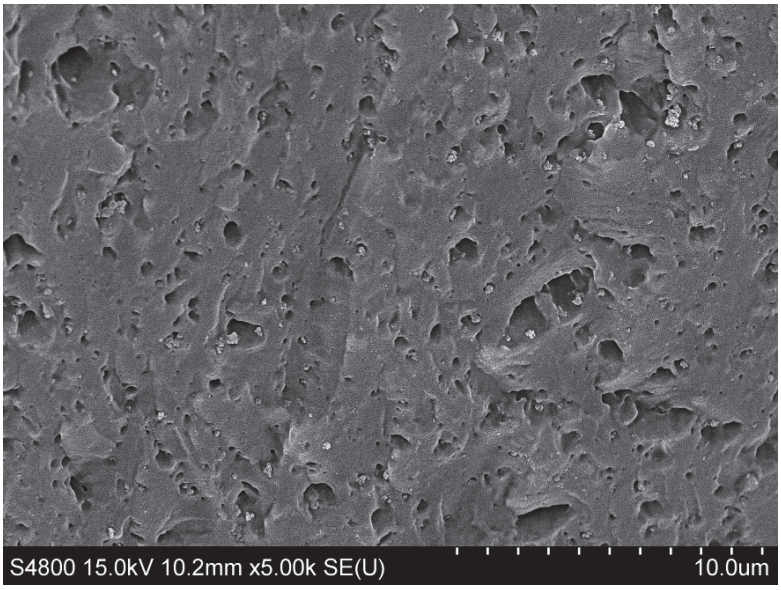
TSM values for SPI-based blends calculated by method 1 were determined by drying specimens in an oven before immersion in water. It is well known that heating, along with the reagent chemical structure, influence Maillard reaction. Therefore, heating of specimens prior to immersion in water could cause cross-linking, leading to underestimation of TSM values. For this

reason, TSM values were also measured by method 2, using different specimens to determine initial and soluble dry matter. As shown in **Table 7.4**, the samples without sugar did not show significant difference ($P > 0.05$) between the two methods employed due to the fact that only physical interactions take place between protein and gelatin. For the blends with sucrose, the results obtained by the two methods were also similar; however, method 2 resulted in greater TSM values than method 1 for the blends with lactose due to the fact that lactose is a reducing disaccharide that present a higher degree of Maillard reaction than sucrose, which leads to insoluble polymeric compounds. On the other hand, TSM values increased when sugar content was increased ($P < 0.05$), which was more pronounced for blends with sucrose where the degree of Maillard reaction is lower.

7.3.7. Morphological analysis

SEM micrographs taken at the break surface of SPI samples fractured in liquid nitrogen are shown in **Figure 7.7**. Samples are characterized by minor surface heterogeneities, which is typical in a brittle fracture. Some porosity was detected in SPI samples, although these cavities seem to be closed pores **(Figure 7.7a)**. Porosity decreased with the incorporation of gelatin **(Figure 7.7b)**, indicating that blending with gelatin cannot completely eliminate the structural defects of SPI. These micrographs are in accordance with the increase of SME values in the extrusion process and the increase of the intensity peak in XRD. Morphologies for SUC30 and LAC30 showed more compact structures. Moreover, crosslinking reactions (Maillard reaction) occurred between lactose and SPI during extrusion lead to more compact structures for LAC30 than SUC30 **(Figure 7.7c)**. In the case of lactose **(Figure 7.7d)**, white spots indicate some local heterogeneities that lead to some surface irregularities where lactose particles comprise dense agglomerates of smaller subunits combined together to form large clusters.

a)



b)

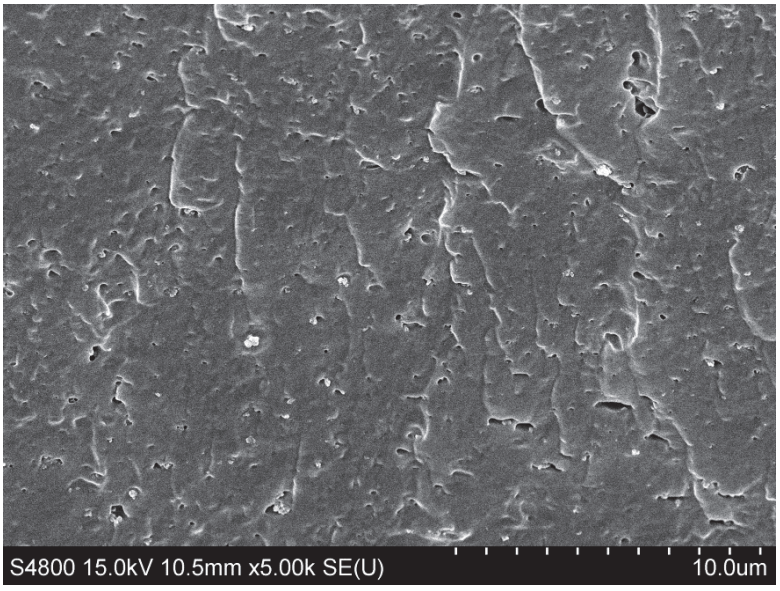
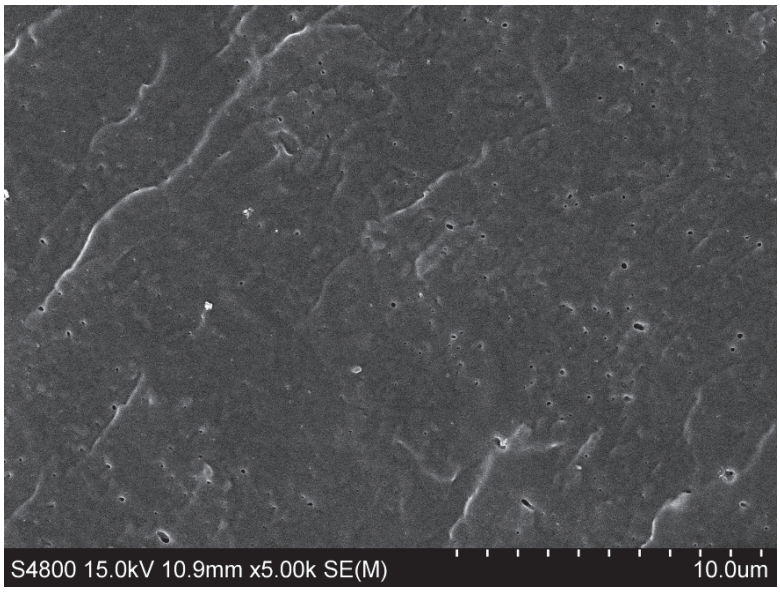


Figure.7.6. SEM micrographs taken at the break surface of SPI-based products fractured in liquid nitrogen: a) SPI20, b) SPI-GE.

c)



d)

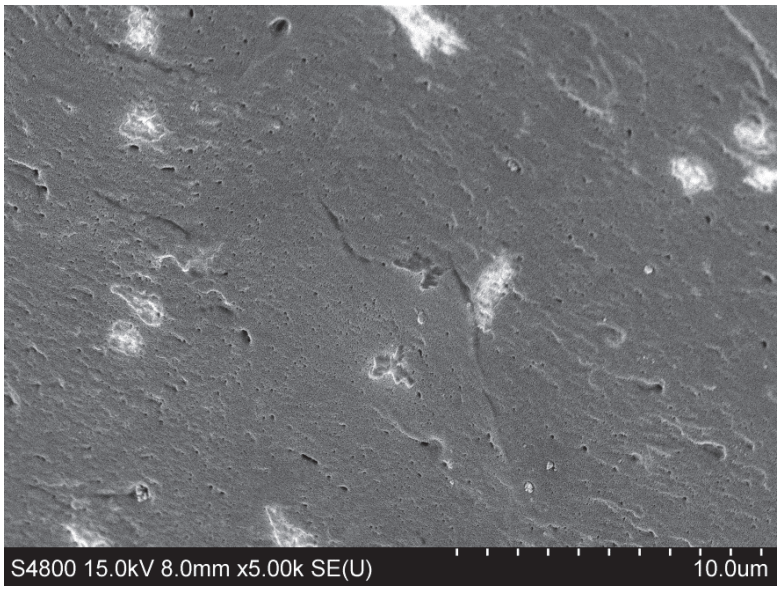


Figure.7.6. SEM micrographs taken at the break surface of SPI-based products fractured in liquid nitrogen: c) SUC30 and d) LAC30**.**

7.4. Conclusions

Extrusion parameters for SPI-based materials with gelatin and sugars using twin-screw extruder at low moisture content were optimized in order to be able to prepare biocomposites in the future. It was observed that water was an important factor for extrusion processing due to its effect on the heat transfer during extrusion and lubricating effect. When SPI/glycerol mixtures were extruded without water, it was no possible to obtain product; however, SME decreased when water was added. The optimum water content chosen in this study was 16% due to the fact that the 20% glycerol used in this study also helped to retain moisture.

The incorporation of sugars also resulted in a decrease of SME values and the colour of the product changed depending on the type of sugar employed. In the case of lactose, cross-linking reactions occurred between sugar and protein during processing of SPI-blends due to Maillard reaction. In the early stage of the reaction, the formation of the protein-sugar conjugates between the carbonyl group of the reducing disaccharide with an available amine group in the protein lead to highly coloured and insoluble polymeric compounds, referred as melanoidins, leading to a more ordered structure as it was shown by XRD and SEM.

Maillard reaction could be evaluated by the changes of the amide I and amide II bands which reflected that hydroxyl groups in sugars and amine groups in SPI were consumed during the blending process at elevated temperatures. It was observed that the degree of Maillard reaction was higher for the materials with lactose than the ones with sucrose due to the presence of the free hydroxyl group in the anomeric carbon of the lactose. These FTIR results were in accordance with the lower TSM values obtained for materials with lactose.

GENERAL CONCLUSIONS

8. General conclusions

- SPI films with glycerol content between 30 and 40% wt are the most highly recommended in order to improve mechanical properties. A lower amount of plasticizer produced brittle films and a higher content resulted in sticky films.

- The optimum film-processing method was compression, which enhanced both tensile strength and elongation at break. Moreover, this method allowed a much shorter period of time for film preparation and the use of conventional techniques, which are more convenient for industrial applications than casting.

- The use of freeze-drying allowed us to modify protein structure at different pHs and process films by compression. The best mechanical properties were obtained when basic pHs were used due to a major unfolding of the protein, which allowed the exposition of the polar groups to be able to interact with small glycerol molecules.

- Mechanical properties remained invariable after two months of storage, which is of crucial importance for packaging purposes.

- Mechanical properties of SPI-based films were improved by the addition of gelatin, especially when 15% wt bovine gelatin with 200 bloom index was added in the system plasticized with 30% wt glycerol, showing an increase in tensile strength with the maintenance of elongation at break.

- Gelatin-incorporated SPI-based films showed excellent barrier properties to UV light, suggesting the potential preventive effect of SPI films on the retardation of product oxidation induced by UV light, and

transparency values similar to the ones measured for OPP and better than the ones obtained for LDPE, commercial films used for packaging purposes.

- The addition of lactic acid produced an increase of the hydrophobic character of the film, maintaining WVP, and a decrease in the yellowness of the films, maintaining excellent barrier properties to UV light.

- Water, glycerol, and sugar contents were optimized for SPI-based materials processed by extrusion in order to decrease SME values.

- Cross-linking reactions occurred between lactose and protein during extrusion of SPI-blends due to Maillard reaction and highly coloured and insoluble polymeric compounds.

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